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Neurobiological Consequences of Fluoxetine Exposure during Adolescence

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NEUROBIOLOGICAL CONSEQUENCES OF
FLUOXETINE EXPOSURE DURING ADOLESCENCE

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To my Peach
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ABSTRACT

Little is known regarding the mechanisms underlying the neurobiological consequences of antidepressant exposure during adolescence. Therefore, in this dissertation, I assessed the long-lasting effects of adolescent exposure to Fluoxetine (FLX), a selective serotonin reuptake inhibitor, on behavioral reactivity to emotion-eliciting stimuli in adulthood using rodent animal models. To do this, in chapters two and three, male Sprague-Dawley rats and c57BL/6 mice are exposed to FLX throughout adolescence (postnatal days 35-49), and tested in adulthood (postnatal day 70+) on a battery of behavioral tasks designed to assess sensitivity to stress- (i.e., forced swimming and the social defeat procedure), and anxiety-inducing situations (i.e., elevated plus-maze, novelty induced hypophagia, and open field test). Because a proposed mechanism by which FLX exerts its therapeutic effect(s) has been linked to the modulation of intracellular signaling pathways involved in the regulation of cell survival, in chapter three, I also assessed how FLX exposure influenced extracellular signal-regulated protein kinase 1/2 (ERK)-signaling within the ventral tegmental area (VTA) of the midbrain. The VTA was selected given the role this brain region plays in regulating mood and motivation under normal conditions. FLX exposure during adolescence resulted in an enduring paradoxical behavioral response in which rodents exhibit a stress-resistant behavioral phenotype, along with an increase in sensitivity to anxiety-inducing situations. In addition, a persistent decrease in ERK-related signaling was observed within the VTA of rats and mice exposed to FLX. Given that chronic exposure to FLX resulted in an enduring downregulation of ERK signaling, it was imperative to determine whether exposure to stress, a major predisposing factor for depression, would result in oppositional regulation of this signaling pathway. Thus, in chapters three and four I assessed the behavioral and biochemical effects of exposure to stress in adult rats and mice using complementary behavioral, molecular, and gene transfer approaches. Here, exposure to chronic unpredictable stress resulted in depressive-like phenotypes, including a reduced ability to experience pleasure (i.e., anhedonia, as inferred from the sucrose preference test) and increased vulnerability to subsequent stress (i.e., forced swim test and the social defeat procedure). This stress-induced behavioral profile was also accompanied by an increase in ERK and its related signaling within the VTA – biochemical results opposite to those observed after FLX exposure. The functional significance of this oppositional effect was further confirmed using viral vectors: increasing ERK activity within the VTA increased sensitivity, whereas decreasing ERK resulted in decreased sensitivity to stress. Together, the data presented in this dissertation strongly implicate ERK signaling within the VTA as a modulator of behavioral responsivity to stress and antidepressant efficacy in adolescent and adult rodents. Lastly, in chapter five, the potential clinical implications, as well as future directions of this work are discussed.
CHAPTER ONE

INTRODUCTION

Mood disorders are among the most prevalent forms of mental illness. It is estimated that 2-5% of the adult population in the United States is afflicted by major depressive disorder (MDD), and around 20% suffer from milder forms of the illness (Blazer et al., 1994). Epidemiologic studies show that roughly 40-50% of the risk for MDD is genetic (Fava and Kendler, 2000), but unlike the progress made on other medical conditions such as diabetes and certain cancers, no genetic abnormality associated with MDD has been identified with certainty (Burmeister, 1999). Despite our limited understanding about the etiology and pathophysiology of depression, there are effective treatments, as the large majority (~80%) of adults with depression show some improvement with antidepressant drug treatment.

The acute mechanisms of action of antidepressant medications have been delineated: inhibition of serotonin and norepinephrine reuptake transporters and inhibition of monoamine oxidase (a major catabolic enzyme for monoamine neurotransmitters) lead to an increase in the extrasynaptic levels of serotonin and/or norepinephrine. However, it is well known that all available antidepressants exert their mood-elevating effects only after prolonged administration (weeks to months) indicating that increased levels of serotonin or norepinephrine by itself is not sufficient for the clinical actions of these drugs. To add to the complexity, these neurotransmitter systems extensively innervate mesolimbic, striatal, and cortical brain regions known to play critical roles in the regulation of emotional states and sensitivity to natural rewards (e.g. food and sex) and drugs of abuse, where they interact, and influence other neurotransmitter systems, such as dopamine and acetylcholine (Graybiel, 1990; Richelson, 1996).

Epidemiologic reports indicate that mood disorders in pediatric populations are quite common, with up to 70% of depressed children and adolescents experiencing a recurrence within 5 years of the onset of MDD (Kovacs, 1996; Rao et al., 1995). Early-life MDD highly correlates with negative functional outcomes. It is estimated that juveniles who suffer from this illness often develop conduct and anxiety disorders (Hughes et al., 1990), that 20-25% develop substance abuse disorder (Birmaher et al., 1996a), and that 5-10% are likely to complete suicide within 15 years of their initial episode of MDD (Pfeffer, 2001; Rao et al., 1993). The necessity for acute treatment of symptoms often outweighs the potential for long-term side effects resulting from treatment (Pliszka, 1998; Vaswani et al., 2003), and decisions regarding antidepressant treatment in pediatric populations have been largely based on data from adults (Bylund and Reed, 2007; Emslie and Mayes, 2001). This is evidenced by available epidemiologic data indicating that antidepressant prescriptions given to juveniles increased (>19-fold) between 1984-1994 (Zito et al., 2002), well before controlled studies on the efficacy of antidepressant treatment for children and adolescent mood disorders had been implemented (Coyle et al., 2003). Although clinical studies for childhood depression treatment have slowly emerged (Jureidini et al., 2004), reliable evidence-based indications for antidepressant use, and its potential long-term consequences in pediatric populations, is critically lacking (Coyle et al., 2003; Emslie and Mayes, 2001).
Despite the urgent need for this type of information, it is surprising that the long-term consequence(s) of such pharmacological treatment in the developing brain has yet to be carefully studied at the clinical or preclinical levels. This is somewhat remarkable given that the therapeutic action for some of the major psychiatric disorders occurs in monoamine systems known to regulate mood under normal conditions (Nestler and Carlezon, 2006), which undergo substantial neuronal adaptations during developmental periods before adulthood (Seeman et al., 1987; Spear, 2000). For example, it has been shown in rhesus monkeys that monoaminergic storage capacity and synthesis continues to develop through puberty (Goldman-Rakic and Brown, 1982). In addition, serotonergic connectivity within the basal forebrain exhibit increases and decreases (i.e., pruning) throughout development (Dinopoulos et al., 1997), and these innervations to the prefrontal cortex continue to develop into adulthood (Moll et al., 2000). In this ontogenetic context, it has also been demonstrated that these neuronal changes result in adaptations which have been correlated, at least in part, with regulation of cognitive processes and responsiveness to emotional stimuli (Hyman, 2001). Thus, it is not surprising that environmental, emotional, and pharmacological perturbations during these developmental periods result in long-lasting alterations in behavioral response to a variety of rewarding and aversive stimuli (Bolaños et al., 2003a; Spear, 2000). In this context, it is not difficult to contemplate the notion that antidepressant exposure during early life may interfere with normal development of brain pathways associated with the mediation of emotional states and responsiveness to rewards that can dramatically influence neurobehavioral functioning later in life (Spear, 2000).

There is a need for systematic assessment of the long-term neurobiological consequences of psychotropic drug treatment in the immature brain. Thus, the experiments outlined in this dissertation are designed around the central hypothesis that antidepressant treatment during adolescence in male rats and mice alters intracellular signaling pathways within the mesolimbic dopamine circuit, a system known to mediate behavioral responsiveness to natural and drug reward, as well as other emotion-eliciting stimuli. Specifically, the aim of this dissertation is to characterize behavioral and molecular changes induced by chronic fluoxetine (FLX) treatment during adolescence by assessing behavioral sensitivity to natural-reward, as well as anxiety- and stress-eliciting situations in adulthood. The selective serotonin reuptake inhibitor FLX was chosen because it is currently the only antidepressant medication approved by the United States Food and Drug Administration (USFDA) for the treatment of pediatric MDD. An additional goal is to determine the biochemical changes induced by FLX exposure within the ventral tegmental area (VTA), a major component of the mesolimbic dopamine system. The VTA was selected given the increasing evidence that mesolimbic reward pathways are part of the neural circuitry that control mood under normal conditions. (Bolaños et al., 2003b; Iñiguez et al., 2010a; Krishnan et al., 2008; Naranjo et al., 2001; Nestler and Carlezon, 2006; Yadid et al., 2001). I hypothesize that exposing immature neural pathways to FLX may lead to alterations in second messenger signaling likely to induce potential functional impairments later in life.
CHAPTER TWO

LONG-TERM EFFECTS OF EXPOSURE TO FLUOXETINE DURING ADOLESCENCE ON MOOD-RELATED STIMULI

Adapted from:


INTRODUCTION

Until relatively recently, the existence of major depressive disorder (MDD) in pediatric populations was not well recognized. Epidemiologic reports now indicate that mood disorders are quite common early in life, affecting approximately 2–8% of children and adolescents, respectively (Birmaher et al., 1996b; Kapornai and Vetro, 2008). Pediatric MDD can lead to impairments in various psychiatric and functional domains such as antisocial personality, bipolar disorder, substance abuse, homelessness, self-harm and up to 75% risk of recurrent depressive episodes in adulthood (Kovacs, 1996; Patten et al., 2001; Rihmer, 2007; Weissman et al., 1999). These observations are indicative of an adverse impact of MDD on the development of neural substrates mediating cognitive, emotional and social functioning (Andersen and Teicher, 2008; Rohde et al., 1994). Thus, depression is a serious disorder necessitating timely and appropriate therapeutic intervention.

Fluoxetine (FLX) (Prozac®), a selective serotonin reuptake inhibitor (SSRI), is the first drug approved for the treatment of pediatric MDD (Safer, 2006). Although data about the effectiveness and safety of pharmacotherapy in youngsters are sparse, it is conceivable that treatment decisions for acute management of symptoms are made under the assumption that limiting dysfunction outweighs the potential for long-term side effects (Emslie et al., 2000; Kapornai and Vetro, 2008; Pliszka, 1998; Vaswani et al., 2003). Decisions regarding antidepressant use in early-life have been largely based on data from adults (Coyle et al., 2003; Emslie and Mayes, 2001). Although reliable evidence-based indications for SSRI use and its potential long-term consequences in youngsters are lacking, prescription rates are on the rise (Zito et al., 2002; Zito et al., 2006).

The acute effects of SSRI antidepressant medications are well defined: they increase the brain’s serotonin neurotransmission; however, they exert their mood-elevating effects after prolonged (i.e., weeks) administration (de Montigny and Blier, 1984). Serotonin is pivotal in the regulation of adolescent brain development in both rodents and humans (Azmitia, 2007; Blakemore and Choudhury, 2006a). There is extensive serotonergic innervation of key brain regions involved in the control of emotional, cognitive, and motivated behaviors (Borue et al., 2007; Molliver, 1987; Whitaker-Azmitia, 2005), and dysregulation of this neurotransmitter system has been correlated with deficits in behavior and emotional regulation (Ansorge et al., 2008; Ansorge et al., 2004; Cools et al., 2008). Because SSRI exposure in youngsters occurs at a
time of ongoing neuronal adaptations (Andersen and Navalta, 2004; Seeman et al., 1987; Spear, 2000), and such treatments can last for years (Bhagwagar and Cowen, 2008; Patten et al., 2001), it is possible that antidepressant treatments may impact the development of brain pathways that can dramatically influence neurobiological functioning later in life. Given the prevalence of prescription antidepressant use during adolescence and the scarcity of knowledge regarding long-term effects of such treatments, it is essential that the neurobehavioral consequences associated with FLX exposure be characterized. Thus, this study was designed to assess the short- and long-term behavioral responsivity to a range of emotion-eliciting stimuli after FLX exposure during adolescence [postnatal day (PD) 35-49] in male rats.

METHODS

Subjects. Male Sprague-Dawley rats were obtained from Charles River (Raleigh, NC). For the initial experiment (Fig. 2.1), rats arrived on the same day at PD30 (adolescent) and PD60 (~250-275 g, adults). For all other experimental conditions, rats arrived on PD30 and treatment started at PD35 or PD65 as depicted in Figure 2.2. The age at the start and duration of the experimental manipulations in adolescent rats (PD35-49) was selected because it roughly approximates adolescence in humans (Andersen and Navalta, 2004; Spear, 2000; Spear and Brake, 1983). Rats were housed in pairs in clear polypropylene boxes containing wood shavings in an animal colony maintained at 23-25˚C on a 12 hr light-dark cycle in which lights were on between 07:00 and 19:00 hr. Rats were provided with food and water ad libitum.

Drug treatment and experimental design. Fluoxetine hydrochloride (FLX) was obtained from Sigma-Aldrich (St Louis, MO), dissolved in sterile distilled water (VEH) and administered in a volume of 2 mL/kg. An initial experiment was conducted using the forced swim test to establish the FLX dose that would reliably decrease immobility as characterized in adult (250-275 g) rats (Porsolt, 2000). The forced swim test consists of two swimming sessions over two days. PD35 and PD65 rats were forced to swim on day 1, and then received intraperitoneal injections of FLX (0, 2.5, 5, 10 or 20 mg/kg) 23, 5, and 1 hr prior to forced swim re-exposure (day 2). Based on the results from this experiment (Fig. 2.1), separate groups of PD35 rats were treated with FLX (0 or 10 mg/kg) twice daily (4 hr apart) for 15 consecutive days. Rats were randomly assigned to treatment and behavioral conditions, and schedule of behavioral testing was counterbalanced among all groups (see Table 2.1). Because rodents metabolize FLX about 10 times faster than humans (Wegerer et al., 1999), this drug schedule was selected to approximate FLX levels observed clinically. Short-term behavioral testing began 24 hr after the last injection, whereas long-term assessments started when subjects reached adulthood (see Figure 2.2a). Rats assigned to receive FLX in adulthood (treatment starting at PD65, see Fig. 2.2b) were used as positive controls (matched for drug-treatment and testing-time) only for the forced swim test. Rats treated with FLX during adolescence and re-exposed to FLX as adults were tested on a single behavioral paradigm (i.e., novelty induced hypophagia; see Table 2.1). Behavioral observations and analyses were performed by observers with no knowledge of the treatment conditions of each rat. All experiments were conducted in compliance with the 1996 Guidelines for the Care and Use of Laboratory Animals and approved by Florida State University Animal Care and Use Committee.
Sucrose preference. The sucrose preference test consisted of a two-bottle choice paradigm (Willner et al., 1987). This paradigm has been used extensively to assess the effects of stress-induced anhedonia. Rats were habituated to drink water from two bottles for 5 days. At the start of the experiment, rats were exposed to ascending concentrations of sucrose (0, 0.125, 0.25, 0.5, and 1% wt/vol) for 2 days per sucrose concentration. Water and sucrose consumption were measured at 8:00 and 17:00 hr each testing day at which time the position of the sucrose bottle (left or right) was counterbalanced between the FLX- and VEH-treated groups, across cages and days. The preference for sucrose over water was used as a measure for rats’ sensitivity to reward.

Locomotor activity. Spontaneous locomotor activity was assessed in an open field apparatus (30 min) that consisted of a square box (63 x 63 x 26 cm) that rats can explore freely. This apparatus is fully automated (Florida State University Psychology Department Engineering Group), and records the rats’ locomotor activity as ‘distance traveled’ in cm (Iñiguez et al., 2009).

Novel object approach. This test was conducted over two days. Rats were introduced to the open field for 30 min (day 1). On day 2, rats were brought back to the open field for a 5 min re-acclimation period, and immediately after, a novel object [a white PVC plastic rod (5 cm diameter, 7.5 cm height)] was placed in the center of the apparatus. Rats were allowed to explore the object for 5 min (light intensity: 5 lx). Latency to approach and time spent exploring the object, on initial approach, were measured. Exploration was scored only when the rat’s nose or front paws touched the object. Longer latencies were interpreted as an anxiety-like response, while exploration time was interpreted as being associated with reward (Bevins and Besheer, 2005; Friedman et al., 2009).

Food approach in a novel environment. Novelty induced hypophagia was modified from (Ansorge et al., 2004), and performed under red light at the beginning of the dark phase (testing time: 5 min). At 17:00 hr rats were single housed with access to water. At the start of the dark phase (19:00 hr), rats were placed in a corner of the open field containing a single food pellet (familiar rat chow) placed on a circular white filter paper (12 cm) positioned in the center of the apparatus. Latency to approach the food and begin feeding was scored. The test ended immediately after rats started feeding or if they failed to approach food after 5 min, at which time they were placed back in their home cage with normal access to food and water.

Elevated plus-maze. FLX- and VEH- treated rats were tested for 5 min on the elevated plus-maze, a behavioral model of anxiety-like behavior. The maze was made of gray plastic and consisted of two perpendicular, intersecting runways (12 cm wide X 100 cm long). One runway had tall walls (40 cm high) or “closed arms,” and the other one had no walls or “open arms.” The arms were connected together by a central area, and the maze was elevated 1 m from the floor. Testing was conducted between 9:00 and 13:00 hr under controlled light conditions (~90 lux). At the beginning of the 5-min observation, animals were placed in the central area, facing one of the open arms, and the cumulative time spent and number of entries into the open arms was recorded (Iñiguez et al., 2009).

Forced swim test. The forced swim test is a two-day procedure in which rats are forced to swim under conditions in which they cannot escape. On the first day, rats are forced to swim.
Initially, they 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. When retested 24 hr later, rats become immobile very quickly; however, antidepressant treatment between the forced swim exposures can significantly increase their escape-like behaviors, an effect that has been correlated with antidepressant activity in humans (Porsolt et al., 1977). At the start of the experiment, rats were placed in plastic cylinders (75 x 30 cm) filled to 54 cm depth with 25°C water and forced to swim for 15 min. At the end of this period, rats were removed from the water, dried with towels, and placed in a warmed enclosure for 30 min. All cylinders were emptied and cleaned between rats. Twenty-four hr after the forced swim, rats were retested for 5 min under identical conditions, and sessions were videotaped. In this study, the latency (sec) to become immobile, total immobility (sec), and behavioral counts (floating, swimming, and climbing) were the dependent variables (Cryan et al., 2005b). Behavioral counts were rated at 5 sec intervals during the 5 min retest. 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. To qualify as immobility, this posture had to be clearly visible and maintained for ≥2.0 sec.

*Sexual behavior.* The sexual behavior experiments were carried out as previously reported (Wallace et al., 2008). Rats were housed in a separate room maintained on a 12-hr light/dark cycle (lights on between 24:00 and 12:00 hr). Sexual behavior was assessed under red light conditions between 13:00-18:00 hr in a circular arena (60 cm) containing wood chips on the floor. Each male was given a 5 min acclimation period to the testing arena. Testing started at the end of the acclimation period by the introduction of a receptive female to the arena. Testing sessions (at PD80 and PD90, respectively) lasted 90 min. Behaviors recorded were mount latency, elapsed time between introduction of the female and the first display of mounting, ejaculation latency, time between first mount and first ejaculation, ejaculation frequency, and total number of ejaculations. For rats that either did not display mounting behavior or failed to reach an ejaculation during the test session, the mount latency and ejaculation latency was recorded as 90 min. Sprague-Dawley ovariectomized female rats (Charles River, Raleigh, NC) were used in these experiments. Receptivity of the females was induced by injection of estradiol benzoate (50 mg, subcutaneously) and progesterone (500 mg, subcutaneously) 48 and 4-6 hr before testing, respectively. One week prior to the experiment, the females were tested for one intercourse session with an experienced male. Prior to testing, female receptivity was verified by the exhibition of lordosis, in the presence of the experienced male, and accepted intromission. Each female was used to test only one experimental male.

*Statistical analyses.* Assignment of subjects to the various testing conditions was random. Behavioral data were analyzed using one-way or mixed-design (between and within variables) repeated analyses of variance (ANOVA) followed by Fisher Least-Significant Difference post hoc test. When appropriate, additional Student’s t tests were used to determine statistical significance of preplanned comparisons. Data are expressed as the mean ± SEM. Statistical significance was defined as p<0.05.
RESULTS

Establishing forced swimming behavioral reactivity. FLX increased latency to immobility in adolescents ($F_{4,39}= 5.43$, $p<0.001$; Fig. 2.1a, left panel; n= 8-9/group). Rats receiving 10 or 20 mg/kg FLX displayed longer latencies to immobility when compared to controls ($p<0.05$). FLX had a tendency toward decreasing total immobility ($p= 0.07$; Fig. 2.1b), and dose-dependently increased swimming-counts ($F_{4,39}= 3.77$, $p<0.01$; Fig. 2.1c), while having no effect on climbing- or floating-counts (Fig. 2.1d-e).

