

**ADOLESCENT DEVELOPMENT DETERMINES THE EFFECTS OF
AGONISTIC SOCIAL STRESS ON RAT BEHAVIOR AND LOCUS
COERULEUS PHYSIOLOGY**

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Dedication

To my family- you guys are the best!

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-Brian

ABSTRACT**ADOLESCENT DEVELOPMENT DETERMINES THE EFFECTS OF
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Stress is a causal factor in the development of many psychological disorders such as depression, anxiety, drug addiction, and conduct disorder. The degree to which stress affects the development of these disorders depends on several factors including the nature of the stressor, its timing with respect to critical periods in brain development, and genetic predispositions towards resilience or vulnerability. Adolescence is a period of development during which stress can have an enduring impact on behavior and susceptibility for affective disorders. The experiments in this thesis used the rat resident-intruder stress to model the interaction between adolescent development and the consequences of social stress on behavior and brain physiology. Rats representing 3 stages of adolescent development, early adolescent (EA, p28-p35), Mid-adolescent (MA, p42-p49) and adult (p63-p70), were placed in the cages of aggressive Long-Evans retired breeder rats daily for 7 days and tested in behavioral models of affective disorders 24-72h later. In EA rats selectively, social stress increased active coping behaviors in the defensive burying and forced swim tests. Because the locus coeruleus (LC)-norepinephrine system has been implicated in these active behaviors, LC neuronal activity was also quantified. Socially stressed EA rats had elevated LC spontaneous

discharge rates and diminished phasic responses to sensory stimuli compared to controls, similar to the effects produced by the stress-related neuropeptide, corticotropin-releasing factor (CRF). Moreover, microinjection of a CRF antagonist into the LC selectively inhibited neurons of stressed EA rats, suggesting that exposure to social stress during early adolescence induces tonic CRF release onto LC neurons, shifting the mode of discharge to a high tonic state that may promote active coping. Interestingly, opposing behavioral and neuronal consequences were seen in adults as well as in EA rats exposed to social stress but tested in adulthood. Taken together, these results demonstrate that social stress interacts with adolescent development to alter coping strategies to novel challenges, with both immediate and long-lasting effects. The data also reinforce the fact that adolescence is physiologically and behaviorally distinct from adulthood and that treatments for stress-induced psychopathologies should reflect those differences.

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CHAPTER ONE: GENERAL INTRODUCCION

Social Stress and humans

The term stress, in a biological sense, owes its definition to the physical world, where it is defined as, “a force exerted when one body or body part presses on, pulls on, pushes against, or tends to compress or twist another body or part” (Webster’s dictionary: Stress). In colloquial terms, the imagery and context of the physical definition lends itself well to the subjective experience of stress on the mind and body. As such, Webster’s dictionary also defines stress as a state of “bodily or mental tension resulting from factors that tend to alter an existent equilibrium”. Although adaptive for dealing with the environment, this change in psychological and physiological balance can have deleterious effects on the long-term functioning of critical systems in the body. Indeed, chronic stress has been linked to heart disease, diabetes, and digestive disorders (McEwen and Stellar, 1993). Stress in humans, particularly social stress, is a major predictor of later psychopathologies including depression, anxiety, post-traumatic stress disorder (PTSD), and substance abuse (Pelcovitz et al., 1994; Felitti et al., 1998b; Andersen and Teicher, 2008; Ernst and Mueller, 2008)

Social stress, development, and the brain

Social stress is a primary stressor in humans and includes physical and psychological threats, abandonment, and other relationship conflicts. Extreme social stress often is a combination of physical abuse, or threat of physical abuse involving assault, sexual abuse, physical intimidation, with emotional and verbal abuse (Follingstad et al., 1990; Edwards et al., 2003). These stressors have been shown to increase the risk of depression, relapse to substance abuse, PTSD, and other anxiety disorders in adults