FLX dose-dependently increased latency to immobility in adults ($F_{4,39}= 8.88$, $p<0.001$; Fig. 2.1a, right panel; n= 8-10/group). Rats receiving 5, 10, or 20 mg/kg FLX displayed longer latencies to immobility ($p<0.05$) and decreased total immobility ($F_{4,39}= 3.12$, $p<0.02$) compared to controls ($p<0.05$; Fig. 2.1b). FLX increased swimming-counts ($F_{4,39}= 2.72$, $p<0.04$; Fig. 2.1c), without affecting climbing- or floating-counts.

Effects of FLX on body weight. Based on the results above, 10 mg/kg FLX was selected to treat adolescent and adult rats for 15 days (twice daily). Figure 2.3 shows the effects of FLX on body weight-gain in PD35 (n= 18/group) and PD65 (n= 7-8/group) rats. A mixed-design repeated measures ANOVA revealed that FLX significantly decreased weight-gain across days (main effect: $F_{14,476}= 930.75$, $p<0.0001$), drug (main effect: $F_{1,34}= 11.67$, $p<0.002$; Fig. 2.3a inset), and as a function of day by drug (interaction: $F_{14,476}= 25.31$, $p<0.0001$) in adolescent rats (Fig. 2.3a). Although body weight increased with age, the FLX-treated adolescents displayed lower weights than controls ($p<0.05$). Similarly, FLX reduced body weight in adult rats (Fig. 2.3b) as a function of injection day ($F_{14,182}= 14.93$, $p<0.0001$), drug ($F_{1,13}= 25.11$, $p<0.0001$; Fig. 2.3b inset), and day by drug ($F_{14,182}= 18.05$, $p<0.0001$). FLX-treated adult rats displayed lower weights than controls ($p<0.05$).

Effects of chronic FLX on sucrose preference. FLX did not influence total fluid intake (water plus sucrose; Fig. 2.4b) 24 hr after treatment (n= 13/group; Short-term). Conversely, there was a main effect of sucrose ($F_{1,24}= 4.71$, $p<0.04$; Fig. 2.4a), with FLX-treated rats preferring sucrose only at the 0.25% concentration ($p<0.05$). A separate ANOVA revealed that FLX treatment during adolescence increased sucrose preference in adulthood (Fig. 2.4c; Long-term), without affecting total fluid intake (Fig. 2.4d; n= 15/group). Sucrose preference varied by sucrose concentration (main effect: $F_{4,112}= 145.56$, $p<0.05$) and drug (main effect: $F_{1,28}= 9.08$, $p<0.05$). FLX treatment increased sucrose preference only at the 0.125, 0.25, and 0.5% concentrations ($p<0.05$, respectively; Fig. 2.4c).

Elevated plus-maze. FLX induced anxiety-like behaviors 24 hr after the last injection (short-term; n= 8/group) and in adulthood (long-term; n= 8/group). FLX significantly decreased percent time spent ($F_{1,14}= 11.03$, $p<0.005$; Fig. 2.5a, left panel) and percent entries ($F_{1,14}= 9.63$, $p<0.008$; Fig. 2.5b, left panel) in the open arms of the elevated plus-maze. Similarly, rats tested in adulthood spent significantly less percent time in the open arms ($F_{1,14}= 21.93$, $p<0.0001$; Fig. 2.5a, right panel); but did not differ in percent entries into the open arms of the elevated plus-maze (Fig. 2.5b, right panel).
**Novel object approach in a familiar environment.** There were significant differences in the latency to approach a novel object 24 hr after treatment ($t_{17} = -2.16, p<0.05$). FLX-treated rats took significantly longer to approach the object than controls (Fig.2.6a; n= 9-10/group). Additionally, once the FLX-treated rats first approached the object, they spent significantly more time exploring it (Fig. 2.6b) than controls ($t_{17} = -3.59, p<0.02$). Somewhat similar behavioral pattern was observed in rats tested in adulthood: FLX-treated rats displayed longer latencies to approach (Fig. 2.6c; n= 14-15/group) ($t_{27} = -2.32, p<0.03$), but show no differences in time spent exploring the object (Fig. 2.6d).

**Novelty induced hypophagia.** FLX-treated rats had significantly longer latencies to approach food in a novel environment 24 hr after treatment ($t_{16} = -4.24, p<0.05$; n= 9/group; Fig. 2.6e, Short-term) or in adulthood ($t_{10} = -2.35, p<0.05$; n= 6/group; Fig. 2.6e, Long-term). We also assessed whether FLX could reverse these effects in a separate group of adult rats pretreated with FLX during adolescence. Repeated (5 days; $t_{10} = -3.8$, p<.05), but not acute (1 day), FLX (10 mg/kg) reversed the aberrant latency to approach food in these rats (Fig. 2.6f; n= 6/group).

**Effects of FLX on the forced swim test.** We used forced swimming to assess rats’ responsiveness to stress 24 hr after treatment (Fig. 2.7a-c), or when they reached adulthood (Fig. 2.7d-f). FLX-treated rats displayed longer latencies to immobility ($t_{9} = -6.1, p<0.05$) and decreased total immobility ($t_{9} = 3.01, p<0.05$) compared to controls 24 hr after treatment (Fig. 2.7a-b; n= 5-6/group). FLX induced higher swimming- ($t_{9} = -3.87, p<0.05$) and climbing-counts ($t_{9} = -2.67, p<0.05$), with lower floating-counts ($t_{9} = 9.16, p<0.05$; Fig. 2.7c) than controls. FLX-treated rats during adolescence and tested in adulthood also displayed a behavioral profile similar to the short-term group (Fig. 2.7d-f; n= 15/group): longer latencies to immobility ($t_{28} = -2.39, p<0.02$; Fig. 2.7d), lower total immobility ($t_{28} = 3.40, p<0.05$; Fig. 2.7e), higher swimming- ($t_{28} = -3.78, p<0.001$), climbing- ($t_{28} = -3.34, p<0.05$), and lower floating-counts ($t_{28} = 3.35, p<0.002$; Fig. 2.7f).

A separate group of adult rats was tested on the forced swim test after chronic FLX (matched drug treatment and testing-schedule; Fig. 2.8a-f; as with the adolescent group above) to determine whether these FLX-induced effects on the forced swim test are specific to adolescent treatment. These adult FLX-treated rats showed a similar behavioral profile as the FLX-treated adolescents only when tested 24 hr after the last injection (Fig. 2.8a-c; n= 7/group): longer latencies to immobility ($t_{12} = -4.35, p<0.001$; Fig. 2.8a), decreased total immobility ($t_{12} = 3.48, p<0.005$; Fig. 2.8b), higher swimming- ($t_{12} = -4.42, p<0.001$), and climbing-counts ($t_{12} = -4.25, p<0.001$; Fig. 2.8c), with lower floating-counts ($t_{12} = 6.06, p<0.0001$; Fig. 2.8c). FLX had no effects when adult rats were tested 21 days after treatment (Fig. 2.8d-f, n= 7/group).

**Effects of adolescent FLX exposure on sexual behavior.** FLX-exposed rats exhibited deficits in sexual activity when assessed in two separate 90-min sexual behavior sessions (PD80 and PD90, respectively; Fig. 2.9a-c; n= 10/group). A repeated measures (sex session) ANOVA indicated that mount latency varied only as a function of drug ($F_{1,18} = 7.38, p<0.01$; Fig. 2.9a). FLX-pretreated rats displayed longer mount latencies than controls at PD80 (p<0.05; Fig. 2.9a, left panel), but not at PD90 (Fig. 2.9a, right panel). FLX also influenced ejaculation latency between the groups ($F_{1,18} = 28.31, p<0.0001$), with FLX-exposed rats displaying longer times to reach the first ejaculation at PD80 (p<0.05; Fig. 2.9b, left panel) and PD90 (p<0.05; Fig. 2.9b,
right panel). Ejaculation frequency was affected by FLX ($F_{1,18} = 20.01, p<0.0001$; Fig. 2.9c), with FLX-exposed rats showing lower number of ejaculations than controls at both PD80 ($p<0.05$) and PD90 ($p<0.05$) sessions.

**Effects of FLX on basal locomotor activity.** Chronic VEH- or FLX (10 mg/kg, bi-daily) exposure during adolescence did not influence distance traveled (cm) in the open field 24 hr after treatment (Short-term; Figure 2.10a; n= 10/group), or in adulthood (Long-term; Fig. 2.10b; n= 14-15/group).

**Basal locomotor activity 24 hr after day 1 of forced swimming.** Because changes in forced swimming performance can be influenced by differences in motor activity, separate groups of VEH- and FLX-treated rats were tested in the open field 24 hr after day 1 of forced swimming (n= 6/group). No changes in locomotor activity were evident in rats tested either short- or long-term (Figure 2.10c-d) after treatment.

**DISCUSSION**

Antidepressants are often prescribed to pediatric populations (Zito et al., 2006), yet there is a scarcity of knowledge regarding the short- and/or long-lasting neurobiological consequences of such treatments during early life (Safer, 2006). Thus, this study was designed to assess enduring behavioral outcomes in response to rewarding and aversive situations resulting from repeated FLX exposure during adolescence in male rats. This approach was taken because serotonin and compounds that regulate its function interact with mesolimbic reward systems, part of the circuitry controlling emotional and motivated behaviors (Naranjo et al., 2001; Nestler and Carlezon, 2006; Yadid and Friedman, 2008). We report that exposure to FLX during PD35-49 leads to decreased responsiveness to stressful situations, increased sensitivity to natural reward and anxiety-eliciting situations, including deficits in sexual behavior, in adulthood.

Exposure to FLX during adolescence increased rats’ normal sensitivity to sucrose (a natural reward) in adulthood, while only inducing a minimal increase in preference (at the 0.25% concentration) in rats tested 24 hr after treatment. Because antidepressants reduce body weight and caloric intake in animals and humans (Halford and Blundell, 2000; Halford et al., 2005; Simansky and Eberle-Wang, 1993), decreases in sucrose preference were expected. However, the lack of changes in overall liquid intake (sucrose plus water) between the groups indicates that increases in preference are likely due to FLX’s ability to alter rats’ responsiveness to the rewarding effects of sucrose in adulthood. Therefore, it is possible that the young rats tested short-term did not respond robustly to sucrose because of FLX’s ability to decreases caloric intake and palatability of sweet solutions (Asin et al., 1992; Popa et al., 2008). To further explore reward sensitivity after FLX administration, time spent exploring a novel object in a familiar environment was measured (Besheer and Bevins, 2000; Bevins and Besheer, 2005; Hughes, 2007). FLX-treated adolescent rats spent significantly longer exploring the object 24 hr after treatment indicating that interacting with the novel object was rewarding (Doremus-Fitzwater et al., 2009). However, no changes in object exploration were observed long-term and consequently failed to compliment the sucrose preference findings. Brain reward pathways, such as the nucleus accumbens (NAc) and its dopaminergic input from the ventral tegmental area (VTA), mediate responses to natural rewards (Hajnal and Norgren, 2001; Kelley and Berridge, 2002;
Wallace et al., 2008). Ingesting sweet solutions and exploring novel objects activate this circuit (Berlyne, 1955; Kelley and Berridge, 2002; Klebaur and Bardo, 1999) and disruption of this neural projection decreases interest for sucrose and novelty (Hajnal and Norgren, 2001; Shimura et al., 2002; Wise, 2004; Wise, 2006). As in the present study, research assessing the effects of antidepressant treatment on reward-related behavior reveals a complex picture. Antidepressants can decrease (Katz and Carroll, 1977; Lee and Kornetsky, 1998), increase (Konkle and Bielajew, 1999; Redgrave and Horrell, 1976), or have no effects (Matthews et al., 1996) on responding for rewarding brain stimulation, with equivocal results when assessing responding for natural rewards (Popa et al., 2008; Qi et al., 2008). Nevertheless, antidepressants do sensitize brain reward pathways (Collu et al., 1997; Collu et al., 1996; Subhan et al., 2000): they increase the firing activity of VTA dopamine neurons (Sekine et al., 2007), increase dopamine neurotransmission in the striatum (Bolaños et al., 2002; Bolaños et al., 2000; Serra et al., 1992), and enhance cocaine and morphine reward (Deslandes et al., 2002; Nomikos et al., 1991). Therefore, it is conceivable that FLX exposure during adolescence enhances reward processes that are likely discernable only in adulthood, however more detailed studies assessing this notion are needed.

Our findings further indicate that FLX enhances reactivity to anxiogenic stimuli as measured in the elevated plus-maze 24 hr after treatment in adolescent rats. This anxiety-like response was long-lived because the FLX-treated adolescent rats tested in adulthood showed similar anxiety-like responding. We also used latency to approach a novel object in a familiar environment and novelty-induced hypophagia as additional indexes of anxiety-like behaviors. When exposed to novel environments, rats face a conflict between their motivation to explore the environment (novelty preference) and fear of potential negative consequences (Kabbaj et al., 2000; Ohl, 2003). Thus, longer latencies to approach a novel object or to start feeding have been interpreted as indicative of higher levels of anxiety (Ansorge et al., 2004). Similar to the elevated plus-maze findings, FLX-exposed rats took longer to approach a novel object in a familiar environment and to start feeding in a novel environment at both short- and long-term testing time points. Because familiarity of environment increases novelty seeking, and the FLX-treated rats had longer latencies to approach the novel object in a familiar environment, it is conceivable that FLX-exposure during adolescence induces ‘trait’ and not situational anxiety (Belzung et al., 2001a; Ohl, 2003); however, an alternative explanation could be that they have increased caution and less impulsivity (Hyman, 2003). These results are supported by reports indicating that administration of SSRIs early in life results in long-lasting anxiogenic phenotypes (Ansorge et al., 2008; Ansorge et al., 2004; Norcross et al., 2008). We also show that chronic, but not acute, re-exposure (i.e., 5 days) to FLX in adulthood alleviates the FLX-induced anxiety-like behavior observed in the novelty-induced hypophagia test, findings consistent with previous reports (Karpova et al., 2009). Furthermore, these findings are supported by studies showing that initial exposure to antidepressants, which have been used successfully for the management of anxiety disorders, exacerbate anxiogenic-like behaviors in humans (den Boer et al., 1987; Nutt et al., 1990; Papp et al., 1998) and animals (Bagdy et al., 2001; Belzung et al., 2001b; Drapier et al., 2007), but these alterations dissipate after prolonged exposure (Silva and Brandao, 2000; To et al., 1999). Under the appropriate conditions, behavioral reactivity in the open field can also be used as an index of anxiety (Britton and Britton, 1981), thus it must be noted that the overall activity observed in the open field (Fig. 2.10) does not complement our findings of increased anxiety-like behaviors. Nevertheless, reports show that emotionality-related behavior from the
open field and the elevated plus-maze do not produce a common anxiety-related factor in adolescent rats (McCormick et al., 2009), indicating that emotionality is multidimensional, and that these tests do not always complement each other (Archer, 1973; Ramos, 2008; Ramos et al., 1998; Ramos and Mormede, 1998).

FLX-treated rats showed lower levels of behavioral despair when exposed to forced swimming. Rats tested 24 hr after treatment showed coping patterns commonly categorized as antidepressant-like behaviors (Porsolt et al., 1977), and this effect was also present in the long-term group (i.e., those treated during adolescence, and tested in adulthood). These findings were not due to FLX-induced changes in motor activity because rats tested 24 hr after day 1 of forced swimming showed no differences in distance traveled in the open field (Fig. 2.10c-d). An antidepressant-like phenotype after adolescent FLX counters reports showing that early-life (PD4-21) FLX administration renders mice vulnerable to stressful situations in adulthood (Ansorge et al., 2008; Ansorge et al., 2004; Popa et al., 2008). However, other studies using similar age and treatment regimen in mice also find equivocal results (de Jong et al., 2006; Hefner and Holmes, 2007; Karpova et al., 2009; Norcross et al., 2008; Oh et al., 2009). To determine if these effects were specific to age of FLX exposure, we treated adult rats and exposed them to forced swimming, 24 hr or 21 days after the last injection (i.e., matched drug regimen and testing time as the adolescents). Only those adult rats tested 24 hr after treatment displayed reduced behavioral despair in the forced swim test, while the long-term group did not differ from controls. Our results suggest that the FLX-induced effects in the forced swim test may be specific to adolescent FLX treatment, and this assumption is supported by studies demonstrating that altered behavioral profiles induced by antidepressants are dependent on age of exposure (Ansorge et al., 2008; Karpova et al., 2009; Popa et al., 2008). The mechanism(s) underlying these effects are unknown. In adults, antidepressants regulate complex cellular and intracellular signaling mechanisms such as brain derived neurotrophic factor (BDNF), extracellular signal-regulated kinase 1/2 (ERK), and cAMP response element binding protein (CREB) activity, factors associated with the regulation of mood and motivation resulting in lasting synaptic changes influencing behavioral functioning (Bolaños and Nestler, 2004; Duman and Monteggia, 2006; Krishnan and Nestler, 2008). FLX actions in the nervous system are complex, and more detailed assessments of these phenomena, accounting for length of exposure and discontinuation, and developmental periods are clearly needed (Adriani and Laviola, 2004; Carlezon and Konradi, 2004).

Lastly, we assessed whether FLX exposure during adolescence influences sexual behavior later in adulthood. FLX-exposed rats showed increased latencies to mount and ejaculate, and deficits in ejaculation frequency. Antidepressant treatments interfere with sexual functioning in both humans and rodents (Cantor et al., 1999; Ferguson, 2001; Sukoff Rizzo et al., 2008); however, these findings were unexpected, as the drug washout period for this particular group of animals was over 30 days, and the behavioral deficits were observed at both PD80 and PD90 sessions. The mechanism(s) underlying these effects are also unknown. Serotonin interacts in a complex manner with several of its receptors to inhibit various aspects of sexual and ejaculatory functioning (Sukoff Rizzo et al., 2009). Therefore, it is conceivable that early-life FLX induces long-lasting changes in receptors (e.g., increased sensitivity and/or density) known to inhibit sexual behavior (Li et al., 1996). Alternatively, it is possible that sustained FLX exposure dysregulates second messenger systems since others have shown that altered CREB
activity within the NAc of adult rats leads to impairments in the initiation of sexual behavior, but not the rewarding aspects of sex, in addition to increases in anxiety-like behavior (Barrot et al., 2002; Barrot et al., 2005; Wallace et al., 2009). These findings parallel our results after adolescent FLX exposure: longer latencies to initiate sexual activity and increased sensitivity to anxiety-inducing situations in adulthood. Unfortunately, our results cannot discern whether the “appetitive” aspects of sexual behavior were influenced by FLX because the dependent variables assessed do not differentiate between “interest” and “performance.” Nevertheless, it is unlikely that the longer latencies to initiate sexual activity were due to a reduced reward valence, because FLX-treated rats initiated sexual behavior no differently than controls on the PD90 session, thus indicating that this impairment is likely due to increased anxiety and not reduced reward sensitivity (Barrot et al., 2002; Barrot et al., 2005; Wallace et al., 2009). In fact, the NAc has recently been found to exert a significant influence on anxiety-related behaviors in both rodents and humans (Barrot et al., 2005; Sturm et al., 2003; Wallace et al., 2009).

Results from our study indicate that treatment with FLX during adolescence can influence responsiveness to rewarding and aversive stimuli in adulthood. These complex functional outputs are likely regulated by many factors, including the emotional valence of the stimulus, the environment in which it is experienced, and the brain circuitry likely being engaged by it. Our findings also demonstrate that FLX-induced anxiety-like behavior can be alleviated by re-exposure to FLX itself. However, it is imperative to note that the FLX-induced effects described in this study were derived from “normal” animals, and similar FLX treatment using established animal models for depression might yield different results. Given that our subjects were purchased, it is impossible to determine if and/or how stress of shipping may have influenced our results. Another caveat is that we did not include female subjects in our study, further limiting the interpretability of our results. Indeed, the results from this study should be interpreted with caution because FLX remains a safe and effective treatment for pediatric MDD.
Figure 2.1. Acute effects of fluoxetine on forced swimming behaviors in adolescent (PD35; n= 8-9/dose) and adult (PD70+; n= 8-10/dose) male rats. (a) Latency to become immobile, (b) total immobility, (c) swimming-, (d) climbing- and (e) floating-counts of rats tested on the forced swim test after three injections (same dose) of fluoxetine (0, 2.5, 5, 10, or 20 mg/kg) 1, 5, and 23 hrs between swims. Data were analyzed using individual one-way ANOVAs between the age groups. *Significantly different from VEH controls within the same age group (p<0.05).
Figure 2.2. Timeline of developmental experimental procedures. All rats arrived in the laboratory on postnatal day (PD) 30. Rats were randomly assigned to receive fluoxetine (FLX, 10 mg/kg, bi-daily) from (a) PD35-49 (adolescence) or from (b) PD65-79 (adulthood). All rats received intraperitoneal injections of FLX (10 mg/kg) or VEH bi-daily 4 hr apart (900 and 1300 hr, respectively). Behavioral testing of rats treated during adolescence (a) was conducted either 24 hr after the last injection (PD50; Short-term) or when they reached adulthood (PD70; Long-term). Rats receiving FLX in adulthood (b) were tested either 24 hr (PD80; Short-term) or 21 days (PD100; Long-term) after the last injection.
Figure 2.3. Effects of repeated (15 days) exposure to fluoxetine (FLX; 10 mg/kg, bi-daily) on weight gain. Data were analyzed by mixed-design (within: day of injection, between: FLX) repeated measures ANOVA followed by post hoc test. (a) Adolescents (n= 18/group): body weight increased across days regardless of condition, and FLX treatment resulted in significantly lower weight gain, starting on day 5 of drug exposure, when compared to control rats. (b) Adults (n= 7-8/group): similar pattern of results was obtained from the adult rats treated with FLX, resulting in lower weight gain starting on day 3 of drug exposure as compared to controls. *Significantly different when compared to VEH-treated controls (p<0.05). Data are presented as average weight gain across days and drug treatment (mean + SEM, in grams).
Figure 2.4. Fluoxetine (FLX; 10 mg/kg, bi-daily) exposure during adolescence regulates responses to sucrose reward. (a) Exposure to FLX significantly increased sucrose preference when compared with vehicle (VEH)-treated controls (at the 0.25% concentration) 24 hr after treatment ($p<0.05$; $n=13$/group). (c) Rats treated with FLX during adolescence and tested in adulthood (Long-term) show a significant increase in sensitivity to the rewarding effects of sucrose ($p<0.05$; $n=15$/group). No differences in total fluid intake (sucrose + water) were detected regardless of treatment or time of testing (b and d). *Significantly different than VEH-treated controls ($p<0.05$). Data are presented as percent preference or total mL consumed between VEH- and FLX-exposed rats (mean ± SEM).
Figure 2.5. Fluoxetine (FLX; 10 mg/kg, bi-daily) exposure during adolescence regulates anxiety-like behavior in the elevated plus-maze. Short-term (n= 8/group): FLX significantly reduced time spent (a, left panel) and entries (b, left panel) into the open arms of the elevated plus-maze 24 hr after the last FLX injection (p<0.05). Long-term (n= 8/group): FLX also reduced time spent (a, right panel) in the open arms of the maze, without influencing entries (b, right panel) as compared to VEH-treated controls. Data are presented as percent time spent and percent entries (mean + SEM) into the open arms.
Figure 2.6. Effects of fluoxetine (FLX; 10 mg/kg, bi-daily) exposure during adolescence on the latency to approach a novel object in a familiar environment (a-d), and novelty induced hypophagia (e-f). Short-term (n= 9-10/group): FLX-treated rats had significantly longer latencies to approach (a), and spent significantly more time exploring (b) the novel object 24 hr after the last FLX injection. Long-term (n= 14-15/group): FLX-treated rats displayed significantly longer latencies to approach (c), but spent similar time exploring (d) the novel object as compared to controls. (e) FLX increased latency to feed in a novel environment at both, short- (n= 9/group) and long-term (n= 6/group), time points of behavioral assessment. (f) Acute exposure to FLX (10 mg/kg) did not decrease latency to feed (f, left panel; n= 6/group), while chronic exposure (5 days) to FLX (10 mg/kg) reversed this effect to control levels (f, right panel; n= 6/group), in a separate group of adult rats pretreated with FLX during adolescence. *Significantly different as compared to VEH-treated controls (p<0.05).
Figure 2.7. Effects of fluoxetine (FLX; 10 mg/kg, bi-daily) on behavioral responsivity to forced swim stress. Short-term (n= 5-6/group): FLX-treated rats displayed significantly longer latencies to immobility (a), lower total immobility (b), higher swimming-, climbing-, and lower floating-counts (c) when compared to VEH-treated controls. Long-term (n= 15/group): FLX-treated rats displayed similar behavioral profile (d-f) as those tested in the short-term condition when compared to their VEH-treated controls. *Significantly different than VEH-treated rats (p<0.05). Data are presented as latencies to become immobile and total immobility (in seconds), and as cumulative 5-sec intervals of swimming, climbing and floating counts (mean + SEM).
Figure 2.8. Effects of fluoxetine (FLX; 10 mg/kg, bi-daily) treatment in adult rats (matched control group) on behavioral responsivity to forced swim stress. Short-term (n= 7/group): FLX-treated rats displayed significantly longer latencies to immobility (a), lower total immobility (b), higher swimming-, climbing-, and lower floating-counts (c) when compared to VEH-treated controls. Long-term (n= 7/group): no differences were observed in any of the measures assessed between the groups (d-f). *Significantly different than VEH-treated rats (p<0.05). Data are presented as latencies to become immobile and total immobility (in seconds), and as cumulative 5-sec intervals of swimming, climbing and floating counts (mean + SEM).
Figure 2.9. Effects of adolescent fluoxetine (FLX; 10 mg/kg, bi-daily) exposure in adult male rat sexual behavior (n= 10/group). Rats were given two 90-min sessions (at Postnatal day [PD] 80 and 90 respectively) to copulate with a receptive female. FLX treatment during adolescence increased the latency to mount an estrous receptive female (a), latency to reach the first ejaculation (b), and the total number of ejaculations (c), as compared to VEH-treated controls, in the first sex session (PD80). During the second sex session (PD90), FLX treatment during adolescence increased latency to ejaculate (b, right panel) and decreased ejaculation frequency (c, right panel), without affecting latency to mount (a, right panel). *p<0.05.
Figure 2.10. Exposure to fluoxetine (FLX; 10 mg/kg, bi-daily) during adolescence did not affect total basal locomotor activity in the open field in rats tested either 24 hr (a: Short-term; n=10/group) after the last injection, or when tested in adulthood (b: Long-term; n=14-15/group). Similarly, FLX treatment did not affect locomotor activity 24 hr after day 1 (c and d) of forced swimming (n=6/group). FST, forced swim test.
Table 2.1. Rat experimental/treatment groups. Adolescent rats received fluoxetine (FLX, 10 mg/kg) or vehicle (VEH) injections (bi-daily; intraperitoneal) from PD35-49. Rats were tested in no more than 2 behavioral paradigms in the order of testing and time interval between tests as depicted in the Table. Rats were tested either 24 hr (Short-term; PD50) or 20+ days after the last injection (Long-term; PD70+). Rats assigned to sex behavior were tested at PD80 and PD90, respectively. Rats in groups 8 and 9 were re-exposed to FLX as adults (>PD60): those in the acute condition (group 8) received a single injection of FLX or VEH at PD69, whereas those in the chronic condition (group 9) received a single injection of FLX or VEH for 5 consecutive days (PD65-69). Novelty induced hypophagia started at PD70.

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CHAPTER THREE

EXPOSURE TO FLUOXETINE DURING ADOLESCENCE RESULTS IN DECREASED ERK-SIGNALING WITHIN THE VENTRAL TEGMENTAL AREA

Adapted from:


INTRODUCTION

Major depressive disorder (MDD) affects up to 8% of youths below 20 years of age (Kapornai and Vetro, 2008), often resulting in enduring negative consequences (Birmaher et al., 1996b). Currently, the selective serotonin reuptake inhibitor (SSRI) Prozac (Fluoxetine hydrochloride; FLX) is the only USFDA approved medication for the treatment of pediatric MDD. This has led to a significant increase in the prescription rate of FLX in this population (Zito et al., 2006), despite that the majority of testing has been done in adults (Olivier et al., 2010). This is surprising given the pivotal role that the serotonin system plays in the regulation of brain development (Azmitia, 2007; Blakemore and Choudhury, 2006b), that pharmacodynamic differences between adult and developing organisms are consistently reported (Correll et al., 2011; Neville et al., 2011), and that such pharmacological treatments result in enduring neurobiological alterations in adulthood (Bolaños et al., 2003a; Brandon et al., 2003; Halladay et al., 2009; Iñiguez et al., 2009; Oberlander et al., 2009).

The long-lasting consequences of FLX exposure during adolescence have recently begun to be elucidated. Juvenile FLX treatment enhances sensitivity to anxiety-eliciting environments, while decreasing responsiveness to inescapable stressful conditions in adulthood (Iñiguez et al., 2010b; Karpova et al., 2009; Warren et al., 2011). The neurobiological mechanisms underlying these enduring paradoxical effects are unknown. Nevertheless, the antidepressant effects of FLX have been linked to adaptations in post-receptor signaling since chronic, not acute, administration is required for its therapeutic effects (Racagni and Popoli, 2008). For example, brain derived neurotrophic factor (BDNF) is highly implicated in the pathophysiology of MDD, as its expression and downstream signaling are compromised in animal models of stress as well as in MDD patients, whereas traditional antidepressant treatments normalize these biochemical alterations (Duman, 2004; Kalueff et al., 2006). The extracellular signal-regulated protein kinase-1/2 (ERK), a signaling substrate of BDNF (Numakawa et al., 2010), has recently been implicated in mediating the deleterious effects of stress (Einat et al., 2003; Gourley et al., 2008b; Iñiguez et al., 2010a), a major predisposing factor for MDD (de Kloet et al., 2005). ERK is activated by the binding of BDNF to tyrosine kinase receptor-B via the Ras-dependent cascade, inducing phosphorylation of transcription factors, such as the cAMP response element binding-
protein (CREB) (Mazzucchelli and Brambilla, 2000). Therefore, it is conceivable that the behavioral adaptations observed after juvenile treatment may be mediated by FLX-induced changes in ERK signaling.

Given the prevalence of antidepressant prescriptions to adolescents and the scarcity of knowledge regarding long-term effects of such treatments, it is essential that the neurobiological consequences associated with FLX exposure be characterized. Thus, this study was designed to assess the impact of FLX exposure during adolescence on behavioral responses to emotional related stimuli and to assess whether this drug treatment results in lasting changes of ERK signaling within the ventral tegmental area (VTA), a neural substrate implicated in the regulation of mood-related behaviors (Eisch et al., 2003; Iñiguez et al., 2010a; Nestler and Carlezon, 2006).

METHODS

Animals. For all experiments, animals were male, fed ad libitum, and housed at 23-25°C on a 12 hr light/dark cycle (lights on between 07:00 and 19:00 hr). Adolescent (postnatal day [PD] 35) c57BL/6 male mice and Sprague-Dawley rats were obtained from the breeding colony at Florida State University. The age at the start and duration of the experimental manipulation in both mice and rats (PD35-49) was selected because it roughly approximates mid-adolescence in humans (Abreu-Villaca et al., 2010), a developmental stage in which first onset of MDD is most often reported (Burke et al., 1990). Rats (two per cage), c57BL/6 mice (four per cage), and CD1 retired breeders (1 mouse per cage; Charles River) were housed in clear polypropylene boxes containing wood shavings. Experiments were conducted in compliance with the 1996 National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval of the Institutional Animal Care and Use Committee at Florida State University.

Experimental design. Fluoxetine hydrochloride (FLX) was obtained from Sigma-Aldrich (St. Louis, MO), dissolved in sterile distilled water (VEH), and administered in a volume of 2 mL/kg. The different experimental groups utilized in this study are listed in Table 3.1. Separate groups of adolescent mice were treated with VEH or FLX (10 mg/kg, twice daily) 4 hr apart for 15 consecutive days (PD35-49; see Fig. 2.2a), in order to assess how this treatment would influence behavioral responses to mood- (i.e., social defeat, forced swimming), anxiety- (i.e., elevated plus maze), and memory- (i.e., object recognition) related stimuli in adulthood (PD70+). To examine how FLX treatment during adolescence influenced ERK expression within the VTA, separate groups of mice and rats were treated under the same drug regimen, and killed 24 hr or 3 weeks after drug exposure. This drug regimen (10 mg/kg, bi-daily) was selected because the typical dose of FLX in humans is around 20-80 mg/day (Koran et al., 1996; Mandrioli et al., 2006; Wood et al., 1993), and this dose yields significant effects on behavior and gene expression in rodents (Englander et al., 2005; Hodes et al., 2010; Iñiguez et al., 2010b; LaPlant et al., 2010; Vialou et al., 2010). To assess how stress alone influenced ERK-signaling within the VTA, a separate group of adolescent mice was treated with VEH (PD35-49), exposed to ten days of social defeat stress in adulthood (PD70-79), and killed at PD80. Also, to more directly assess the role of ERK signaling in mediating responses to mood-related behaviors (i.e., forced swim test and social defeat), we selectively modulated ERK activity within the VTA of rodents by microinfusing a pharmacological inhibitor of ERK (U0126, Sigma-Aldrich) or a herpes virus vector (HSV). The vectors used in this investigation encoded green fluorescent protein (HSV-
GFP, control), a dominant negative mutant of ERK2 (HSV-dnERK2), or a wild type (HSV-wtERK2) form of ERK2 (see virus vectors section below). Specifically, we assessed whether increasing ERK activity would reverse the stress-resistant phenotype induced by FLX exposure, by infusing HSV-wtERK2 into the VTA of adult c57BL/6 mice pretreated with FLX during adolescence, and assessed their behavioral reactivity in a submaximal social defeat procedure (see reversal experiments section for details). This submaximal defeat procedure is commonly used to assess enhanced sensitivity to stress (Iñiguez et al., 2010a; Krishnan et al., 2008). Lastly, to characterize the ontogenic role of ERK in the mediation of behavioral responses to aversive stimuli within the adolescent naïve VTA (i.e., prior to FLX treatment), separate groups of PD47 rats were microinjected with the different HSV vectors, and tested on the forced swim test or elevated plus-maze three days after virus infusion. The age of the rats microinjected with HSV vectors was selected in order to manipulate ERK expression at PD50, in a similar fashion as in animals undergoing chronic FLX treatment. All behaviors were video recorded and scored by video tracking software (Noldus), except for the forced swim- and tail suspension-tests, which were scored by observers blind to the treatment conditions of each subject.

**Social defeat stress and social interaction test.** Social defeat was performed as previously described (Iñiguez et al., 2010a; Krishnan et al., 2008). CD1 mice were screened for aggressive behavior after intrusion into their home cages. Those mice exhibiting aggression were used to defeat the experimental c57BL/6 mice. Specifically, adult (PD70) c57BL/6 mice pretreated during adolescence (PD35-49) with VEH or FLX were exposed to a 10-min long defeat episode and then housed for the remainder of the day with the aggressor, but separated by a Plexiglas screen (Florida State University Psychology Department Engineering Group). This process was repeated daily for 10 consecutive days (PD70-79) with a different CD1 aggressor each day (see Fig. 3.1a for schematic of experiment). Social avoidance behavior toward an unfamiliar CD1 mouse was assessed the following day (day 11; PD80) in a two-stage social interaction test. In the first 2.5 min trial (target absent), the c57BL/6 mouse exposed to FLX during adolescence was allowed to freely explore a square-shaped open-field arena (42 X 42 cm) containing a wire-mesh cage (10 X 6 cm) apposed to one side (see Figure 3.1b). During the second 2.5 min trial (target-present), the c57BL/6 mouse was reintroduced into this arena now containing an unfamiliar CD1 mouse within the cage. The time spent in the interaction zone (14 X 26 cm) and the corner zones (10 X 10 cm) were the dependent variables.

**Forced swim test.** The forced swim test is a behavioral procedure in which rodents are forced to swim under inescapable conditions. Initially, rodents engage in escape-like behaviors but eventually adopt a posture in which they make only the movements necessary to maintain their head/nose above water; however, antidepressant treatment can significantly increase their escape-directed behaviors, an effect that has been correlated with antidepressant efficacy in humans (Porsolt et al., 1987). In mice, this task was carried out according to published protocols (Vialou et al., 2010). Briefly, mice were forced to swim once in a 4 L Pyrex glass beaker containing 3 L of water (24±1°C) for 6-min. For rats, the forced swim test is a two-day procedure. Here, rats were placed in plastic cylinders (75 X 30 cm) filled to 54 cm depth with 24±1°C water and forced to swim for 15 min. At the end of this period, rats were removed from the water, dried with towels, and placed in a warmed enclosure for 30 min, then returned to their home cage. All cylinders were emptied and cleaned between rats. Twenty-four hr later (PD50), rats were retested for 5 min under identical conditions. For both mouse- and rat- forced
swimming sessions, latency to become immobile (sec) and total immobility (sec), were the dependent variables. Latency to immobility was defined as the time at which the animal first initiated a stationary posture that did not reflect attempts to escape from the water. To qualify as immobility, this posture had to be clearly visible and maintained for ≥2.0 sec (Detke and Lucki, 1996; Iñiguez et al., 2009).

**Elevated plus-maze.** The elevated plus-maze is a classic test of anxiety-like behavior (Montgomery, 1955). The mazes were made of gray plastic and consisted of two perpendicular, intersecting runways (12 cm wide X 100 cm long for rats, and 7 cm wide X 30 cm long for mice). One runway had tall walls or “closed arms” (30 cm for rats, and 25 cm for mice), and the other one had no walls or “open arms.” The arms were connected together by a central area, and the maze was elevated (1 m for rats; 55 cm for mice) from the floor. At the beginning of the test, under controlled light conditions (~90 lux), rodents were placed in the central area, facing one of the open arms, and the cumulative time spent in the closed- and open-arms was recorded (Iñiguez et al., 2009; Warren et al., 2011). We also recorded the number of fecal boli produced by rats as an additional index of anxiety (Ghisleni et al., 2008).

**Novel object recognition test.** Because ERK is highly implicated in learning and memory mechanisms (Adams and Sweatt, 2002; Iñiguez et al., 2011), we assessed object memory recognition in separate groups of adult (PD70) c57BL/6 mice pretreated with VEH or FLX during adolescence (PD35-49). Prior to testing, mice were handled for five days (5 min each day; PD63-67). Mice were also habituated to the testing apparatus (42 x 42 cm white open field box) for three days (PD67-69), in the absence of any object, for 3 min each day. No data were collected during habituation. This protocol was adopted in order to reduce levels of stress/anxiety prior to testing. In the training trial (24 hr later; PD70), animals were re-habituated to the testing box for 1 min, and then placed in a holding cage while two identical objects (clear plastic cups; 9 cm in diameter, 4 cm in height) were placed in the left and right corners (~1.5 cm from the wall) of the box. Mice were immediately placed back into the testing box and allowed to freely investigate the identical objects until they accumulated 30 sec exploring the objects. If a subject did not reach 30 sec of cumulative exploration time within 7 min, they were excluded from the experiment. Twenty-four hr later (PD71), for object recognition memory, one copy of the familiar object (clear plastic cup) and a new object (yellow plastic square; 9 cm in length, 4.5 cm in height) were placed in the same location as in the training trial. The location of the novel object was counterbalanced (left vs. right) across mice to control for possible side-preference bias. Dependent variables recorded were the latency (sec) to approach, as well as the total time (sec) spent with the novel object during the 5 min object recognition test.

**Quantitative real-time reverse transcription-PCR (qPCR).** To assess the effects of adolescent FLX administration on VTA-ERK gene expression, separate groups of mice and rats were killed either 24 hr (PD50; Short-term) or three weeks post last FLX injection (PD70; Long-term). The brains were rapidly removed, VTA punches (1 mm diameter for mice, and 1.25 mm for rats) were collected, and samples were immediately placed on dry ice and stored at -80°C until assayed. Total RNA was isolated using RNeasy Micro kits (Qiagen) and cDNA was created from these samples using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qPCRs were performed in triplicate using 96 well PCR plates and RealMasterMix (Eppendorf) with an Eppendorf MasterCycler Realplex² according to manufacturer’s instructions. Threshold cycle
(Ct) values were measured using the supplied software and analyzed with the ΔΔCt method (Warren et al., 2011). Primer sequences for ERK1, ERK2, CREB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are presented in Table 3.2.