(Pelcovitz et al., 1994; Felitti et al., 1998; Kaplan et al., 1998; Edwards et al., 2003; Reinherz et al., 2003; Fergusson et al., 2008). However, children and adolescents may be at particular risk for the effects of social stress because of the cerebral and social development that occur during this time period (Andersen et al., 2008). Similar to the perinatal period, the transition from childhood to adolescence is a critical time in which structural and functional rearranging of different brain regions occurs, including the prefrontal cortex, amygdala, hippocampus, cerebellum, and white matter tracts such as the corpus callosum (Thompson et al., 2000; Gogtay et al., 2006; Shaw et al., 2008). The maturation process of each of these areas occurs in specific time windows. Cortical maturation typically proceeds from posterior to anterior with respect to age and phylogeny (Gogtay et al., 2004). The average thickness of the cortex peaks at about 9.5 years old, however, depending on the brain area, peak thickness occurs anywhere from age 7 (visual cortex) to age 13 (frontal cortex). Subsequent pruning patterns, as determined by cortical thickness, are either linear, quadratic, or cubic, depending on the type of cortex and complexity of its laminar structure (Shaw et al., 2008). The corpus callosum and other white matter tracts also continue to thicken and grow in a region by age manner which follows the transitions noted in cortical development (Thompson et al., 2000). Many changes in these regions are also sexually dimorphic and lateralized (Giedd et al., 1997; Durston et al., 2001). For example, males have larger right amygdala than females, whereas females have larger left hippocampus (Giedd et al., 1996). Hippocampal volume changes across development also vary by anterior-posterior level within the hippocampus itself (Gogtay et al., 2006).

Social stress in adolescence is associated with altered structural development in each of these age sensitive brain areas, dependent on the state of maturity at the timing of the insult (Teicher et al., 2003; Andersen and Teicher, 2008). Andersen, et al. demonstrated that the locations of structural changes in the brains of adult women, caused by childhood sexual abuse, are dependent on the age at which the abuse occurred. Decreases in hippocampal volume were noted if the abuse occurred between the ages of 3-5 and 9-11. The size of the corpus callosum was decreased if it occurred between the ages of 9-10, whereas the frontal cortex was attenuated if the abuse happened later in adolescence from the ages of 14-16 (Andersen et al., 2008). A meta-analysis of MRI studies on depressed adults show that depression is associated with a 8-10% decrease in hippocampal volume (Videbech and Ravnkilde, 2004); however, the effect of childhood and adolescent stress on hippocampal size is less clear. Gender, duration and type of stressor, development, and ultimate psychopathology may all interact to determine the effect of childhood and adolescent stress on hippocampal volume (Bremner et al., 1997; Vythilingam et al., 2002; Tupler and De Bellis, 2006).

Stress can also alter the functional properties of neural circuits (Teicher et al., 2003). Interactions between the amygdala and prefrontal cortex mediate the emotional and cognitive response to emotionally laden images. Stress and stress-related disorders such as post-traumatic stress disorder (PTSD) and major depressive disorder are generally associated with increased amygdala activity (Roberson-Nay et al., 2006), however this activation seems to be dependent on past stressors and the type of psychopathology present (Taylor et al., 2006; Beesdo et al., 2009). Various regions on the prefrontal cortex, like the dorsolateral prefrontal cortex (DLPFC), inhibit amygdala

activity during conflict tasks where the person is asked to perform a task in the face of emotionally distracting stimuli. In these tasks, people with depression show an increase in amygdala activity as well as a failure to increase DLPFC activity, which occurs in controls (Roberson-Nay et al., 2006; Fales et al., 2008) However, Fales and colleagues found that successful treatment with citalopram normalized the DLPFC and the amygdala activity in this task (Fales et al., 2009). As adolescents mature, the frontal cortex becomes more active in response to fearful stimuli (Yurgelun-Todd and Killgore, 2006), therefore, it stands to reason that stress during this time of development may alter cortical activity and subsequent corticolimbic regulation.

Social stress and psychopathology

As previously noted, early life social stress is a major risk factor for the development of psychiatric disorders, both during adolescence and in later adulthood (Pine et al., 2002). As the family is the primary social unit during childhood and adolescence, it is not surprising that dysfunctional family relationships are a leading risk factor for psychiatric disease (Reinherz et al., 2003; Herrenkohl et al., 2009). According to the US Department of Health and Human Services, 90% of child maltreatment, especially sexual and physical abuse, occurs in the home by a parent, a parent's partner, or other family member (Administration for Children and Families, n.d.) Bullying, taunting, and threats outside of the home are another potentially damaging form of social stress for children and adolescents (Gladstone et al., 2006; Sourander et al., 2007). In fact, experience both as the victim and as the bully increases risk for later depression and suicide (Kaltiala-Heino et al., 1999). These stressors in early life increase the risk for

adolescent depression, anxiety, conduct disorder, PTSD and substance abuse (Kaplan et al., 1998).