**Western blotting.** Tissue punches of VTA (1.25 mm diameter) from adult rats (PD70) pretreated with FLX during adolescence (PD35-49) were collected and immediately placed on dry ice and stored at -80°C until assayed. Samples were then sonicated in a standard lysis buffer and then centrifuged at 14,000 rpm for 15 min. Samples (20 µg; estimated via Bradford assay) were treated with β-mercaptoethanol and subsequently electrophoresed on precast 4-20% gradient gels (Bio-Rad), as previously described (Iñiguez et al., 2011). Proteins were transferred to a polyvinylidene fluoride membrane, washed in 1X Tris-buffered saline with 0.1% Tween 20 (TBST), and blocked in milk dissolved in TBST (5% w/v) for 1 hr at 25°C. Blots were probed (overnight at 4°C) with antibodies against the phosphorylated forms of ERK1/2, ribosomal S6 kinase of 90 kDa (RSK), mitogen- and stress-activated protein kinase-1 (MSK), CREB, and mammalian target of rapamycin (mTOR), stripped with Restore (Pierce Biotechnology), and reprobed with antibodies against total ERK1/2, CREB, mTOR, tyrosine hydroxylase (TH), and GAPDH. All antibodies were from Cell Signaling and were used according to the manufacturer’s instructions in 5% milk dissolved in TBST. After further washes, membranes were incubated with peroxidase-labeled goat anti-rabbit IgG or horse anti-mouse IgG (1:40,000; Vector Laboratories). Bands were visualized with SuperSignal West Dura substrate (Pierce Biotechnology), quantified using NIH ImageJ, and normalized to GAPDH.

**Animal surgery.** To establish a causal relationship between decreased levels of VTA-ERK and antidepressant efficacy (as in FLX treatment), separate groups of c57BL/6 mice received an infusion of the ERK inhibitor U0126 into the VTA. Specifically, adult mice were placed under a combination of ketamine (100 mg/kg) and xylazin (10 mg/kg) anesthesia prior to implantation of a 26-gauge unilateral guide cannulae (side counterbalanced across animals) fitted with obturators (Plastics One, Roanoke, VA) that were secured to the skull after being positioned 1 mm directly above the VTA (anteroposterior, -3.2; mediolateral, 0.4; dorsoventral, -3.6 mm). After 7-8 days of post-operative recovery, microinjections of U0126 or dimethyl sulfoxide (DMSO; control) were delivered through an injector cannula in a total volume of 0.5 µL at a continuous rate of 0.1 µL/min. Concentration of U0126 (2 µg dissolved in 2 µL DMSO) was selected based on earlier in vivo studies (Huang and Lin, 2006). After the delivery of the ERK inhibitor, the infusion needle remained in the guide cannula for 1 additional min, to ensure proper delivery (see Fig. 3.5c). Twenty-four hr after behavioral experiments, 0.5 µg of 4% methylene blue in saline was infused as described above, and animals were then killed 1 hr later and their brains extracted and stored in Formalin for histological localization of infusion sites. For the reversal study in mice (see reversal experiments section below), adult c57BL/6 mice pretreated with FLX during adolescence were anesthetized with ketamine (100 mg/kg) and xylazin (10 mg/kg), and bilaterally infused 0.5 µL of either HSV-GFP or HSV-wtERK2 over 5-min into their VTA. We used established mouse VTA coordinates (anteroposterior: -3.2 mm, lateral: +1.0 mm, dorsoventral: -4.6 mm, 7° angle) (Krishnan et al., 2008). For stereotaxic delivery of viruses in adolescent rats, PD47 rats were anesthetized with a cocktail of ketamine (90 mg/kg) and xylazin (10 mg/kg; intramuscular) and given atropine (0.25 mg/kg; subcutaneously) to minimize bronchial secretions. Rats received bilateral microinjections (1 µL per side over 10 min of either HSV-GFP, HSV-wtERK2, or HSV-dnERK2) into the rostral
region of the VTA (anteroposterior: -4.9, lateral: +2.2, dorsoventral: -7.6 mm below dura, 10° angle) (Iñiguez et al., 2010c). The local anesthetic bupivacaine was applied directly along the wound edges to minimize potential post-operative discomfort.

**Virus vectors.** We used HSV vectors to directly increase (HSV-wtERK2) or decrease (HSV-dnERK2) ERK signaling within the VTA and assess responses to stress- and anxiety-inducing situations. The construction of the vectors (HSV-GFP, HSV-wtERK2, and HSV-dnERK2) has been thoroughly described (Neve et al., 1997; Robinson et al., 1996), and the HSV-ERK2 viruses have been previously validated in vivo and in vitro (Iñiguez et al., 2010c; Krishnan et al., 2007; Russo et al., 2007; Warren et al., 2011). All behavioral experiments were commenced on day 3 after viral surgery, a time at which maximal transgene expression is observed (Barrot et al., 2002; Carlezon et al., 1998). Expression of the HSV-encoded transgenes was limited to an area of ~1 mm³ around the injection site (Russo et al., 2007). The VTA injection sites were confirmed in all animals.

**Histology and transgene detection.** Immediately after behavioral testing, HSV-treated animals were given an overdose of pentobarbital and perfused transcardially with 0.9% saline, followed by cold 4% paraformaldehyde. The brains were removed, postfixed overnight in 4% paraformaldehyde and stored in 20% glycerol. Coronal sections (rat: 45 µm, mice: 30 µm) through the midbrain were taken on a microtome and stored in 0.1 M sodium phosphate buffer with 0.05% azide. Sections were processed to examine the ability of HSV construct to drive expression of GFP and TH within the VTA as previously described (Russo et al., 2007). Midbrain free-floating coronal sections were processed for immunohistochemistry using rabbit anti-GFP (1:1000; Abcam, Cambridge, Massachusetts), and TH (mouse; 1:5000, Chemicon, Temecula, CA), a marker of VTA dopamine neurons. Adjacent sections were blocked in 3% normal donkey serum (NDS) and incubated overnight in one of the primary antibodies mentioned above, along with 0.3% Triton X-100 (Fisher Scientific, Pittsburgh, PA) and 1% NDS. Sections were incubated with anti-rabbit or -mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 2 hr at room temperature. Stained sections were then slide mounted (Fisher Scientific), dehydrated in ethanol and citrosolv, and coverslipped with clear DPX adhesive (Sigma-Aldrich). Slides were then visualized and photographed using a fluorescence microscope and a digital camera. Data obtained from animals with placements outside the intended brain regions (<10% of all experimental animals) were not included in the analyses.

**Reversal experiments.** To directly assess the functional significance of decreased ERK activity as mediator of the FLX-induced stress-resistant behavioral phenotype we conducted two separate experiments in c57BL/6 mice pre-exposed to VEH or FLX during adolescence (PD35-49) and infused with HSV-wtERK2 in adulthood. In the first experiment, the FLX-pretreated c57BL/6 mice were infused with HSV-wtERK2 and exposed to the submaximal social defeat test (see Fig. 3.5d for schematic). In this test, mice were subjected to three defeat episodes over 1 day (PD69), as described above. The c57BL/6 mice were placed into the CD1 aggressor’s cage for 5 min, followed by a 15 min break (in their home cage). Each defeat episode was done with different aggressors. This “submaximal” social defeat stress procedure has been successfully used to assess whether drug exposure, or genetic manipulation (i.e., virus vectors) results in enhanced susceptibility to stress (Iñiguez et al., 2010a; Krishnan et al., 2008). Social interaction
was assessed 24 hr later (see social defeat and social interaction test section above). In a second experiment, using the tail suspension test (Steru et al., 1985), a separate group of (PD67) c57BL/6 mice pretreated with VEH during adolescence received HSV-wtERK2 microinjections into their VTA. Three days later (PD70), the mice were suspended by their tail using adhesive tape (attached ~2 cm from the tip of the tail) secured to the edge of a table in a quiet room. The duration of immobility during the 6 min test was the dependent variable. If mice climbed up their tail, they were gently returned to the hanging position; animals that climbed up the adhesive tape more than two times were excluded from the experiment.

Statistical analyses. Assignment of subjects to the various drug, behavioral testing, and virus conditions was random. Behavioral data were analyzed using one-way ANOVAs, followed by Tukey post hoc test. A t-test was used for all other analyses implicating only a two-groups comparison. Data are expressed as the mean ± SEM. In all cases, statistical significance was defined as p<0.05.

RESULTS

Exposure to FLX during adolescence regulates responses to stress in adulthood.

Effects of FLX exposure during adolescence on the social defeat procedure in adulthood. Figure 3.1 shows the long-lasting effects of adolescent VEH or FLX exposure on reactivity to social defeat stress in c57BL/6 mice (n= 9/group). Mice were subjected to 10 days (PD70-79) of social defeat (see Figure 3.1a), which induces a robust social avoidance in the majority of animals (Krishnan et al., 2007). Consistently, 24 hr after the last defeat (PD80), the avoidant phenotype was evident in the VEH-treated mice, as they spent significantly less time in the interaction zone (Fig. 3.1c) in the presence of the social target (target absent vs. target present; t<sub>16</sub> = 2.92, p<0.01). On the contrary, mice pretreated with FLX during adolescence, spent as much time in the interaction zone in the absence, as in the presence, of the target (p>0.05), thus suggesting that exposure to FLX during adolescence induced a resilient-like behavioral phenotype in adulthood. Indeed, the FLX pretreated group spent more time in the interaction zone when compared to VEH-pretreated controls in the presence of the social target (t<sub>16</sub> = 2.92, p<0.01; Fig. 3.1c). Assessing time in the corner zones (Fig 3.1d), an additional measure of social avoidance (Krishnan et al., 2007), yielded similar results: VEH-pretreated mice spent significantly more time in the corners in the presence of the target (target absent vs. present; t<sub>16</sub> = 2.57, p<0.01), or when compared to the FLX-pretreated group (t<sub>16</sub> = 2.62, p<0.01).

Effects of FLX exposure during adolescence in the forced swim test in adulthood. The long-lasting effects of adolescent FLX exposure (PD35-49) on behavioral reactivity to forced swimming in PD70 c57BL/6 mice are shown in Figure 3.1e-f. Similar to the results observed in the social defeat procedure, adolescent FLX exposure (n= 9) resulted in an enduring antidepressant-like response in adulthood, as evidenced by a significant increase in the latency to become immobile (t<sub>17</sub> = 3.89, p<0.001; Fig. 3.1e), and an overall decrease in total immobility (t<sub>17</sub> = 2.27, p<0.03; Fig. 3.1f) when compared to VEH-pretreated controls (n= 10). Separate groups of VEH or FLX-pretreated mice (n= 5/group) were tested on spontaneous locomotor activity (PD70) in order assess whether forced swimming could be confounded by drug-induced
Changes in general locomotor activity (Fig. 3.1g). No differences in total distance traveled (cm) were observed between the groups in the 6 min test (p>0.05).

**Effects of FLX exposure during adolescence in the elevated plus-maze in adulthood.** Chronic FLX exposure during adolescence (PD35-49) resulted in a long-lasting increase in sensitivity to anxiogenic stimuli as measured in the elevated plus-maze (see Fig. 3.2; n= 10/group) in adult c57BL/6 mice (PD70). Mice exposed to FLX during adolescence spent more time in the closed arms (t_{18} = 3.09, p<0.006; Fig. 3.2a) and less time in the open arms (t_{18} = 3.53, p<0.002; Fig. 3.2b) of the maze when compared to controls. No differences in total distance traveled during the 5 min test were observed between the groups (p>0.05; Fig. 3.2c).

**Exposure to FLX during adolescence does not influence novel object memory performance in adulthood.** Chronic exposure to FLX during adolescence (PD35-49) did not influence object memory performance in c57BL/6 mice when assessed in adulthood (PD70); see Figure 3.3. Specifically, FLX pre-exposed mice did not take longer to approach the novel object (p>0.05; Fig.3.3a), nor they differ in total amount of time (p>0.05; Fig. 3.3b) exploring the novel object when compared to the VEH-pretreated controls (n= 10-11/group). Furthermore, no differences in total locomotor activity were observed between the groups (p>0.05; Fig. 3.3c).

**Opposing role between FLX and stress in the regulation of ERK within the VTA.**

**Effects of FLX or stress on ERK-related signaling within the VTA.** Figure 3.4a-c shows the effects of chronic FLX administration (PD35-49) on VTA gene expression, 24 hr and three weeks post drug administration, in male c57BL/6 mice (n= 8-9/group). Chronic administration of FLX resulted in a downregulation of ERK2- (t_{14} = 2.72, p<0.01) and CREB- (t_{14} = 2.46, p<0.01) mRNA, 24 hr after the last injection (Short-term; see Fig. 3.4b-c), when compared to VEH-pretreated mice. Three weeks after the last injection (PD70), FLX treatment still induced a decrease of ERK2 mRNA when compared to VEH-pretreated controls (t_{15} = 2.28, p<0.05; Fig. 3.4b). In addition, a strong trend toward decreased CREB mRNA (t_{15} = 1.7, p= 0.056) was observed three weeks after FLX exposure when compared to VEH-pretreated controls (Long-term; Fig. 3.4c). No differences in ERK1 mRNA as a function of FLX treatment were observed at either point of assessment (see Fig. 3.4a).

To determine how stress alone influenced ERK mRNA within the VTA, two separate groups of mice (n= 7/group) were treated with VEH during adolescence, and then exposed to either 10 days of social defeat in adulthood or left undisturbed (stress vs. no stress). Twenty-four hrs after (PD80), mice were killed, and VTA was extracted and processed for qPCR. No differences in ERK1 mRNA (Fig. 3.4d) were detected between defeated and non-defeated mice (p>0.05). Conversely, defeated mice exhibited increased ERK2 mRNA (t_{12}=4.92, p<0.01) when compared to the non-defeated controls (Fig. 3.4e). Together, these findings indicate that stress and FLX modulate ERK mRNA within the VTA in an opposing manner.

**Inhibition of VTA-ERK induces a stress-resistant phenotype.**

**U0126 induces an antidepressant-like effect in the mouse forced swim test.** To further explore whether downregulation of ERK within the VTA results in an antidepressant-like
behavioral phenotype, separate groups of c57BL/6 mice were locally infused with the ERK inhibitor U0126 into the VTA and tested (35 min post infusion) in the forced swim test (see Fig. 3.5a-c). Mice infused with the ERK inhibitor (n= 15) showed longer latency to immobility ($t_{24} = 2.39$, $p<0.05$; Fig. 3.5a), as well as decreased total immobility ($t_{24} = 2.31$, $p<0.05$; Fig. 3.5b) when compared to the DMSO-treated controls (n= 11), thus displaying a classic antidepressant-like effect (Porsolt et al., 1977). The infusion cannula tip locations are depicted in Figure 3.5c.

Virus-mediated overexpression of ERK2 within the VTA reverses the long-lasting FLX-induced stress-resistant phenotype in mice. The effects of viral-mediated overexpression of ERK2 within the VTA of adult c57BL/6 mice exposed to VEH or FLX during adolescence, and tested in the submaximal social defeat procedure, or a tail suspension test are shown in Figure 3.5e-f. Mice overexpressing HSV-wtERK2 (n= 8) spent significantly less time in the interaction zone in the presence of the social target (present vs. absent; $t_{16} = 2.96$, p<0.01) 24 hr after the last defeat. When assessing time spent in the corner zones, a similar avoidance behavioral phenotype was observed: HSV-wtERK2-infused mice spent significantly more time in the corners in the presence, versus the absence of the social target ($t_{16}=2.20$, p<0.05; Fig. 3.5e). To demonstrate that infusions of HSV-wtERK2 alone result in depressive like phenotypes regardless of developmental drug pre-exposure, we microinjected HSV-wtERK2 (n= 9) or HSV-GFP (n= 11) into the VTA of mice treated with VEH during adolescence, and assessed reactivity to behavioral despair in the tail suspension test. Mice receiving HSV-wtERK2 spent significantly longer time immobile when compared to the HSV-GFP-treated mice ($t_{18}= 2.49$, p<0.02), thus demonstrating that increasing ERK2 activity within the VTA results in a depressive-like phenotype.

Functional role of ERK in the mediation of responses to stress in the adolescent male rat.

Thus far, the data indicate that exposure to FLX during adolescence results in behavioral changes indicative of a stress-resistant phenotype accompanied by an enhanced sensitivity to anxiety-inducing situations in adulthood. In addition, our results point to alterations in ERK-signaling as a mediator in the responses to stress and antidepressant efficacy. To more directly examine the functional role of ERK within the adolescent VTA, we assessed how FLX would influence ERK mRNA 24 hr and 3 weeks after drug administration in adolescent male rats. Also, we increased or decreased the expression of ERK signaling using the viral-mediated gene transfer approach in the naïve (i.e., untreated) adolescent rat VTA. We selected to use rats for these experiments for three specific reasons. First, to assess if chronic FLX administration results in a sustained decrease in VTA-ERK expression in a separate, yet complimentary animal species. Second, to complement previous behavioral results in male rats under similar experimental conditions (Iñiguez et al., 2010b). And lastly, because of the larger anatomy of the rat makes it easier to target the VTA.

Effects of FLX exposure during adolescence on ERK mRNA within the VTA of rats.

Separate groups of rats were exposed to either VEH or FLX during adolescence (PD35-49) and killed either 24 hr or 3 weeks after drug exposure (see Fig. 3.6). When compared to VEH-treated controls, adolescent FLX exposure significantly decreased ERK2- (Fig. 3.6b), but not ERK1- (Fig. 3.6a) mRNA 24 hr (Short-term, n= 9/group, $t_{16}= 2.02$, p<0.03) as well as 3 weeks (Long-term, n= 7-8/group, $t_{13}= 2.05$, p<0.05) after drug administration. Similarly, there was a
significant decrease of CREB mRNA (Fig. 3.6c), a signaling substrate of ERK, at the short- \((t_{16}=2.31, p<0.01)\) and long-term \((t_{13}=2.21, p<0.05)\) points of assessment, when compared to the VEH-treated controls.

**FLX exposure during adolescence results in enduring decreases of ERK-related protein phosphorylation in the VTA of rats.** ERK-related signaling was assessed three weeks after VEH or FLX exposure (Long-term; \(n=8/\)group), as inferred from the phosphorylation (p) levels of ERK1/2, as well as its downstream targets, RSK, MSK, CREB, and mTOR, within the VTA of rats (see Fig. 3.7a). FLX-treated rats had a significant decrease in pERK2 \((t_{14}=1.89, p<0.04)\), pRSK \((t_{14}=3.28, p<0.01)\), pCREB \((t_{14}=1.87, p<0.04)\), and pmTOR \((t_{14}=4.91, p<0.01)\), but not in pERK1 \((p>0.05)\) or pMSK \((p>0.05)\) when compared to controls. No changes in total protein levels of ERK1 \((p>0.05)\), ERK2 \((p>0.05)\), CREB \((p>0.05)\), mTOR \((p>0.05)\), or TH \((p>0.05)\) were detected when compared to VEH-treated controls (all normalized to GAPDH; Fig. 3.7b).

**Validation of virus-mediated gene transfer in the VTA.** Figure 3.8a shows the region of the anterior portion of the VTA to which virus vectors (HSV-GFP, HSV-wtERK2, HSV-dnERK2) were aimed. We found that virus expression was maximal on day 3 after infusion (data not shown), declining thereafter, and non-detectable seven days after the microinjection as previously reported (Barrot et al., 2002; Carlezon et al., 1997; Iñiguez et al., 2010c; Warren et al., 2011). Confocal microscopy (Fig. 3.8b) revealed that the percentage of TH-positive neurons overexpressing GFP in the VTA (~50%) was similar to previous findings (Iñiguez et al., 2008b; Russo et al., 2007).

**Virus-mediated expression of ERK2 in the adolescent VTA mimics the long-lasting effects of FLX on the forced swim test.** The effects of HSV-GFP \((n=10)\), HSV-wtERK2 \((n=9)\), and HSV-dnERK2 \((n=9)\) on day 2 of forced swimming in adolescent rats are shown in Figure 3.8c-d. We found that the amount of time (latency to immobility; Fig. 3.8c) rats engaged in escape-directed behaviors was dependent on virus treatment \((F_{2,25}=23.37, p<0.001)\). Specifically, HSV-wtERK2-treated rats displayed shorter latencies to become immobile when compared with HSV-GFP-treated controls \((p=0.051)\). Conversely, HSV-dnERK2-treated rats displayed longer latencies to become immobile when compared with the HSV-GFP-treated controls \((p<0.05)\). Virus treatment also influenced total immobility \((F_{2,25}=13.51, p<0.01;\) Fig. 3.8d), with HSV-wtERK2 showing higher (interpreted as increased susceptibility to stress) and dnERK2-treated rats showing lower (decreased susceptibility to stress) total immobility when compared with the HSV-GFP-treated group \((p<0.05,\) respectively).

**Virus-mediated expression of ERK2 in the adolescent VTA mimics the long-lasting effects of FLX in the elevated plus-maze.** The effects of viral-mediated ERK2 activity within the VTA of adolescent rats on anxiety-like behaviors, as measured by the elevated plus-maze, are shown in Figure 3.9 \((n=8-9/\)group). Virus treatment influenced time spent in the closed arms of the maze \((F_{2,22}=18.86, p<0.001)\). Adolescent HSV-wtERK2-treated rats spent less time \((p<0.05)\), whereas the HSV-dnERK2-treated rats spent more time \((p<0.05)\) in the closed arms of the maze when compared to the HSV-GFP-treated controls (Fig. 3.9a). Also, a one way ANOVA revealed that time spent in the open arms (Fig. 3.9b) varied as a function of virus treatment \((F_{2,22}=10.43, p<0.001)\). HSV-wtERK2-treated rats spent significantly more time in the open arms of the maze when compared to the HSV-GFP-treated controls \((p<0.05)\), with no apparent differences
between the HSV-GFP- and -dnERK2-treated rats (p>0.05). The number of fecal boli was also quantified as an additional index of anxiety (see Fig. 3.9c). We found that the number of fecal boli was influenced by HSV vector treatment ($F_{2,22}= 14.68$, p<0.0001). Specifically, rats infused with HSV-dnERK2 produced more fecal boli when compared to the HSV-GFP-treated controls (p<0.05). Conversely, rats infused with the HSV-wtERK2 virus produced significantly less fecal boli than the HSV-GFP-treated controls (p<0.05). No differences in total distance traveled (cm) were observed between any of the groups as a function of virus treatment during the five min test (p>0.05; Fig. 3.9d).

DISCUSSION

The antidepressant FLX is commonly prescribed for the treatment of pediatric MDD, even though little is known about potential treatment-induced neurobiological consequences (Olivier et al., 2010). To address this gap at the pre-clinical level, we assessed behavioral responses to mood-related stimuli in adult c57BL6 mice (PD70+) exposed to VEH or FLX during adolescence (PD35-49). Because the mesolimbic “reward circuit,” which is comprised of dopaminergic neurons that extend from the VTA to the nucleus accumbens (NAc) is highly implicated in emotional processing (Berton et al., 2006; Bolaños et al., 2003b; Krishnan et al., 2008), we examined ERK activity within the VTA as a function of FLX treatment. We selected to examine ERK signaling, given that this signaling cascade has recently been proposed to modulate the expression of depression-like behaviors in clinical populations and animal models (Duric et al., 2010; Fumagalli et al., 2005).

Our findings indicate that, in mice, FLX exposure during adolescence influences responsivity to aversive-stimuli in adulthood, findings similar to those recently reported in rats (Iñiguez et al., 2010a). Specifically, adult mice pretreated with FLX during adolescence displayed decreased sensitivity to the social defeat and the forced swimming procedures, and showed enhanced sensitivity to the elevated plus-maze (see Fig. 3.1c-f and 3.2a-b). Importantly, these FLX-induced behavioral phenotypes observed were not influenced by long-lasting changes in memory-associated performance (see Fig. 3.3), nor in changes in basal locomotor activity as a function of drug pre-treatment (see Fig. 3.1g, 3.2c, and 3.3c). Chronic exposure to FLX during adolescence also resulted in downregulation of ERK2 mRNA within the VTA, 24 hr after the last drug injection. This finding is consistent with recent in vivo (Fumagalli et al., 2005; Galeotti and Ghelardini, 2011; Todorovic et al., 2009) and heterologous expression system reports demonstrating that FLX blocks ERK phosphorylation (Labasque et al., 2010), resulting in overall decreased ERK activation (Fumagalli et al., 2005; Stepulak et al., 2008). Our data, therefore, strongly suggest that the therapeutic effects of FLX are mediated, at least in part, via a downregulation of ERK2-signaling within the adolescent VTA. This conclusion is further supported by our findings in decreased CREB (a transcription factor downstream of ERK) expression, which has been implicated in underlying antidepressant responses within the VTA-NAc circuitry (Carlezon et al., 2005; Nestler and Carlezon, 2006; Newton et al., 2002; Wallace et al., 2009). Surprisingly, this FLX-induced downregulation of ERK-signaling within the VTA persisted into adulthood. Specifically, FLX decreased ERK2 and CREB mRNA along with decreased ERK2 protein phosphorylation (see Fig. 3.7). Conversely, we recently found that depressive-like behaviors (i.e., increased total immobility in the forced swim test) are accompanied by increased levels in protein phosphorylation of ERK2, CREB, and mTOR within
the VTA (Warren et al., 2011). Together, these findings indicate that the enduring decrease in sensitivity to behavioral despair and helplessness measures (i.e., social defeat and forced swimming) may be the result of a lasting FLX-induced downregulation of ERK-related signaling within the VTA, a molecular signature likely promoting resilient-like behaviors (Feder et al., 2009; Krishnan and Nestler, 2010). It is possible that stress-induced increases in ERK activity (see Fig. 3.4e, Iñiguez et al., 2010a), which result in the expression of several proteins involved in neuroplasticity (e.g., CREB), can ultimately influence the function of circuits and/or individual dopaminergic/GABAergic neurons within the VTA. For instance, a key stress-induced adaptation accompanying depressive-like phenotypes in rodents is enhanced pacemaker frequency of VTA dopamine neurons (Berton et al., 2006; Krishnan et al., 2008). Because ERK and its related signaling cascade is highly implicated in the mechanisms underlying neuroplasticity (Adams and Sweatt, 2002), it is likely that the stress-induced enhanced neuronal firing rate of the VTA may be mediated by ERK-dependent mechanisms (Pittenger and Duman, 2008). In support of this hypothesis, we recently have shown that blocking ERK2 activity (via HSV-dnERK2) reduces the firing rate of dopaminergic neurons within the VTA in a similar fashion as FLX administration (Cao et al., 2010), resulting in antidepressant-like responses. It should be noted that ERK activity in the VTA differs from its role in other brain areas such as the hippocampus (Duric et al., 2010) and prefrontal cortex (Dwivedi et al., 2009), where enhanced ERK function has been reported to mediate the effects of antidepressants.

Exposure to FLX during adolescence also resulted in an anxiogenic-like phenotype in adulthood. While this result may appear contradictory at face value, this paradoxical finding has often been reported when assessing the molecular underpinnings of mood regulation within the VTA-NAc circuitry (Carlezon et al., 2005; Green et al., 2006; Iñiguez et al., 2010b; Krishnan et al., 2007). As previously stated, low CREB activity within the NAc results in an overall antidepressant-like effect when assessed in the forced swim test, along with anxiogenic-like responses in the elevated plus-maze (Barrot et al., 2002; Barrot et al., 2005; Green et al., 2010), behavioral responses mimicking those observed here (see Fig. 3.2) and in rats pretreated with FLX during adolescence (see chapter two; Iñiguez et al., 2010b). Given that FLX administration to adolescent mice was systemic, it is expected that FLX will have its pharmacological effects across various brain regions. Therefore it is possible that chronic FLX administration may also result in lowered ERK signaling in other brain areas such as the NAc, thus resulting in paradoxical antidepressant- and anxiogenic-like behavioral responses in adulthood (Carlezon et al., 2005; Green et al., 2006).

Because FLX exposure induces a stress-resistant phenotype along with decreases in ERK-related signaling within the VTA (see Fig. 3.6), it was of interest to determine whether increasing ERK, using virus vectors, would reverse this phenotype in FLX-pretreated mice. We found that increasing ERK signaling in adult mice pretreated with FLX during adolescence enhanced susceptibility to a submaximal exposure to defeat stress, conditions which are not sufficient to induce avoidance behaviors in control mice. More specifically, FLX pretreated mice receiving HSV-wtERK2 spent significantly less time interacting with a social target (interpreted as increased sensitivity to stress), thus reversing the stress-resistance phenotype induced by FLX exposure during adolescence. This finding mimics the depressive/avoidance behavior of mice exposed to ten days of social defeat (Fig 3.1c), or exposed to VEH during adolescence and receiving HSV-wtERK2 in adulthood (Fig. 4.9).
To determine whether ERK activity within the VTA may play an age-specific role in mediating responses to stress (between adolescents and adults), we examined how artificial increases or decreases of ERK2 levels (using virus vectors) would influence behavioral responses to aversive stimuli in naïve (drug-free) adolescent rats (age-matched to those undergoing FLX administration). We found that downregulation of ERK2 activity (via HSV-dnERK2) in adolescent rats results in a stress-resistant phenotype similar to that observed in rodents treated with FLX. In contrast, increasing ERK2 levels (with HSV-wtERK2) results in depressive-like behavioral responses (see Fig. 3.7c-d) similar to those observed in animals exposed to chronic unpredictable stress (Iñiguez et al., 2010a; Krishnan et al., 2008). These findings indicate that modulation of ERK2 activity within the VTA of naïve adolescent rats regulates responses to aversive stimuli in an oppositional manner, mimicking the behavioral responses of adolescent rodents either treated with FLX (antidepressant-like effect via decreased ERK) or exposed to stress (depressive-like effect via increased ERK) – findings consistent with the role of ERK within the VTA of adult rodents (Iñiguez et al., 2010a). Interestingly, the role that ERK2 plays in modulating responses to anxiety-inducing situations was age-dependent (compare Figures 3.9 and 4.6b). For instance, viral-mediated increases in ERK2 levels within the VTA results in an anxiogenic-like behavioral profile (Iñiguez et al., 2010a), whereas in adolescent rats this behavioral response is observed after HSV-dnERK2-treatment (along with increased fecal boli as an additional index of anxiety; see Fig. 3.9). Together, these age-dependent conflicting results indicate that ERK-signaling within the VTA may play a unidirectional role on depression-like sequelae of stress in adolescents and adults, but not on the anxiogenic sequelae, suggesting that manipulation of ERK may be most effective as prophylaxis to stress during adolescence, a hypothesis that now requires detailed investigation.

Overall, the results of this investigation indicate that administration of FLX during adolescence results in behavioral and molecular alterations that influence responsivity to aversive situations in adulthood. Specifically, a sustained stress-resistant behavioral response is observed that is accompanied by enhanced sensitivity to anxiety-inducing stimuli (Iñiguez et al., 2010b; Karpova et al., 2009; Warren et al., 2011). This paradoxical behavioral phenotype is likely mediated, at least in part, via dysregulation of ERK signaling within the VTA, as regulation of this signaling cascade using HSV gene transfer mimics the behavioral responses observed after stress or FLX treatment. Importantly, these age-specific behavioral effects in response to ERK modulation within the VTA may give insight into the potential biomarkers underlying adolescent MDD and/or antidepressant response, which may assist in delineating safety guidelines for pharmacological treatment and discontinuation windows in adolescent populations.
Figure 3.1. Enduring effects of fluoxetine (FLX; 10 mg/kg, bi-daily) exposure during adolescence on responsivity to behavioral despair measures in adulthood. (a) Schematic timeline of the chronic social defeat procedure in which adolescent c57BL/6 mice treated with FLX (PD35-49) and later defeated 10 consecutive days (10 min/episode) in adulthood (PD70-79). Mice were tested for social avoidance behaviors on day 11 (PD80). (b) Schematic diagram of the social interaction/avoidance arena illustrating the geographic location and size of the interaction and corner zones with respect to the enclosure in which a social target (CD1 mouse) is positioned. (c) Adolescent FLX exposure resulted in a long-lasting resilient-like behavioral phenotype 21-days post drug washout (n= 9/group). Whereas a clear avoidance phenotype was observed in mice treated with saline (VEH) (p<0.05; target absent vs. target present), FLX treatment during adolescence prevented social avoidance (p>0.05; target absent vs. target present) and displayed higher social interaction when compared to VEH-treated mice (p<0.05). (d) When assessing time in the corner zones, VEH-treated mice spent more time in the corners in the presence of the target (p<0.05), as well as when compared to mice exposed to FLX during adolescence (p<0.05). (e-f) In the forced swim test (n= 9-10/group), adult mice (PD70) exposed to FLX during adolescence (PD35-49) displayed higher latency to become immobile and an overall decrease in total immobility when compared to VEH-pretreated mice. (g) There were no group differences when locomotor activity (distance traveled in centimeters), rather than swimming was quantified on an open field (n= 5/group). Data are presented as mean + SEM. *p<0.05; PD, postnatal day.
Figure 3.2. Effects of fluoxetine (FLX; 10 mg/kg, bi-daily) exposure during adolescence on the elevated plus-maze in adulthood (n= 10/group). Mice exposed to FLX during adolescence (n= 10) spent significantly less time in the open arms (b), and more time in the closed arms (a), of the elevated plus-maze, when compared to saline (VEH) treated controls (n= 10). No differences in locomotor activity were observed between the groups (c). Data are presented as mean ± SEM. *p<0.05.
Figure 3.3. Fluoxetine (FLX; 10 mg/kg, bi-daily) exposure during adolescence did not influence behavioral responses to a novel object recognition test in adulthood (n= 10-11/group). C57BL/6 mice pretreated with FLX did not differ in the latency (in seconds) to initially approach (a), nor total time spent exploring a novel object when compared to saline (VEH) controls (b). Furthermore, no differences in total locomotor activity were observed between the groups (c). Data are presented as mean ± SEM.
Figure 3.4. Opposing regulation of ERK mRNA within the ventral tegmental area (VTA) of c57BL/6 mice between antidepressant administration and 10 days of social defeat stress. Chronic Fluoxetine (FLX; 10 mg/kg, bi-daily) exposure on gene expression within the VTA of mice. Exposure to FLX during adolescence (n= 9/group) reduced ERK2- and CREB-, but not ERK1-, mRNA at both time-points of assessment when compared to respective saline (VEH) controls (a-c; Short- and Long-Term). Exposure to stress (10 days of social defeat) resulted in a significant increase in ERK2-, but not ERK1 mRNA, when compared to non-stressed controls, in adult c57BL/6 mice (d-e). Data are presented as fold change normalized to GAPDH + SEM. *p<0.05; βp= 0.05.
Figure 3.5. ERK2 within the ventral tegmental area of c57BL/6 mice regulates behavioral responses to stress. (a) Infusion of the ERK inhibitor U0126, into the VTA of adult c57BL/6 mice (n= 15), results in longer time (sec) to become immobile, (b) as well as a total reduction in the time spent immobile, when compared to controls (n= 11). (c) Injection sites of the intra-VTA infusion are represented systematically (Franklin and Paxinos, 2008). Black dots represent the sites of cannula tips in the VTA of mice infused with DMSO (control) or U0126. (d) Schematic/timeline of the submaximal social defeat procedure, in which c57BL/6 mice were first pretreated with FLX during adolescence and later (PD66) received an infusion of HSV-wtERK2 (n= 8). Three days later (PD69), the mice were socially defeated three times (5 min each) in a single day, and tested on the social interaction test 24 hr later (PD70). (e) FLX pretreated animals infused with HSV-wtERK2 spent significantly less time in the interaction zone (e, left panel) and more time in the corners (e, right panel) in the presence, versus the absence, of the social target. (f) Selectively increasing VTA ERK2 via microinfusion of HSV-wtERK2 (n= 9), results in enhanced vulnerability to stress as depicted by a significant increase of total immobility in the tail suspension test, when compared to HSV-GFP controls (n= 11). *p<0.05. Data presented as mean time in seconds ± SEM.
Figure 3.6. Short- and long-term effects of fluoxetine (FLX; 10 mg/kg, bi-daily) exposure during adolescence on gene expression within the ventral tegmental area of male rats. When compared to saline-treated (VEH) controls, exposure to FLX during adolescence, resulted in a significant decrease in ERK2- (b) and CREB- (c), but not ERK1-mRNA (a) either 24 hr, or three weeks after the last injection. Data are presented as fold change normalized to GAPDH + SEM. *p<0.05.
Figure 3.7. Long-term effects of adolescent exposure to saline (VEH) or fluoxetine (FLX; 10 mg/kg, bi-daily) on ERK-related protein phosphorylation within the ventral tegmental area of male rats (n= 8/group). Exposure to FLX significantly decreased the levels of phospho (p) ERK2, pRSK, pCREB, and pmTOR (p<0.05, respectively), without affecting total (t) levels of protein (tERK1, tERK2, tCREB, tmTOR, and TH) when compared with the VEH-pretreated controls. *Significantly different from VEH-pretreated controls (p<0.05). Data are presented as a ratio of total protein normalized to GAPDH (mean ± SEM).
Figure 3.8. ERK2 in the ventral tegmental area (VTA) regulates behavioral responses in the adolescent rat forced swim test. (a) Rostral region of the VTA to which microinjections of virus vectors were targeted (Paxinos and Watson, 1997). (b) Merged confocal photomicrograph (magnification, 400x) of a representative brain slice from the rostral VTA (~5 mm caudal to Bregma) double-labeled for tyrosine hydroxylase (TH) (red: cyanine 3) and HSV-wtERK2-GFP (green: cyanine 2) fluorescence. Arrows indicate double-labeled cells. (c) Latencies to become immobile were decreased in rats treated with HSV-wtERK2 (βp= 0.05) and significantly increased in rats treated with HSV-dnERK2 (p<0.05) when compared with HSV-GFP controls (n= 9-10/group). (d) Total immobility was also influenced by virus treatment, with HSV-wtERK2 showing higher, and dnERK2-treated rats showing lower total immobility when compared to controls (HSV-GFP). Data are presented as mean ± SEM. *p<0.05.
Figure 3.9. ERK2 in the ventral tegmental area (VTA) of adolescent rats regulates responses to the elevated plus-maze (n= 8-9/group). (a) Rats overexpressing HSV-wtERK2 spent less time, while HSV-dnERK2 spent more time in the closed arms of the elevated plus-maze, when compared to HSV-GFP-treated control rats. (b) HSV-wtERK2 rats also spent more time in the open arms of the maze when compared to controls. (c) The number of fecal boli produced by rats was also influenced by virus treatment, with HSV-wtERK2 rats producing less, and HSV-dnERK2 rats producing more fecal boli when compared to controls. (d) No differences in total locomotor activity were observed between the groups as a function of HSV vector. Data are presented as mean ± SEM. *p<0.05.
Table 3.1. Mouse and rat experimental/treatment groups

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PD, postnatal day; qPCR, real-time polymerase chain reaction.
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ERK, extracellular signal-regulated protein kinase; CREB, cAMP response element binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qPCR, real-time polymerase chain reaction.
CHAPTER FOUR

ERK WITHIN THE VENTRAL TEGMENTAL AREA REGULATES MOOD-RELATED BEHAVIOR

Adapted from:


INTRODUCTION

Acting in several brain regions, neurotrophins and their signaling pathways play significant roles in neuronal excitability, synaptic transmission, models of learning and memory (Alonso et al., 2002; Poo, 2001; Schuman, 1999), and responses to drugs of abuse (Bolaños and Nestler, 2004; Horger et al., 1999; Lu et al., 2004; Pierce and Bari, 2001; Schoenbaum et al., 2007). Neurotrophin signaling cascades have also been shown to play crucial roles in regulating responses to stress in several animal models of depression and antidepressant action (Berton et al., 2006; Duman and Monteggia, 2006; Eisch et al., 2003).

Midbrain dopamine neurons of the ventral tegmental area (VTA) and their projections to the nucleus accumbens (NAc), which together form the mesolimbic dopamine system, express neurotrophins and their Trk receptors (Conner et al., 1997; Numan and Seroogy, 1999; Yan et al., 1997). The VTA-NAc pathway is best known for its role in encoding the incentive-motivational valence of drug and natural rewards (Kelley and Berridge, 2002; Koob and Le Moal, 2001). Chronic exposure to drugs of abuse has been shown to alter neurotrophin-signaling within the VTA, including upregulation of the extracellular signal-regulated kinase (ERK) cascade (Berhow et al., 1996; Nestler et al., 1996; Ortiz et al., 1995). ERK consists of two isoforms (ERK1 and ERK2), and is one of several mitogen-activated protein kinases (MAPKs) involved in numerous cellular processes, including long-term neuronal plasticity, maintenance, and survival (Hetman and Gozdz, 2004; Subramaniam and Unsicker, 2010). More recently, ERK has also been implicated in adaptive responses to stress and antidepressant treatments, although in brain regions other than the VTA-NAc circuitry (Duman et al., 2007; Dwivedi et al., 2006; Einat et al., 2003; Gourley et al., 2008a).

Beyond addiction, the mesolimbic dopamine system has been increasingly implicated in the regulation of mood- and depression-related behaviors (Naranjo et al., 2001; Nestler and Carlezon, 2006; Willner, 1983). Chronic exposure to stress activates the VTA-NAc pathway (Bertolucci-D'Angio et al., 1990; Horger and Roth, 1996; Jensen et al., 2003). Moreover, prior exposure to stress sensitizes an individual to the effects of drugs of abuse and vice versa (Covington and Miczek, 2005; Shaham et al., 2000), and chronic stress causes many of the same long-term molecular and cellular adaptations seen after acute or chronic administration of drugs.
Based on these shared actions of drugs of abuse and stress, the goal of the present investigation was to assess whether acute or chronic stress, like drugs of abuse, alters the ERK pathway within the VTA and, if so, whether such adaptations can influence an animal’s functional responses to stress and other mood-related stimuli.