Exposure to violence, both as a recipient and a witness increases aggression and violence perpetuation especially in boys (Dodge et al., 1990). Violence in the form of physical and sexual abuse in the home is especially damaging with respect to violence perpetuation and self-mutilation or suicide (Duke et al., 2010). Presence of conduct disorders and behavioral problems in adolescence, as a result of early social stress, may be a compounding factor for the formation of other psychiatric disorders as their presence also increases the risk for both unipolar and bipolar depression, as well as social maladjustment and personality disorders (Rowe et al., 1996; Endrass et al., 2007; Biederman et al., 2008). While most children are able to overcome the effects of these disorders, early life stress and stress-related psychiatric disorders markedly increase the risk for adult psychiatric disease (Kaplan et al., 1998; Pine et al., 1998; Hofstra et al., 2002; Pine et al., 2002; Roza et al., 2003; Turner and Lloyd, 2004; Herrenkohl et al., 2009). Early psychosocial stress also has long term health consequences in the form of depression, sexual promiscuity, high cholesterol, obesity, and other metabolic disorders as well as drastically increased risk for substance abuse (Felitti et al., 1998; Danese et al., 2009).

Animal social stress as a model of human stress

While many retrospective human studies have shown a correlation between stress and psychological disorders, and prospective studies have suggested causality, investigations into the mechanisms of stress-induced psychopathology in humans are

limited by obvious ethical considerations. For these reasons, animal models are created that take advantage of conserved biology across species to allow inference to the underlying mechanisms of disease in the human condition. However, for complex human psychiatric conditions like depression and anxiety, it is impossible to recreate the syndrome in its entirety with animal models. Therefore, models are devised that use experimental conditions designed to model aspects of a much more complex disorder. This allows the researcher to alter genetic, pharmacological, and environmental conditions to investigate the underlying mechanisms behind complex disorders.

To model the effects of human social stress, several types of experimental paradigms have been designed using rodents, including social isolation (Hatch et al., 1963), visible colony systems (Blanchard et al., 1995), and resident-intruder stress (Miczek, 1979). Investigators can take advantage of the dominant-submissive relationships that are naturally established between rodents to model elements of human social conflict (Björkqvist, 2001; Huhman, 2006). Hamsters are especially amenable to dominance based stressors as they are naturally territorial and solitary. Rats and mice are amenable to both isolation and dominance-based stressors because they readily form colonies with defined dominant relationships.

Resident-intruder stress (also referred to as social defeat) has proven to be an especially useful model of social stress in adult rats. Initially developed by Von Holst and Raab in tree shrews to model the physiological and psychological effects of social conflict, it was adapted for rats by Miczek in 1979 as a model to investigate the effects of psychomotor stimulants on aggression (Holst, 1972; Miczek, 1979; Raab and Oswald, 1980; Walletschek and Raab, 1982). In this model an intruder animal is placed in the

home-cage or territory of another, often larger or more aggressive resident animal. The two are allowed to freely interact for a period of time and, because of the territorial nature of these animals, a fight will generally ensue with the larger, more aggressive animal dominating the other. Once this domination is established or a set period of time has elapsed, the animals are again separated. As a model of social conflict, social defeat produces a constellation of effects in the subjugated animal that are similar to specific clinical manifestations in depression including anhedonia, weight loss, social anxiety, and alterations in the hypothalamic-pituitary-adrenal axis (HPA) that facilitates the endocrine response to novel stressors (Koolhaas et al., 1997; Von Frijtag et al., 2000; Bhatnagar and Vining, 2003; Rygula et al., 2005; Bhatnagar et al., 2006). Many of the effects of social defeat on metabolism, anxiety, and the functioning of the HPA functioning are long-lasting but can also be tempered by social housing and anti-depressant treatment (Ruis et al., 1999; Von Frijtag et al., 2000; de Jong et al., 2005; Rygula et al., 2008).