**METHODS**

*Animals.* For all experiments, animals were male, fed ad libitum, allowed a 1-week habituation period before experimental manipulation, and housed at 23-25°C on a 12 hr light/dark cycle (lights on between 07:00 and 19:00 hr). Sprague-Dawley rats (275-300 g; Charles River, Raleigh, North Carolina), 9 week-old c57BL/6 mice (Jackson Labs, Bar Harbor, MA), and CD1 retired breeders (Charles River), were used in this study. Rats (two per cage), c57BL/6 (four per cage) and CD1 (one per cage) mice were housed in clear polypropylene boxes containing wood shavings. Experiments were conducted in compliance with the guidelines for the *Care and Use of Laboratory Animals* (Council, 2003), and approved by the Florida State University Animal Care and Use Committee.

*Acute and chronic unpredictable stress.* An initial experiment was performed to assess how stress affects ERK phosphorylation within the VTA. More specifically, rats were randomly assigned to either an acute or chronic unpredictable stress regimen. Rats in the acute condition were stressed one time only: forced to swim for 15 min under conditions they could not escape (18°C water; light cycle). Under chronic stress, rats were subjected to single daily episodes of unpredictable stress for 4 weeks (Willner et al., 1987). Chronic stress consisted of randomized periods of food or water deprivation (overnight), continuous cage shaking (1 hr on an automatic shaker; light cycle), forced swim stress (15 min, in 18°C water; light cycle), continuous overnight illumination (12 hr), intermittent illumination (12 hr; 2 hr light/2 hr dark), overcrowded caging (10 rats per cage; overnight), wet cage (12 hr; light cycle), exposure to cold temperature (1 hr at 4°C; light cycle), and acute restraint stress (40 min; light cycle) using plastic DecapiCones (Briantree Scientific, Braintree, MA). Immediately after the last episode of stress (i.e., forced swimming in the acute, or physical restraint in the chronic, stress condition) VTA punches were collected and maintained at -80°C until immunoassayed.

*Western blotting.* Tissue punches of VTA (1.25 mm diameter) from rats were sonicated in a standard lysis buffer and then centrifuged at 14,000 rpm for 15 min. Samples (20 µg; estimated through the Bradford assay) were treated with β-mercaptoethanol and subsequently electrophoresed on precast 4%-20% gradient gels (Biorad, Hercules, CA). Proteins were transferred to a PVDF membrane, washed in 1X Tris-buffered saline with 0.1% Tween-20 (TBST), and blocked in milk dissolved in TBST (5% weight/volume) for 1 hr at 25°C. Blots were probed (overnight at 4°C) with antibodies against the phosphorylated forms of the protein [except tyrosine hydroxylase (TH) and GAPDH] then stripped (Restore, Thermo Scientific) and probed with antibodies against total protein of the same type. Antibodies were from Cell Signaling (Beverly, MA; ERK1 and ERK2, 90RSK [ribosomal S6 kinase of 90 kD], MSK1 [mitogen- and stress-activated protein kinase 1] phospho- and total-protein, except total MSK1) and were used according to manufacturer’s instructions (in 5% milk dissolved in TBST). After further washes, membranes were incubated with peroxidase-labeled goat anti-rabbit IgG or horse
anti-mouse IgG (1:40,000; Vector Labs, Burlingame, CA). Bands were visualized with SuperSignal West Dura substrate (Pierce Biotechnology, Rockford, IL) and quantified (normalized to GAPDH) using ImageJ (NIH).

Viral manipulation, behavioral design, and procedures. Based on the results from the acute and chronic stress experiment described above, separate groups of rats were microinjected with herpes simplex virus (HSV) vectors encoding green fluorescent protein (GFP), wildtype ERK2-GFP (HSV-wtERK2), or a dominant negative mutant of ERK2-GFP (HSV-dnERK2) to determine whether direct viral-mediated gene manipulation modulates ERK signaling and, if so, assess if the viral-induced adaptations influence the firing rate of dopamine neurons within the VTA. After these experiments, separate groups of rats and c57BL/6 mice received microinfusions of HSV -GFP, HSV-wtERK2, or HSV-dnERK2 within the VTA to further assess how manipulation of ERK2 itself influences an animal’s reactivity to stress. Thus, subjects were tested on different behavioral procedures designed to assess functional responses to stressful situations and other emotion-eliciting stimuli. All viral-mediated experiments were performed three days after virus infusion.

Virus vectors. The construction of the HSV vectors has been described previously (Neve et al., 1997; Robinson et al., 1996), and the ERK2 vectors have been validated in vivo and in vitro (Krishnan et al., 2007; Russo et al., 2007). The average titer of the recombinant virus stocks was $4.0 \times 10^7$ infectious units/ml. Titers did not differ by >10% among preparations. All behavioral experiments were commenced on day 3 after viral surgery, a time at which maximal transgene expression caused by these vectors is observed (Barrot et al., 2002; Carlezon et al., 1998). Expression of the HSV-encoded transgenes was limited to an area of ~1 mm$^3$ around the injection site, and viral expression was not apparent in either afferent or efferent regions of the injected area. Thus, we found no detectable HSV expression in either the NAc or the dorsal raphe.

Animal surgery. For stereotaxic delivery of the viruses, rats were anesthetized with a ketamine/xylazine cocktail (80/10 mg/kg; intramuscular) and given atropine (0.25 mg/kg) subcutaneously to minimize bronchial secretions; afterward, rats were given bilateral microinjections (1.0 µl per side over 10 min of either HSV-GFP, HSV-wtERK2, or HSV-dnERK2) into the rostral region of the VTA, anteroposterior: -4.9, lateral: +2.2, dorsoventral: -7.6 mm below dura (Paxinos and Watson, 1997), using a 32 gauge Hamilton syringe angled at 10° from the midline, to avoid piercing the sinus system (Franklin and Paxinos, 2008). Similarly, mice were anesthetized with ketamine/xylazine (100/10 mg/kg; intraperitoneal) and received bilateral infusions (0.5 µl over 5 min) of virus into established mouse VTA coordinates (anteroposterior: -3.2, lateral: +1.0, dorsoventral: -4.6; 7° angle). The VTA injection sites were confirmed in all animals by standard histology methods (described below). The local anesthetic bupivacaine was applied directly along the wound edges to minimize any potential post-operative discomfort.

Histology and transgene detection. At the end of the behavioral experiments, rats and mice were given an overdose of pentobarbital and perfused transcardially with 0.9% saline, followed by cold 4% paraformaldehyde. The brains were removed, postfixed overnight in 4% paraformaldehyde and stored in 20% glycerol solution. Coronal sections (45 µm) through the
midbrain were taken on a microtome and stored in 0.1 M sodium phosphate buffer with 0.05% azide. Sections were processed to examine the ability of HSV construct to drive expression of GFP and TH within the VTA as previously described (Russo et al., 2007). Midbrain free-floating coronal sections were processed for immunohistochemistry using the following antibodies: rabbit anti-GFP (1:1000; Abcam, Cambridge, MA), and TH (mouse; 1:5000, Chemicon, Temecula, CA), a marker of VTA dopamine neurons. Adjacent sections were blocked in 3% normal donkey serum (NDS) and incubated overnight in one of the primary antibodies mentioned above, along with 0.3% Triton X-100 (Fisher Scientific, Pittsburgh, PA) and 1% NDS. Sections were incubated with anti-rabbit or -mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 2 hr at room temperature. Stained sections were then slide mounted (Fisher Scientific), dehydrated in ethanol and citrosolv, and coverslipped with clear DPX adhesive (Sigma, St. Louis, MO). Slides were then visualized and photographed using a fluorescence microscope and a digital camera. Data obtained from animals with placements outside the intended brain regions (<10% of all experimental animals) were not included in the analyses.

**Forced swimming and tail suspension tests.** The forced swim test is a two-day procedure in which rats are forced to swim under conditions in which they cannot escape. On the first day, rats are forced to swim. Initially, they 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. When retested 24 hr later, rats become immobile very quickly; however, antidepressant treatment between the forced swim exposures can significantly increase their escape-like behaviors, an effect that has been correlated with antidepressant activity in humans (Porsolt et al., 1977; Steru et al., 1985). At the start of the experiment, rats received intra-VTA injections of HSV-GFP, HSV-wtERK2, or HSV-dnERK2 and were left undisturbed to recover for two days. Twenty-four hr later (three days after viral infusion), rats were placed in plastic cylinders (75 X 30 cm) filled to 54 cm depth with 25°C water and forced to swim for 15 min. At the end of this period, rats were removed from the water, dried with towels, and placed in a warmed enclosure for 30 min, then returned to their home cage. All cylinders were emptied and cleaned between rats. Twenty-four hours later, rats were retested for 5 min under identical conditions, and sessions were videotaped. In this study, the latency to become immobile, total immobility, and behavioral counts (i.e., floating, climbing, and swimming) were the dependent variables. 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. To qualify as immobility, this posture had to be clearly visible and maintained for ≥2.0 sec. Behavioral counts were taken at 5 sec intervals during the 5 min retest (Lucki, 1997).

A related test of behavioral despair, the tail suspension test, was carried out in c57BL/6 mice. This test involves the stress of being suspended by the tail in a fashion in which they cannot escape, rather than forced to swim (Cryan et al., 2005a; Steru et al., 1985). Mice were microinjected with HSV vectors into the VTA, and three days later were suspended by their tail for 5 min and the total time spent immobile (sec) was measured.

**Basal locomotor activity after day 1 of forced swimming.** Spontaneous locomotor activity was indexed as distance traveled (cm) in an open-field (63 x 63 cm) that consisted of a square box that rats can explore freely (Iñiguez et al., 2009). This task was performed 24 hr after the forced swim test on day 1, in order to assess whether the effects observed in the forced swim test...
(day 2) could be confounded by changes in general locomotor activity after viral-mediated gene transfer.

**Social defeat stress.** To further delineate how ERK2 modulation within the VTA influences responsiveness to stressful situations, we conducted social defeat experiments in c57BL/6 male mice. CD1 mice with consistent attack latencies (≤30 sec on three consecutive screening tests) were housed in cages fitted with perforated Plexiglas separators (Florida State University Psychology Department Engineering Group), which allow sensory contact without permitting physical contact, and used to defeat c57BL/6 mice in two separate experiments (Berton et al., 2006; Krishnan et al., 2007). In the first experiment, naïve mice were microinjected with HSV-GFP or HSV-wtERK2 and were exposed to a submaximal defeat protocol: three 5 min long defeat episodes interspersed by 15 min rest periods (see Figure 4.4a) on a single day. Social avoidance behaviors toward an unfamiliar CD1 mouse were assessed the following day in a two-stage social interaction test. In the first 2.5 min trial (“target absent”), the experimental c57BL/6 mouse was allowed to freely explore a square-shaped open-field arena (44 X 44 cm) containing a wire-mesh cage (10 X 6 cm) apposed to one side (see Figure 4.4b). During the second 2.5 min trial (“target-present”), the mouse was reintroduced into this arena, which now contained an unfamiliar CD1 mouse within the cage. Video-tracking software (Noldus®) was employed to measure the time spent in the “interaction zone” (14 X 26 cm) and the “corner zones” (10 X 10 cm). For the second experiment (see Figure 4.4c), a separate group of naïve mice were defeated for 10-days (10 min/day). After the social interaction test (day 11), the defeated mice (those showing social avoidance) were randomly assigned to receive HSV-GFP or HSV-dnERK2 vectors into the VTA. Three days after surgery, avoidance behaviors (social interaction test) toward an unfamiliar CD1 mouse were assessed (Krishnan et al., 2007, 2008).

**Sucrose preference.** Two separate sucrose preference tests were conducted using a two-bottle choice procedure under red light at the beginning of the dark phase. In the first experiment, rats were habituated to drink a 1% sucrose solution for 3 days (from two drip-controlled bottles). On day 4, the sucrose solution was replaced with tap water for an additional 2 days. Two hr (1700 hr) before the test (at the end of day 5), rats were singly housed with access to food. At the start of the dark phase (19:00 hr), rats were given access to the two bottles (containing water or 1% sucrose). The position of the water and sucrose bottles (left or right) was switched every 30 min from 1900 to 2200 hr. After the 3 hr period, rats were left undisturbed and their overnight fluid consumption was measured the next morning (0800 hr). The preference for sucrose over water (i.e., sucrose intake divided by the sum of total water plus sucrose intake [sucrose/(water + sucrose)]) was used as a measure for rats’ sensitivity to reward (Willner et al., 1987). No differences in sucrose preference or liquid intake were found during the 3 hr period, thus the water and sucrose consumption data analyzed were those taken at 1900 and 0800 hr. At the end of the testing period, rats were again housed in pairs. The total amount of fluid (water or sucrose) intake was considered baseline preference. Only rats showing a ≤60% preference to sucrose over water were used for the rest of the experiment. Four days after the pretest (i.e., baseline preference), animals received intracranial HSV microinjections into the VTA. On day 3 after surgery, the sucrose preference test was repeated exactly as performed on day 5 of baseline (Bolaños et al., 2003b). The second experiment was conducted to assess whether ERK2 could influence preference for sucrose after chronic unpredictable stress. After baseline preferences
(procedure as described above), rats were exposed to 7 days of chronic unpredictable stress (Willner et al., 1987). Twenty-four hr after the last day of stress (forced swimming), animals received intracranial HSV or sham microinjections into the VTA. On day 3 after surgery, the sucrose preference test was performed (as described above).

**Nociceptive stimuli.** In this test, rats were exposed to an electric foot-shock session in an apparatus consisting of a computerized box with a grid three days after viral infusions into the VTA (Barrot et al., 2002). The threshold of foot-shock intensity required to induce a behavioral response was determined. After 2 min of habituation to the testing chamber, rats received a foot shock every 30 sec starting at 0.05 mA, with a 0.05 mA increment between each shock (to a maximum of 1.0 mA). The intensity at which a flinch, an audible vocalization, or a jump first appeared was recorded. The test session was terminated after all three behavioral responses were observed in each animal (Bonnet and Peterson, 1975).

**Elevated plus-maze.** Rats receiving HSV microinjections into the VTA were tested for 5 min on the elevated plus maze, a test of anxiety-like behavior (Montgomery, 1955). The maze was made of gray plastic and consisted of two perpendicular, intersecting runways (12 cm wide X 100 cm long). One runway had tall walls (40 cm high) or “closed arms,” and the other one had no walls or “open arms.” The arms were connected together by a central area, and the maze was elevated 1 m from the floor. At the beginning of the test, under controlled light conditions (~90 lux), rats were placed in the central area, facing one of the open arms, and the cumulative time spent in the open arms was videotaped. We also assessed self-grooming in the “closed arms” because rats engage in repetitive grooming in response to anxiogenic stimuli (Spruijt et al., 1988).

**VTA slice cultures.** Slice cultures were prepared as described previously (Han et al., 2006; Krishnan et al., 2007). Acute coronal brain slices (300 µm) containing the VTA were obtained in sucrose-aCSF (artificial cerebrospinal fluid, for composition see Electrophysiology below). Slices were trimmed as small as possible and were maintained for up to 3 days in 34°C incubator. We used Gibco MEM medium containing 30 mM Heps, 20 mM D-glucose, 5% B27, 5.0 mM L-glutamine, and 25 unit/ml streptomycin/penicillin. This culture medium successfully maintained stable pacemaker activity of the dopamine neurons in the VTA slice cultures.

**Electrophysiology.** Brain was quickly removed and put into cold sucrose-aCSF, which was derived by fully replacing NaCl with 254 mM sucrose in aCSF – it contained [mM]: 128 NaCl, 3 KCl, 1.25 NaH2PO4, 10 D-glucose, 24 NaHCO3, 2 CaCl2, and 2 MgCl2 (oxygenated with 95% O2 and 5% CO2, pH 7.35, 295-305 mOsm). Brain slices containing VTA were cut using a microslicer (DTK-1000, Ted Pella Inc, Redding, CA) in cold sucrose-aCSF. Slices were kept for recovery in a holding chamber with aCSF for one hr at 37°C, and HSV vectors were pipetted onto the VTA area of the slice surface. VTA slice cultures were transferred into a recording chamber, in which a constant flow of aCSF (~2.5 ml/min) was maintained throughout the experiment at 34°C. Single-unit extracellular potentials were recorded by the use of loose patch recording mode (Han et al., 2006) with glass pipettes filled with (in mM) 115 potassium gluconate, 20 KCl, 1.5 MgCl2, 10 phosphocreatine, 10 Heps, 2 ATP-Mg and 0.5 GTP (pH 7.2, 285 mOsm), and monitored through high-input impedance amplifier (axoclamp 2B, Axon Instruments). VTA dopamine neurons in these experiments were identified by location and well-
established electrophysiological criteria: regular spontaneous firing, action potential (AP) with triphasic waveforms (positive, negative, positive), and AP width (from start to trough) >1.1 ms under the filter conditions of 300Hz-0.8 KHz as previously reported (Ungless et al., 2004; Werkman et al., 2004). The waveform of AP (see Figure 4.7a) for measuring AP width was obtained by averaging 30-40 AP episodes. The firing rate of dopamine neurons were recorded in the bridge mode of amplifier AxoClamp 2B, and data acquisition and on-line analysis of firing rate were realized with Digidata 1322A digitizer and pClamp 8.2 (Axon Instruments).

Statistical analyses. Assignment of subjects to the various testing and viral conditions was random. Behavioral data were analyzed using one- or two-way analysis of variance (ANOVA) followed by Tukey post hoc test. A t-test was used for all other analyses implicating only a two-groups comparison. Data are expressed as the mean ± SEM. In all cases, statistical significance was defined as p<0.05.

RESULTS

Stress increases ERK signaling within the VTA. Immunoblot analysis was used to examine the effects of acute or chronic unpredictable stress on ERK activity, as inferred from the phosphorylation of ERK1/2 and of two downstream targets, 90RSK and MSK1, within the VTA. Figure 4.1 shows immunoblots of VTA homogenates representing averages of expression levels of pERK1, pERK2, p90RSK, pMSK1, and total (t) TH under acute (a) and chronic (b) stress conditions. Acute stress (n= 10/group) significantly increased levels of pERK1 (t_{18}= 2.89, p<0.001), pERK2 (t_{18}= 4.71, p<0.0002), p90RSK (t_{18}= 4.49, p<0.0003), and pMSK1 (t_{18}= 6.63, p<0.0001), with no changes in tERK1, tERK2, t90RSK, and tTH (p>0.05, respectively) when compared to controls (all normalized to GAPDH). Chronic unpredictable stress (n= 5/group) similarly increased levels of pERK1 (t_{8}= 2.03, p<0.04), pERK2 (t_{8}= 2.11, p<0.03), p90RSK (t_{8}= 2.05, p<0.03), and pMSK1 (t_{8}= 3.72, p<0.01) with no changes in total levels of these enzymes, including TH (p>0.05, respectively), when compared to controls.

Validation of viral-mediated gene transfer in the VTA. Figure 4.2a shows the region of the VTA to which microinjections of HSV vectors (HSV-GFP, HSV-wtERK2, or HSV-dnERK2) were targeted. As reported previously, we found that virus expression was maximal between days 3 and 4 after injection (data not shown), significantly declining thereafter, and nondetectable one-week after the microinjection (Barrot et al., 2002; Olson et al., 2005). Confocal microscopy (Fig. 4.2b-d) revealed that the percentage of TH-positive neurons overexpressing GFP in the VTA (~53%) was similar to previous findings (Olson et al., 2005; Russo et al., 2007) with no detectable expression of the viral-encoded transgenes in glial cells (data not shown).

We also demonstrated that our viral-mediated gene transfer manipulation modulates ERK signaling in the VTA. Three days after viral microinjections, VTA punches were obtained from infected rats (n= 6/group) for immunoblotting studies in which we examined a panel of antibodies against molecules involved in ERK signaling (Fig. 4.2e). Separate one-way ANOVAs (for protein of interest) showed significant differences in pERK2 (F_{2,15}= 6.31, p<0.01) and other pERK-dependent signaling proteins as a function of virus treatment (p90RSK: F_{2,15}= 5.71, p<0.01 and pMSK1: F_{2,15}= 28.77, p<0.0001, respectively). Post-hoc analyses showed that HSV-
wtERK2 significantly increased pERK2, p90RSK, and pMSK1 levels, while HSV-dnERK2 significantly decreased pERK2, p90RSK, and pMSK1 levels (p<0.05, respectively) as compared to their respective HSV-GFP controls. On the other hand, no differences in levels of pERK1 as a function of virus were detected (p>0.05). Similarly, no differences in tERK1, tERK2, t90RSK, or tTH as a function of virus treatment were observed (p>0.05).

**ERK2 regulation of behavioral responses to emotion-eliciting stimuli.** Based on the ability of acute and chronic unpredictable stress to increase ERK2 signaling in the VTA, it was of interest to study the functional consequences of altered ERK activity in this brain region on emotional behavior. Increased ERK2 function was induced in the VTA of adult rats or mice by local injection of HSV-wtERK2, while decreased ERK2 function was induced by HSV-dnERK2 injection.

**ERK2 activity in the VTA regulates behavioral reactivity to forced swimming and tail suspension stress.** We first used the forced swim test in rats, which is used widely as a screen for antidepressant efficacy as well as a measure of behavioral despair (Cryan et al., 2002). In this test, animals initially struggle in an attempt to escape, but within 1-2 min become immobile. The effects of HSV-GFP, HSV-wtERK2, and HSV-dnERK2 on day-two of forced swimming are shown in Figure 4.3a-b (n= 12-13/group). We found that the amount of time rats engaged in escape-directed behaviors in the forced swim test was dependent on viral treatment. Latency to become immobile varied as a function of virus treatment (F\(_{2,34}\) = 42.04, p<0.0001; Fig. 4.3a). HSV-wtERK2-treated rats had shorter latencies to become immobile (interpreted as increased susceptibility to stress) when compared to HSV-GFP-treated rats (p<0.05). Conversely, HSV-dnERK2-treated rats displayed significantly longer latencies to become immobile (decreased susceptibility to stress) when compared to HSV-GFP-treated controls (p<0.05). Virus treatments also influenced total immobility (F\(_{2,34}\) = 8.81, p<0.001), with HSV-dnERK2-treated rats showing significantly lower total immobility when compared to the HSV-GFP-treated group (p<0.05). This HSV-dnERK2-induced stress-resistant phenotype was correlated with lower floating counts (F\(_{2,34}\) = 7.16, p<0.003) and higher climbing counts (F\(_{2,34}\) = 9.25, p<0.001) and a tendency for higher swimming counts (p= 0.07) when compared to controls (Fig. 4.3b). In contrast to the VTA, HSV microinjections into the substantia nigra (n= 5/group) did not alter the responses (p>0.05) of the animals in the forced swim test (see Figure 4.3c).

A separate group of rats receiving HSV-wtERK2, HSV-dnERK2, or HSV-GFP into the VTA (n= 4/group) were tested in the open field box to assess whether the effects observed in the forced swim test could be confounded by changes in general locomotor activity after viral-mediated gene transfer. As can be seen in the insert of Figure 4.3b, no significant differences were apparent between the groups when locomotor activity was assessed 24 hr after day one of forced swimming.

To complement the findings from the forced swim test in a second species, we studied the effect of viral infusions into the VTA of c57BL/6 mice in the tail suspension test (n= 6/group), in which total immobility has been widely validated as a measure of antidepressant efficacy and depression-like behavior (Cryan et al., 2005a). We obtained results very similar to those observed in the forced swim test with rats (F\(_{2,15}\) = 16.59, p<0.0002; Figure 4.3d). HSV-wtERK2-treated mice showed higher total immobility (p<0.05; increased susceptibility to stress), whereas
HSV-dnERK2-treated mice showed lower total immobility (p<0.05; decreased susceptibility to stress) when compared to the HSV-GFP controls.

**ERK2 activity in the VTA regulates vulnerability to social defeat stress.** To further assess the causal role of ERK2 activity in the VTA as a mediator of increased vulnerability to stress, we microinjected HSV-GFP (n= 6) or HSV-wtERK2 (n= 8) into the VTA of naïve c57BL/6 mice and assessed their social interaction time after a submaximal period of social defeat (Fig. 4.4a). This behavioral assay has been used previously to study molecular adaptations that promote stress vulnerability (Krishnan et al., 2007). As can be seen in Figure 4.4d, HSV-GFP-treated mice demonstrated significantly higher levels of social interaction when the target is present (target absent vs. present: $t_{10} = 2.275$, p<0.02), similar to responses observed in uninjected control mice (data not shown). In contrast, no change in social interaction was observed in the HSV-wtERK2-treated mice upon exposure to a social target (target absent vs. present: p>0.05). These results indicate that the submaximal social defeat protocol utilized was, as expected, not sufficient to induce social avoidance in the HSV-GFP mice, but did induce significant social avoidance in HSV-wtERK2-treated mice when compared to controls (target present: $t_{12} = 3.19$, p<0.001), thus indicating increased vulnerability to stress under these conditions. Assessing time in the corner zones (Fig. 4.4e), which provides an additional measure of social avoidance (Krishnan et al., 2007), yielded similar results: HSV-wtERK2-treated mice spent significantly more time in the corners in the presence of the aggressor (target absent vs. target present: $t_{14} = 2.61$, p<0.02), or when compared to the HSV-GFP-treated controls ($t_{12} = 2.98$, p<0.01).

In a separate experiment, naïve c57BL/6 mice were subjected to a full 10 days of social defeat (Fig. 4.4c), which induces social avoidance in the majority of animals (Krishnan et al., 2007), an effect that can be reversed by chronic, but not acute, antidepressant treatment (Berton et al., 2006; Tsankova et al., 2006). Only those defeated mice demonstrating significantly high levels of social avoidance were selected to receive microinjections of HSV-GFP (n= 6) or HSV-dnERK2 (n= 7) into the VTA. No differences in the magnitude of social avoidance were detected between these groups prior to surgery (data not shown). Three days after viral infusions (Fig. 4.4f), HSV-GFP-treated mice showed the expected decrease in interaction times in response to a CD1 mouse ($t_{10} = 2.8$, p<0.01). On the other hand, the HSV-dnERK2-treated mice did not display social avoidance in the presence of the aggressor (p>0.05) and, on the contrary, they displayed a tendency toward social interaction (p= 0.06), thus showing that decreasing ERK2 in the VTA ameliorates the effects of chronic social defeat stress. This was further evidenced when assessing the time spent in the corner zones (Fig. 4.4g), as the GFP-treated mice spent significantly more time in the corners when compared to HSV-dnERK2-treated mice ($t_{11} = 2.32$, p<0.05).

**ERK2 modulates preference for sucrose.** To further characterize the functional consequences of altered ERK2 signaling within the rat VTA, we assessed the effects of HSV-wtERK2 and HSV-dnERK2 on behavioral reactivity to a natural reward, namely, sucrose preference (Fig. 4.5a-d; n= 12-15/group). This procedure has been used extensively as an animal model of anhedonia (Papp et al., 1991; Sampson et al., 1992; Willner et al., 1987). Overall analyses indicated that HSV microinjections did not significantly affect the total fluid intake (water + sucrose) during either testing day (Fig. 4.5b). However, sucrose preference varied as a function of ERK2 expression ($F_{2,38} = 14.45$, p<0.003). HSV-wtERK2 overexpression increased
sucrose preference when compared to its pre-test scores (Fig. 4.5a) or the HSV-GFP-treated rats during the post-testing day (p<0.05).

ERK2 activity within the VTA also regulated preference for sucrose after 7 days of chronic unpredictable stress. Viral-mediated gene transfer influenced sucrose preference as a function of virus treatment (F_{3,68} = 7.76, p<0.0001), stress (pre- vs. post-stress: F_{1,68} = 12.67, p<0.001), and virus by stress interaction (F_{3,68} = 10.84, p<0.0001) without affecting total liquid intake (Fig. 4.5d; n= 8-10/group; p>0.05). Post hoc analyses show that chronic unpredictable stress alone decreased sucrose preference (a depression-like effect) in the sham- and GFP-treated rats (p<0.05, pre- vs. post-stress, respectively; Fig. 4.5c). Conversely, we found that HSV-wtERK2 overexpression enhanced preference for sucrose after chronic unpredictable stress when compared to sham- and HSV-GFP-treated rats (p<0.05, respectively). Downregulation of ERK2 did not affect sucrose preference after chronic stress, thus indicating that HSV-dnERK2 blocked the effects of chronic unpredictable stress on sucrose preference (Fig. 4.5c), an antidepressant-like effect (Willner et al., 1987).

ERK2 activity in the VTA regulates responses to nociceptive stimuli. We next assessed the influence of ERK2 in the rat VTA on unconditioned behavioral responses to nociceptive stimuli (Fig. 4.6a; n= 10-11/group). When compared with the GFP-treated group, overexpression of HSV-wtERK2 in the VTA decreased the threshold foot-shock intensity required to elicit jumping (F_{2,29} = 23.08, p<0.001), without affecting the threshold intensity to elicit a flinch reaction. A similar pattern of results was observed for the intensity necessary to evoke vocalization (F_{2,29} = 5.68, p<0.01), with HSV-wtERK2-treated rats showing lower threshold intensity when compared with the HSV-dnERK2-treated (p<0.05), but not the HSV-GFP-treated control group (p>0.05). No differences between controls and HSV-dnERK2-treated rats were observed.

ERK2 activity in the VTA regulates anxiety-like behavior. The effects of ERK2 activity within the VTA on anxiety eliciting situations, as measured by the elevated plus-maze, are shown in Figure 4.6b (n= 13/group). A one-way ANOVA revealed that percent time spent in the open arms varied as a function of virus treatment (F_{2,36} = 10.42, p<0.001). HSV-wtERK2-treated rats spent significantly less time in the open arms (an anxiety-like response) when compared to HSV-GFP controls (p<0.05), while HSV-dnERK2-treated rats did not differ from the HSV-GFP-treated controls (p>0.05). Virus treatment also affected the percentage of entries into the open arms of the maze (F_{2,36} = 7.73, p<0.002). No differences in the percentage of entries into the open arms were observed between the HSV-dnERK2- and HSV-GFP-treated rats (p>0.05), whereas HSV-wtERK2-treated rats had a tendency toward less percent entries onto the open arms as compared to the HSV-GFP-treated controls (p= 0.057). Additionally, virus treatment influenced time spent self-grooming in the closed arms of the maze (F_{2,36} = 10.72, p<0.001). Although the HSV-wtERK2-treated rats displayed higher (p= 0.055) and the HSV-dnERK2-treated rats displayed lower (p= 0.052) self-grooming (when compared to the HSV-GFP-treated rats), these differences were marginally significant.

ERK2 regulation of VTA dopamine neuron firing rate. Our behavioral data indicate that overexpression of HSV-wtERK2 in the VTA increases susceptibility to stress, while overexpression of HSV-dnERK2 produces stress-resistant responses in several assays. Recently, we found that
resistance to chronic social defeat stress is causally related to decreased firing of VTA dopamine neurons (Krishnan et al., 2007). Based on these observations, we determined whether altered ERK2 activity modulates the firing rate of these neurons (Fig. 4.7a-c). Extracellular recordings of rat VTA dopamine neurons (n= 22-24/group) in slice cultures of brain showed that their firing rate was influenced by virus treatment ($F_{3,86}= 3.08, p<0.05$). Consistent with our behavioral data, we found that HSV-dnERK2 significantly lowered the firing rate of VTA dopamine neurons when compared to non-virus treated (GFP-) or HSV-GFP (GFP+) infected VTA slices ($p<0.05$; Fig. 4.7c). However, no differences in firing rate were observed between any of the other groups ($p>0.05$).

**DISCUSSION**

The present study was designed to assess whether acute or chronic exposure to stress alters the ERK pathway within the VTA and, if so, whether such adaptations influence an animal’s behavioral reactivity to stress and other mood-eliciting stimuli. This approach was taken because the mesolimbic dopamine reward pathway is important in the regulation of mood and motivation under normal conditions, and in behavioral abnormalities seen in depression and other mood disorders (Anisman and Zacharko, 1986; Fibiger and Phillips, 1981; Koob, 1996; Nestler and Carlezon, 2006; Willner, 1983; Wise and Bozarth, 1987; Yadid and Friedman, 2008). Although the mechanisms underlying these effects continue to be explored, several reports have implicated brain-derived neurotrophic factor (BDNF) in the behavioral and cellular adaptations that occur in the VTA after exposure to stress (Berton et al., 2006; Eisch et al., 2003; Krishnan et al., 2007), and ERK is one of the key signaling molecules downstream of BDNF (Huang and Reichardt, 2003). We show here that acute or chronic exposure to stress increases ERK1/2 activity in the VTA, and that selectively increasing ERK2 activity within the VTA enhances sensitivity to stressful situations, whereas blockade of ERK2 activity in this brain region exerts behavioral responses similar to those observed after administration of antidepressants (Iñiguez et al., 2010b).

Two major isoforms of ERK, ERK1 and ERK2, are expressed in the VTA and other brain areas. These isoforms may have distinct functions in brain (Lloyd, 2006), however, the specific role that each plays is not understood, as the two isoforms are very similar in sequence (Yoon and Seger, 2006) and the pharmacological tools currently available cannot distinguish between them (Nishimoto and Nishida, 2006). Genetic inactivation of ERK1 results in animals displaying basal locomotor alterations (Selcher et al., 2001) with compensations of enhanced ERK2 signaling in the brain (Ferguson et al., 2006), thus making behavioral interpretation of these mutants difficult. ERK2 deletions result in early embryonic lethality (Aouadi et al., 2006). We therefore took advantage of viral-mediated gene transfer, which makes it possible to assess the functional effects induced by modulation of ERK2 activity selectively within the VTA of adult animals on functional responses in several overlapping behavioral tests that assess mood in rodents.

Our findings with viral-mediated gene transfer demonstrate a clear behavioral phenotype caused by manipulating ERK2 activity within the VTA. Increasing ERK2 activity in this region increased susceptibility to stress. Animals treated with HSV-wtERK2 exhibited decreased latency to immobility and decreased escape-like behaviors in the rat forced swim test and displayed greater total immobility in the mouse tail-suspension test, effects opposite to those
elicited by antidepressant treatments (Cryan et al., 2002, 2005; Porsolt et al., 1977). The decreased latency to immobility observed in the HSV-wtERK2-treated rats did not result from deficits in motor activity because these rats did not show alterations in basal locomotion (inset, Fig. 4.3b). Increased susceptibility to stress induced by HSV-wtERK2 was further supported by our findings in the social defeat procedure. Mice expressing HSV-wtERK2 in the VTA displayed social avoidance in response to submaximal defeat episodes, a behavioral response opposite to control mice, which displayed higher social interaction under these conditions (Krishnan et al., 2007). Further experiments also revealed increased reactivity to anxiogenic stimuli upon activation of ERK in the VTA in that HSV-wtERK2-treated rats spent significantly less time in the open arms of the elevated-plus maze and more time engaged in grooming behavior, a typical behavior in response to stressful or anxiogenic stimuli (Bolaños et al., 2003a; Spruijt et al., 1988), and displayed increased sensitivity to nociceptive stimuli. Conversely, decreasing ERK2 activity in the VTA, achieved by overexpressing HSV-dnERK2, induced potent stress resistant effects in the forced swim test, tail suspension test, and chronic social defeat procedure. HSV-dnERK2 also reversed a decrease in sucrose preference induced by seven days of chronic unpredictable stress, a response categorized as antidepressant-like behavior (Di Chiara et al., 1999; Pucilowski et al., 1993; Tacchi et al., 2008; Willner et al., 1987). In contrast, HSV-dnERK2 did not affect responses to nociceptive or anxiogenic stimuli. Together, these data demonstrate that stress-induced activation of ERK2 within the VTA is both necessary and sufficient to mediate depression-like sequelae of stress, with a possible role in anxiogenic and aversive sequelae as well.

Surprisingly, we also found that elevated ERK2 activity in the VTA enhances sucrose preference, in both normal and stressed animals, a finding that appears contradictory to a depressive-like response seen in several other assays. The explanation for this paradoxical effect remains unknown. One possibility is that HSV-wtERK2 overexpression in this brain region sensitizes the reward circuitry despite inducing depression-like behavior (Yap and Miczek, 2008), thus mimicking enhanced responses to appetitive behaviors observed in rodents after stress (Lu et al., 2006). Chronic stress has been found to both decrease (Zacharko et al., 1983) and increase (Kreibich et al., 2009; Willner et al., 1998) appetitive responses, and therefore the enhanced sucrose preference induced by HSV-wtERK2 could represent a specific characteristic of a subtype of depressive disorder (Kosten et al., 1998; Nestler and Carlezon, 2006; Posternak, 2003). Clearly, further research is needed to delineate the role played by ERK in the VTA in the interactions between responses to natural reward and stress.

Previous work indicates that the dopamine system is highly responsive to stress (Horger and Roth, 1996) as both fear and restraint stress increase dopamine neuron firing rate (Anstrom and Woodward, 2005; Guarraci and Kapp, 1999), and enhanced excitability of these neurons is a signature of susceptibility to social defeat stress (Krishnan et al., 2007). We therefore assessed the effects of HSV-ERK2 treatments on VTA firing rates using a VTA slice culture preparation. We discovered that HSV-dnERK2 treatment significantly decreased the firing rate of VTA dopamine neurons, consistent with the antidepressant-like effects of this manipulation demonstrated here in several behavioral assays, whereas no change in firing rate was evident after HSV-wtERK2 treatment. A possible mechanism by which decreased ERK2 activity reduces VTA firing rate is via GABA_A receptors, since pharmacological inhibition of ERK results in an enhancement of GABA_A-gated currents in heterologous expression systems (Bell-Horner et al.,
Given the present behavioral results, it is surprising that HSV-wtERK2 did not increase VTA firing rate. It is conceivable that increased ERK2 activity does not increase dopamine cell firing per se, but may enhance the neurons’ intrinsic excitability such that the cells are primed to respond excessively in response to stress, a possibility that now requires further in vivo investigation. Also, the viral vectors utilized in the present study, while specific for neurons, target all neurons. Thus, it would be important in future studies to determine which class of VTA neurons (i.e., dopaminergic vs. GABAergic) are responsible for mediating the observed ERK2-induced biochemical and behavioral responses.

Previous studies assessing the role of ERK activity in mediating responses to stress and antidepressant-like effects are equivocal. Pharmacological inhibition of ERK activity in hippocampus, amygdala, or prefrontal cortex has been reported to induce a pro-depressant behavioral response (Duman et al., 2007; Gourley et al., 2008a; Qi et al., 2009; Tronson et al., 2008), while others report antidepressant phenotypes (Creson et al., 2009; Fumagalli et al., 2005; Huang and Lin, 2006; Todorovic et al., 2009). The discrepancies between these studies may be the result of experimental design factors, such as stress conditions, antidepressant exposure (i.e., regimen and dose), and differential effects of various pharmacological inhibitors on several cellular elements (neuronal cell bodies, nerve terminals, glia) within the injected area. In contrast, HSV vectors affect neuronal cell bodies only, and thereby enable more precise hypotheses to be tested. Regardless, the observation that manipulations of the ERK pathway in different brain regions bring about distinct behavioral phenotypes in response to stress is not surprising, since BDNF, which is upstream of ERK, induces opposite behaviors between the hippocampus (Duman and Monteggia, 2006; Shirayama et al., 2002) and the VTA-NAc circuitry (Berton et al., 2006; Eisch et al., 2003).