Social defeat may mediate the aforementioned effects via alterations in the central monoamine and peptide systems associated with the stress response. It increases serotonin in the hippocampus acutely (Keeney et al., 2006) while desensitizing the serotonin 1A receptor (5-HT_{1A}) response both after acute stress and following chronic stress. This desensitization is evident in both the corticosterone and hypothermic response to the 5-HT_{1A} agonist, 8-OH-DPAT as well as hippocampal 5-HT_{1A} binding (Korte et al., 1995; McKittrick et al., 1995). Within the dorsal raphe, where many of the forebrain-projecting serotonin cells reside, social defeat has been shown to increase transcription of genes responsible for neurotransmitter release and signal transductions. Chronic treatment with citalopram, a selective serotonin reuptake inhibitor (SSRI) antidepressant,

has been shown to counter the changes in several of these genes (Abumaria et al., 2006; Abumaria et al., 2007). Social stress also interacts with the mesolimbic dopamine system. Acute exposure to social defeat has been shown to increase phasic dopamine firing in the ventral tegmental area (Anstrom et al., 2009) and increase dopamine release in the accumbens and prefrontal cortex (Tidey and Miczek, 1996). While typically thought of as a reward pathway, activation of the mesolimbic dopamine system also occurs in response to stress and is thought to facilitate behavioral responses to novel stimuli, including social avoidance following social defeat via brain-derived neurotrophic factor (Berton et al., 2006; Miczek et al., 2008)

Acute social defeat has been shown to induce c-fos activation in limbic brain regions crucial to stress reactivity like the amygdala, bed nucleus, dorsal raphe, lateral septum, and locus coeruleus. This regional pattern of activation changed slightly following chronic exposure to social defeat in that the septum, central amygdala, and locus coeruleus no longer expressed c-fos (Martinez et al., 1998). Even though c-fos expression returns to normal in the LC after chronic defeat, the activity of tyrosine hydroxylase is increased, indicating increased norepinephrine synthesis (Watanabe et al., 1995). Chronic social defeat also increases the mRNA levels of preprogalanin in LC (Holmes et al., 1995), a peptide that has been implicated in anxiety and coping behaviors (Echevarria et al., 2005).

Social defeat also modulates corticotropin-releasing factor systems (CRF). Investigations using hamsters have indicated that learned expression of submissive behaviors following an initial experience of social defeat is dependent on activation of the central nucleus of the amygdala (CeA) and CRF release in the bed nucleus of the stria

terminalis BNST (Jasnow et al., 2004; Cooper and Huhman, 2005). Presumably this could indicate that previous experience with social defeat mediates later submission via sensitized CRF input into the BNST from the CeA. Circuits between the CeA and dorsal raphe (DR) may be crucial in the acquisition of this conditioned defeat, as CRF1-receptor antagonists within the DR have been shown to block acquisition and expression, while CRF2 antagonists only block expression (Cooper and Huhman, 2007). CRF1 antagonists into the basolateral amygdala of mice also blocked post-defeat submissive postures, suggesting that this region may also play a role in the CeA-BNST mediation of conditioned defeat. Wood and colleagues have also recently demonstrated that individual susceptibility to exhibit submissive behavior during chronic social defeat is associated with a decrease in CRF protein and mRNA within the paraventricular nucleus of the hypothalamus as well as CRF₁ receptor protein in the pituitary (Wood et al., 2010). This fits well with data from Albeck et al. which indicate a similar decrease in CRF message in the PVN but an increase in CRF message in the amygdala of subordinate rats (Albeck et al., 1997) after defeat. Chronic social stress also increases CRF mRNA and CRF labeled neurons in Barrington's nucleus which mediates the visceral effects of social defeat on urinary pathologies (Wood et al., 2009).

Animal models of adolescent social stress

The majority of the experiments that model social stress do so in adult animals. Fewer studies, have examined the effects of social stress in adolescent animals. This is surprising given that adolescence is a time of unique social and physiological maturation

during which exposure to stress may have unique consequences that are not replicable in adults.

Social Isolation

Social isolation is one of the more commonly used forms of adolescent social stress (Lukkes et al.). The actual paradigms differ widely, but social isolation in pre-adolescent and adolescent rats typically encompasses the 4th-6th week of life and results in rats who are hyperlocomotive in the open field (dependent on strain) with increases in startle response and impaired prepulse inhibition (Heidbreder et al., 2000; Weiss et al., 2004). These behavioral changes are associated with region specific changes in serotonin and dopamine as well as heightened HPA responsivity to stress (Heidbreder et al., 2000; Serra et al., 2005; Lukkes et al., 2009)

Hamster studies

Akin to studies in adults, rodent models of social stress could have high validity in terms of modeling the effects of agonistic social stress during this critical period in development. To date, the majority of studies which investigate social defeat in adolescence in rodents have been done in hamsters; the results of which have helped to clarify the immediate and long-term effects of social stress on aggression and anxiety. In hamsters, adolescence social defeat seems to have divergent effects on aggression when compared to adult defeat. Delville and colleagues found that social defeat increased subsequent aggressive behaviors towards other hamsters of equal or smaller stature while decreasing aggressive behavior towards larger conspecifics (Delville et al., 1998). In a

similar study, Ferris, et al. confirmed that adolescent social defeat increases later aggressive behavior, even initially towards adults. Similar to the Delville report, however, if these adolescent animals were subjugated again in adulthood it caused a lasting decrease in aggressive behaviors (Ferris et al., 2005). In another study by the same group, it was determined that animals who were subjugated as early adolescents transitioned from “play attack” interactions with conspecifics to adult aggression at an earlier age (Wommack and Delville, 2003).

Social stress during adolescence may increase aggression and decrease submission via interactions with testosterone. In hamsters, the timing of adolescent exposure to androgenic steroids helps to program aggressive and submissive social behaviors (Salas-Ramirez et al.; Schulz and Sisk, 2006). Likewise, exposure to social stress during adolescence has long-lasting protective effects against reductions in testosterone caused by later social defeat (Ferris et al., 2005). Combined, these hamster studies support the human literature describing a link between early abuse and adolescent aggression (Dodge et al., 1990). However, hamsters naturally exist in isolated territories, therefore, while studies using hamsters are illustrative of the effects of social stress on aggressive behavior, rats may provide a better model for the human condition because of their social nature. To this end, two studies have been performed using socially subjected adolescent rats to the resident-intruder paradigm.

Rat studies

Watt and coworkers (2009) found that social defeat stress in early adolescence (postnatal days 35-40) increases anxiety and risk assessment behavior in the stressor

context as adults. Interestingly they also found that social defeat increased the locomotor response to novelty in adulthood and altered adult monoamine levels, including decreased medial prefrontal cortex dopamine, increased norepinephrine and serotonin in the ventral dentate gyrus, and decreased norepinephrine in the dorsal raphe. Vidal et al. (2007) performed a similar study in which adolescent animals (p45- p57) were subjected to intermediate defeat for an extended period of time. They found that intermediate adolescent social stress caused a long-lasting social avoidance to an unknown resident rat with no changes in monoamine levels. Both of these studies determined that adolescent social defeat increases inhibited behavior towards contextual reminders of the defeat scenario as adults. The differences between the two studies in terms of monoaminergic content may be due to the different ages at which the stressor was applied and the increased regional specificity of the micro-dissections performed in the Watt paper.

These studies show convincingly that in rat models, social defeat can have enduring consequences on social anxiety related measures. However, until now, there have not been any studies which systematically analyze the effects of agonistic social stress on behavior and brain physiology in rats across adolescent development.

Rodent behavior tests as indices of psychopathology

Because of obvious ethical concerns, it is difficult to design experiments that would establish mechanistic relationships between stress conditions and subsequent psychopathology using human subjects. Therefore, investigators create animal models which take advantage of the similarity in stress systems among different species.

However, these models can't recreate complete human psychological disorders, therefore, many of the behavioral tests used to evaluate the psychological impact of stress models are designed to measure certain aspects of disorders like anxiety (avoidance, fear reactivity, freezing) or depression (anhedonia, helplessness, altered HPA activity). Described below are 4 of the most commonly used tests.

Elevated plus maze

The elevated plus maze was designed by Handly and Mithani in 1984 and is based on previous work by Montgomery in the 1950's (Montgomery, 1955; Handley and Mithani, 1984). It consists of an elevated platform with 4 arms in a '+' shape with two of the arms having enclosed walls and 2 that are open. The premise of the test is based on the approach-avoidance theory of rodent novelty put forward by Montgomery. This theory states that, upon exposure to a novel environment, rodents have 2 competing drives; exploration and avoidance. Treit later refined the practical application of this theory by showing that it's the open space, not novelty, that the rats find aversive (Treit et al., 1993). Hence, the endpoints of this test are typically time spent in the open vs. closed arms, number of entries in each arm and total distance traveled. Using anxiogenic and anxiolytic drugs, Handly and Mithani sought to alter the balance of open arm exploration-avoidance as a model of fear or anxiety. Pellow and coworkers then pharmacologically validated and expanded the model showing selective responses to known anxiolytic and anxiogenic psychotropic drugs over antidepressants, barbiturate sedatives, and antipsychotics (Pellow et al., 1985).

Since its inception, the elevated-plus maze has become one of the most popular tests for anxiety due to its simplicity and ease of use, however its major drawback is that treatment differences can be difficult to replicate across labs (Hogg, 1996; Crabbe et al., 1999). Investigators most commonly use this test to screen novel compounds for anxiolysis and as a model for stressed-induced anxiety.