Our overall findings are in agreement with abundant evidence suggesting that the ERK pathway plays an important role in mediating several domains of complex behavior, including responses to drugs of abuse (Berhow et al., 1996; Girault et al., 2007; Lu et al., 2006; Lu et al., 2009) and mood regulation (Dwivedi et al., 2006; Einat et al., 2003; Manji et al., 2000; Todorovic et al., 2009). More specifically, we establish a novel role for elevated ERK2 levels within the VTA in increasing sensitivity to stressful circumstances, whereas blockade of ERK2 activity in this region results in a stress-resistant phenotype. Thus, our data identify a new neurobiological mechanism underlying stress responsiveness, and point directly to ERK2 and its many downstream signaling molecules as potential therapeutic targets for mood disorders (Manji and Chen, 2002). The present results further substantiate the influence of the VTA-NAc circuit on mood regulation, and underscore the need for a clearer understanding of the mechanisms downstream of ERK2 in the VTA that regulate depression-like behavior.
Figure 4.1. Immunoblots of rat VTA homogenates representing average expression levels of phospho (p) ERK1, pERK2, p90RSK, pMSK1, and total TH under acute (a) and chronic unpredictable (b) stress conditions. Acute stress (n= 10/group) consisted of a single exposure to forced swimming (15 min). Chronic stress (n= 5/group) consisted of daily episodes of stress for 4 weeks, ending with restraint stress (40 min). Tissue was extracted immediately after the last exposure to stress. Both acute and chronic stress significantly increased the levels of pERK1, pERK2, p90RSK, and pMSK1 (normalized to GAPDH) without affecting total levels of these proteins, or total TH when compared to controls. Data are shown as mean + SEM, with *indicating significant (p<0.05) comparisons from respective controls.
Figure 4.2. Viral-mediated gene transfer into the rat ventral tegmental area (VTA). (a) Region of VTA to which microinjections of HSV vectors were targeted. Adapted from The Rat Brain in Stereotaxic Coordinates (3rd Ed), Paxinos and Watson, 1997. (b) Cells expressing HSV-wtERK2-GFP (green: Cy2) fluorescence. (c) Cells expressing tyrosine hydroxylase (TH) (red: Cy3) fluorescence. (d) Merged image of b and c showing dual-labeled neurons in the VTA (magnification, 400X; −5 mm caudal to Bregma). Arrows indicate labeled cells. (e) Infusion of the different virus vectors (HSV-GFP, HSV-wtERK2, or HSV-dnERK2) into the VTA (n=6/group) resulted in differences in phosphorylation (p) levels of ERK1/2, p90RSK, and pMSK1. Infusion of HSV-wtERK2 increased, while HSV-dnERK2 decreased, levels of pERK2, p90RSK, and pMSK1, without altering levels of pERK1 (normalized to GAPDH) or total protein levels of ERK1, ERK2, 90RSK, and TH when compared to respective controls (HSV-GFP). Data are shown as mean ± SEM, with *indicating significant (p<0.05) comparisons from respective controls.
**Figure 4.3.** ERK2 in the ventral tegmental area (VTA) regulates behavior responses in the rat forced swim test and the mouse tail suspension test. In the forced swim test, latencies to become immobile varied as a function of viral vector treatment (n= 12-13/group). Latency to become immobile was significantly decreased in rats treated with HSV-wtERK2, and significantly increased in rats treated with HSV-dnERK2, when compared to HSV-GFP controls (*p<0.05). Total immobility was also influenced by viral treatment with HSV-dnERK2-treated rats showing significantly lower total immobility (a). Behavioral counts were affected by viral treatment (b). HSV-dnERK2-treated rats showed fewer floating- (p<0.05), and greater climbing- (p<0.05) and swimming (√p= 0.07) counts as compared to HSV-GFP controls. There were no group differences when locomotor activity (distance traveled in centimeter), rather than swimming, was quantified during testing day (b: inset; n= 4/group). Viral infusions into the substantia nigra (anatomical control; n= 5/group) did not influence latency to immobility or total immobility in the rat forced swim test (c). In mice, HSV-wtERK2 increased, while HSV-dnERK2 decreased, total immobility in the tail suspension test (n= 6/group) when compared to HSV-GFP controls (d). Data are presented as latencies to become immobile and total immobility (in sec), and as cumulative 5 sec intervals of swimming-, climbing- and floating- counts (mean + SEM).
**Figure 4.4.** ERK2 in the ventral tegmental area (VTA) regulates behavior responses in the social defeat procedure in c57BL/6 mice. (a) Schematic time-line of the submaximal social defeat procedure, where c57BL/6 mice were defeated three times within a single day, three days after HSV-GFP or HSV-wtERK2 infusions into the VTA. (b) Schematic diagram of the social interaction/avoidance arena illustrating the geographic location and size of the interaction and corner zones with respect to the enclosure in which a social target is positioned. (c) Schematic time-line of the chronic social defeat procedure, where c57BL/6 mice were defeated 10 consecutive days (10 min/episode). Mice showing clear avoidance phenotypes 24 hr after the last defeat (day 11) underwent HSV (-GFP or -dnERK2) surgery on day 12, and were tested for social avoidance behaviors on day 15 (3 days after HSV surgery). (d) Naïve mice infused with HSV-GFP (n= 6) and subjected to submaximal social defeat showed significantly higher levels of interaction (p<0.05), whereas HSV-wtERK2 overexpression in the VTA (n= 8) prevented interaction levels (p>0.05; HSV-wtERK2 target absent vs. target present). Furthermore, HSV-wtERK2-treated mice showed significantly less time in the interaction zone when compared to HSV-GFP-treated mice (p<0.05) in the presence of the target. This was also evident when assessing time in the corner zones (e), where HSV-wtERK2-treated mice spent significantly more time in the corners in the presence of the target. In the chronic social defeat experiment, we examined the effects of viral manipulation on two matched groups of socially avoidant mice (n= 6-7/group). Whereas a clear avoidant phenotype was observed (f) after an intra-VTA injection of HSV-GFP (p<0.05), overexpression of HSV-dnERK2 alleviated social avoidance (p>0.05), and showed higher social interaction ("p"= 0.06). When assessing time in the corner zone (g), HSV-GFP-treated mice spent significantly more time in the corners when compared to HSV-dnERK2-treated mice. Data are presented as mean ± SEM. *Indicates p<0.05.
Figure 4.5. ERK2 in the ventral tegmental area (VTA) regulates sucrose preference. Overexpression of ERK2 (HSV-wtERK2) in the VTA of normal rats increased sucrose preference (a), without affecting total liquid (water + sucrose) intake (b). Data are presented as percentage difference of total liquid intake (water + sucrose) between pre- and post-sucrose test (n= 12-15/group). ERK2 activity in the VTA also regulates responses to sucrose preference after one week of chronic unpredictable stress (c-d; n= 8-10/group). Stress alone significantly decreased sucrose intake in Sham (non-viral treated) and HSV-GFP-treated rats. HSV-wtERK2 overexpression increased sucrose preference above levels seen in Sham and HSV-GFP controls (c), without affecting total liquid intake (d). HSV-dnERK2 prevented the decrease in sucrose preference after chronic unpredictable stress (c). αSignificantly different between pre- and post-stress. *p<0.05: from Sham- and HSV-GFP-treated groups.
Figure 4.6. ERK2 in the ventral tegmental area regulates responses to nociceptive (a) and anxiogenic (b) stimuli. Rats with HSV-wtERK2 overexpression jumped at lower foot-shock intensities than rats overexpressing GFP (n= 10-11/group). HSV-GFP and HSV-dnERK2-treated rats did not differ in their responses to foot shock. (b) Rats overexpressing HSV-wtERK2 spent significantly less time in the open arms of the elevated plus-maze than the HSV-GFP controls (n= 13/group). HSV-wtERK2-treated rats also had a tendency toward less open arm entries, and higher grooming time within the closed arms as compared to HSV-GFP treated rats. *Significantly (p<0.05) different from HSV-GFP group. ∨ p= 0.05 when compared to HSV-GFP controls.
Figure 4.7. Decreased ERK2 lowers the excitability of ventral tegmental area (VTA) dopamine neurons (n= 22-24 cells per group). (a) GFP expression of infected dopaminergic neurons within the VTA slice culture, and a sample spontaneous triphasic waveform action potential. (b) Sample recordings from untreated (GFP-), HSV-GFP treated (GFP+), HSV-wtERK2-GFP-treated (wtERK2), and dnERK2-GFP-treated (dnERK2) VTA slice cultures. (c) HSV-dnERK2 infected VTA neurons showed a significantly decreased firing rate when compared to controls (GFP- and GFP+). *Indicates p<0.05.
CHAPTER FIVE
DISCUSSION & FUTURE DIRECTIONS

Mood-related disorders, particularly major depressive disorder (MDD), affect up to 8% of the adolescent population within the United States (Kessler et al., 2001; Olfson et al., 2008). Currently, the selective serotonin reuptake inhibitor Prozac® (Fluoxetine; FLX) is the only antidepressant medication approved by the Food and Drug Administration (FDA) for the treatment of pediatric MDD. As a result, there has been a significant increase in the prescription rate of FLX in this population (Ma et al., 2005), even though concerns have been raised about the possibility of adverse side-effects resulting from chronic exposure during stages prior to adulthood (Olivier et al., 2010). Thus, the major goal of this dissertation was to assess whether exposure to FLX during adolescence would result in enduring functional neurobiological alterations in adulthood, and to delineate the potential mechanism(s) underlying these effects using rodent animal models.

One of the major problems in basic research addressing pediatric MDD is the lack of valid juvenile animal models for the study of depression and antidepressant efficacy (Bylund and Reed, 2007). Therefore, as an initial step in this dissertation, I assessed the behavioral effects of FLX exposure in adolescent (PD35) rats using the forced swim test, one of the most widely used and validated animal models for the study of antidepressant efficacy and depressive-like behaviors in adult rodents (Porsolt et al., 1977). This was necessary to determine an efficacious dose that would result in an antidepressant-like behavioral response in the adolescent rat as it is traditionally exhibited in adult rats (see Fig. 2.1), and because age-related differences in pharmacodynamics and pharmacokinetics are often reported between adult and developing organisms (McLeod and Evans, 1992). In this initial experiment, I found that a 10 mg/kg dose of FLX resulted in significantly higher indices of escape-like behaviors (longer latency to immobility and higher swimming counts) when compared to adolescent rats treated with saline (controls), and paralleling FLX-induced antidepressant-like behaviors in adult rats. The average dose of FLX for the treatment of MDD in humans is around 20-80 mg/day, and therefore decided to administer 10 mg/kg twice daily to adolescent rats for 15 days (PD35-49) in order to assess whether exposure to FLX at this stage of development would result in enduring behavioral alterations in response to mood-related stimuli in adulthood. To do this, I examined behavioral responses to stress (i.e., forced swimming), anxiety (i.e., elevated plus-maze and novelty induced hypophagia), and reward related (i.e., sucrose, and novelty seeking) stimuli 24 hr, or three weeks (PD70+) post drug administration. The behavioral data presented in chapter two revealed that chronic FLX administration during adolescence leads to decreased responsiveness to behavioral despair measures (i.e., a stress-resistant effect), increased sensitivity to natural reward (i.e., increased sucrose consumption and novelty preference) and anxiety-eliciting situations. Interestingly, this overall behavioral phenotype was also observed in rats exposed to FLX during adolescence and tested three-weeks after drug exposure, thus indicating that such drug treatment results in long-lasting neuroadaptations in brain circuits that modulate responsiveness to rewarding and aversive stimuli in adulthood (Iñiguez et al., 2010b; Karpova et al., 2009; Warren et al., 2011).
In chapter three, to further assess whether exposure to FLX during adolescence alters behavioral responses to aversive stimuli in adulthood, in a different, yet complimentary animal species, I exposed male c57BL/6 mice to FLX throughout adolescence (PD35-49), and then exposed them to battery of stressful assays in adulthood. Here, adult c57BL/6 mice treated with FLX during adolescence displayed an enduring paradoxical stress-resistant behavioral phenotype, along enhanced sensitivity to anxiety-inducing situations as measured in the forced swim test, the social defeat procedure, and elevated plus-maze. While these results replicated my previous findings in chapter two, and further shed light on behaviors associated with resilience (Krishnan et al., 2007), they offered little insight into the molecular mechanisms underlying these enduring behavioral responses. Because a proposed mechanism by which FLX exerts its therapeutic effect(s) has been linked to intracellular signaling pathways involved in the regulation of cell survival and cell death, I decided to examine how FLX treatment influenced ERK expression in tissue samples from the VTA of mice and rats 24 hr and three weeks after treatment (to match the behavioral testing time points). As previously stated, the VTA was selected because it is part of the neural circuitry controlling mood and motivation under normal conditions (Bolanos et al., 2003b; Nestler and Aghajanian, 1997; Nestler et al., 2002). Here, I found that chronic FLX administration resulted in a downregulation of ERK2 mRNA 24 hr after treatment (in both mice and rats), along with downregulation of CREB, a well-characterized transcription factor downstream of ERK (McCubrey et al., 2008). Interestingly, this downregulation of ERK2 mRNA persisted three weeks after drug exposure, and resulted in decreased ERK-related signaling within the VTA, as inferred from the phosphorylation of ERK2 and its downstream targets RSK, CREB, and mTOR (Cryan and O'Leary, 2010; Li et al., 2011; McCubrey et al., 2008). These findings suggested that the FLX-induced decrease in ERK signaling within the VTA might mediate the stress-resistant phenotype observed in the forced swim and the social defeat procedures. To test this hypothesis, I pharmacologically decreased ERK activity by infusing the ERK inhibitor U0126 into the VTA of naïve mice and tested them in the forced swim test. As depicted in Figure 3.5b, the mice infused with U0126 displayed decreased total immobility, an antidepressant-like effect, when compared to controls. To this point, the behavioral, pharmacological, and molecular data strongly implicated decreased ERK activity within the VTA as a molecular mediator of FLX's antidepressant effects, but still did not provide causality between decreased ERK activity and antidepressant efficacy. Thus, to establish a causal link between ERK activity and behavioral responses stress, I overexpressed ERK2 (using the virus vector HSV-wtERK2) within the VTA of mice pre-treated with FLX during adolescence. I designed this experiment to examine whether this manipulation would promote depressive-like behaviors in a submaximal social defeat assay, a stress procedure that is not sufficient to induce avoidance behaviors in normal, untreated mice (Krishnan et al., 2007). That is, in this experiment, I assessed whether increasing ERK in the VTA could reverse the enduring FLX-induced “anti-stress” phenotype. Indeed, viral-mediated increases in ERK activity using HSV-wtERK2 in adult mice pretreated with FLX during adolescence resulted in avoidance behaviors after a single day of exposure to social defeat, thus reversing the FLX-induced antidepressant-like effects, providing a direct link between ERK activity within the VTA and behavioral responses to stress.

Because differences in response to pharmacological agents between adult and developing organisms are often reported (Correll et al., 2011; Iñiguez et al., 2008a; Marin et al., 2011; Neville et al., 2011), it became imperative to delineate the functional role of ERK within the
untreated (i.e., naive) adolescent VTA. To do this, I infused HSV vectors designed to increase (HSV-wtERK2) or decrease (HSV-dnERK2) ERK and its related signaling into the VTA of PD50 rats (age matched to those under pharmacological treatment). For these experiments I used rats, rather than mice, because of their larger anatomy, making it easier to target the adolescent VTA, and to further compliment the molecular findings (mRNA and protein phosphorylation) as well as the behavioral data (from chapter two) obtained from rats. Here, I found that blocking ERK activity within the VTA with HSV-dnERK2 resulted in an antidepressant-like behavioral response in the forced swim test, along with an anxiogenic phenotype as measured in the elevated plus-maze, findings identical to those observed in FLX pretreated rats tested 24 hr after drug administration. Conversely, increasing ERK activity with HSV-wtERK2 resulted in a depressive-like phenotype along with an anxiolytic behavioral response in the elevated plus-maze, as these rats spent more time in the open arms (interpreted as less anxious). Lastly, to fully delineate the role of ERK-dependent responses to stress and antidepressant efficacy as a function of age (i.e., adolescents versus adults), I characterized the role of ERK within the VTA of adult rats in chapter four. These experiments revealed that acute and chronic unpredictable stress increase ERK phosphorylation and ERK-related molecules (e.g., RSK) within the VTA. Importantly, this finding supported the behavioral responses in measures of helplessness in which ERK activity had been artificially increased using virus vectors. Thus, modulation of ERK within the adult VTA mirrored the behavioral effects observed in the adolescent VTA in measures of behavioral despair. Increasing ERK activity within the VTA, via chronic stress (Fig. 4.1) or HSV-wtERK2, resulted in a depressive-like effect, while downregulation of ERK, via FLX (Fig. 3.7), U0126 (Fig. 3.5a), or HSV-dnERK2 (Fig. 3.8), resulted in an antidepressant-like effect. Conversely, behavioral responses to anxiogenic stimuli (i.e., elevated plus-maze) were equivocal between adolescents and adults as a function of ERK signaling within the VTA. In adolescent rats, enhancing ERK activity (HSV-wtERK2) resulted in animals spending more time in the open arms of the maze (interpreted as less anxious), while in adults this behavioral phenotype was seen in rats infused with HSV-dnERK2 (compare Figures 3.9b and 4.6b). Together, these data indicate that the role of ERK within the VTA is unidirectional in mediating responses to stress-related measures in both adolescent and adult rats. On the other hand, ERK activity differentially influences responses to anxiety-inducing situations between adolescent and adult rats when assessed in the elevated plus-maze.

The clinical implications of this work are challenging since studies assessing potential enduring FLX-induced side effects in pediatric populations are lacking. This challenge is exacerbated when interpreting a behavioral phenotype in which rodents display paradoxical functional outcomes in response to stress- and anxiety-inducing situations (i.e., rats show stress tolerance, yet are more anxious). While clinical data strongly implicate a close relationship between anxiety and MDD (i.e., share common symptomology and developmental etiology), these are separate disorders (Hale et al., 2009; Kessler, 2007), thus suggesting that the behavioral results of this dissertation could shed light into the specific molecular differences/underpinnings between anxiety and MDD. Indeed, evidence is starting to accumulate showing these paradoxical behavioral phenotypes (Carlezon et al., 2005; Einat et al., 2003; Green et al., 2006; Iñiguez et al., 2010b; Wallace et al., 2009). For instance, decreasing levels of CREB, a downstream target of ERK, within the NAc, induces an antidepressive-like response while at the same time inducing anxiogenic phenotypes (Barrot et al., 2002; Green et al., 2010). Thus, these findings fully support the results presented in this dissertation because MDD is a complex, non-unitary disorder, and it
is very likely that various subtypes of the disease exist, with distinct etiology and pathophysiology (Fava and Kendler, 2000; Manji et al., 2001; Nestler et al., 2002). Many patients with MDD do not exhibit increased anxiety, but do exhibit blunted responses to emotional stimuli. While such subtypes of hyper-reactive and hypo-reactive depression have been described for decades, very little information is available concerning the underlying neurobiological mechanism involved. Nevertheless, the results presented in this dissertation do suggest that the integrity of the ERK pathway within the VTA is critical for normal responses to stress throughout discrete stages of development (adolescence versus adulthood), since this signaling cascade is an integrator of various signals that, in turn, regulate mood (Goldsmith and Dhanasekaran, 2007). Together, my findings could lead to furthering the understanding of the etiology of mood-related disorders, particularly during adolescence, a developmental hallmark for the initial episodes of depression that lead to MDD (Jankord et al., 2011).

**Future directions.** Given the neuronal heterogeneity of the VTA (dopaminergic versus GABAergic), further experimentation is needed to assess the specific phenotype and anatomical location of neurons within the VTA (anterior vs. posterior) responsible for mediating the paradoxical behaviors observed (i.e., anxiety versus stress resistant phenotypes). In addition, the role that the FLX-induced decrease in ERK activity within the VTA plays in the mediation of anxiety-related behaviors needs to be further examined. Evidence from animal research (Qi et al., 2010) indicate that behavioral inhibition (e.g. anxiety-like behaviors like freezing and withdrawal), could represent an adaptive response to threats that promotes survival (Porges, 2003). This suggests that the anxiogenic effect observed in the elevated plus-maze could potentially compliment the stress-resistant phenotype that underlies overall well-being and resilience-like behaviors (Iñiguez et al., 2010a; Krishnan et al., 2007).
MEMORANDUM

TO: Dr. Carlos Bolaños
Department of Psychology

FROM: Dr. Paul Q. Trombley, Chair
Animal Care and Use Committee

SUBJECT: Approval of Protocol #1103

DATE: April 11, 2011

"YOUR NEW PROTOCOL IS APPROVED"

The Animal Care and Use Committee approved new Protocol #1103, “Second Messenger Pathways in the Mesolimbic Dopamine System: Role in Mood, Stress, and Substance Abuse Co-morbid Disorders”, for proposed vertebrate animal use at the March 30, 2011 ACUC meeting. You are approved for the following species and numbers for the proposed protocol approval period.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number Animals Approved</th>
<th>Protocol Approval Expiration Date</th>
<th>Rewrite Due</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (Sprague Dawley)/</td>
<td>2400</td>
<td>March 30, 2011</td>
<td>February 1, 2014</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
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<tr>
<td>Mice (C57)/Mus musculus)</td>
<td>1002</td>
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</tbody>
</table>

Enclosed for your records are:
✓ A copy of the Committee Comments
✓ A copy of the Protocol and supporting documents

When you order animals on this protocol, please remember to convey the ACUC number to the LAR at 644-4262. In addition, if you do not currently have animal housing or procedural space assigned or should you need additional animal housing or procedural space, please make a request for space in writing to the Biomedical Advisory Committee (BAC) care of Kristin Auter at kauter@fsu.edu. Animals will not be ordered unless adequate animal housing/procedural space is confirmed by the LAR Facility Manager.

We appreciate your contribution to assuring that animal research at Florida State University complies with federal guidelines and regulations. Let us know if we can be of further assistance.

PQT/kji
Enclosures
REFERENCES


Bagdy G, Graf M, Anheuer ZE, Modos EA, Kantor S. 2001. Anxiety-like effects induced by acute fluoxetine, sertraline or m-CPP treatment are reversed by pretreatment with the 5-HT2C receptor antagonist SB-242084 but not the 5-HT1A receptor antagonist WAY-100635. Int J Neuropsychopharmacol 4(4):399-408.


BIOGRAPHICAL SKETCH

Sergio Iñiguez completed a Bachelors degree (B.A.) in Psychology in 2002 at California State University San Bernardino. In summer 2007, under the advisement of Professor Cynthia Crawford, he also obtained a Master’s degree (M.A.) in General Experimental Psychology at California State University San Bernardino. He began the doctoral program in Neuroscience at Florida State University in the fall of 2007 under the mentorship of Professor Carlos Bolaños-Guzmán.