Open Field test

Originally developed by Hall in 1934 to measure emotionality in rats, the Open Field test consists of a single open arena bordered by high walls. In Hall's initial experiments the rat was placed in the arena and emotionality was determined by defecation, urination and ambulation (Hall, 1934, 1936; Walsh and Cummins, 1976). In present day, the open field test remains one of the more popular tests for anxiety-like behaviors. It is similar to the elevated plus maze in that it operates on the same internal exploration-avoidance premise. Because of their aversion to open spaces, rats prefer to spend most of their time near the outer wall, a phenomenon called thigmotaxis. Therefore, the arena is often divided into concentric zones and the time spent in the center-most zones and distance traveled in each zone are the most typically used indicators of anxiety (Prut and Belzung, 2003).

Similar to the elevated plus maze, the open field test is sensitive to classical anxiogenic pharmaceuticals as well as stressors and environmental manipulations. Anxiolytic agents such as benzodiazepines and 5-HT_{1A} receptor agonists increase center time while anxiogenic drugs like CRF decrease it (Britton et al., 1982; Prut and Belzung, 2003). The open field is also useful for determining the effect of various treatments on baseline locomotor activity, thus controlling for possible confounding changes in general

locomotion on other tests whose final endpoints have some element of locomotor activity. However, one drawback of the open field is that its predictive validity as a model for generalized anxiety disorders is less than optimal, given that SSRI's, which are clinically effective treatments for generalized anxiety disorders, are generally not detected by this test (Prut and Belzung, 2003).

Defensive Burying test

When faced with a threat or a noxious stimulus, rodents will spray bedding or dirt in the attempt to bury it. Described as a species specific defense response, this behavior complements flight, freezing, and fighting in the repertoire of unconditioned behaviors that rodents use to cope with threats in their environment (De Boer and Koolhaas, 2003). Rodents have been shown to bury shock prods (Pinel and Treit, 1978), mouse-traps (Terlecki et al., 1979), vials with predator odors (Holmes and Galea, 2002), as well as noxious and aversive-conditioned food (Wilkie et al., 1979). Initially described by Hudson in 1950, Pinel, published the paradigm that would come to be known as the defensive burying test in 1978 and it has changed little since (Hudson, 1950; Pinel and Treit, 1978). It uses an electrified shock prod that is inserted into a hole on one side of a cage filled with bedding. Upon investigating the novel prod, the rat receives a small shock. Soon after shock the rodent will begin to bury the probe with bedding by pushing it with the forepaws, nose and head. The duration of burying time, latency to bury, and total height of the pile are typically the measurable endpoints of this test, although, researchers will sometimes measure immobility, grooming, and ambulation as an alternative behaviors.

Because the primary endpoint of this test is an active response to an identifiable threat, the defensive burying test operates on a different premise than the open field or elevated plus maze. In this sense, burying behavior can be considered an active-coping behavior used to deal with an adverse environmental stimulus. Indeed, Korte, et al found that if a rat is deprived of bedding material in the shock test, then the resulting corticosterone levels increase dramatically over the animals who were allowed to bury (Korte et al., 1992). Even though it has proven to be an effective screen for a wide variety of anxiolytic drugs (Treit et al., 1981; Korte et al., 1994; Lopez-Rubalcava et al., 1996), behavior in the defensive burying test does not always coincide with behavior in the elevated plus maze or open field (Pesold and Treit, 1995; Basso et al., 1999; Sandbak and Murison, 2001). This indicates that behaviors in these tests, even though they are all thought to measure the anxiety state of the animal, may be subserved by distinct neuronal circuitry.

Defensive burying behavior is also subject to previous stressors. The presence of bedding from a previous encounter with an aggressive conspecific decreased burying and augmented freezing (Williams and Scott, 1989). Likewise, Williams also found that uncontrollable stress, as well as exposure to bedding from another rat's experience with uncontrollable stress, decreased burying and increased freezing behavior. However, controllable shock was protective against this switch in coping mechanisms (Williams, 1987). Interestingly, if animals are exposed to the defensive burying test prior to uncontrollable shock, and then re-exposed to an inactivated probe, they bury more than controls (Bruce Overmier et al., 1994). This suggests that an interaction may occur whereby uncontrollable stress heightens reactivity to prior stressors while creating a

“learned helpless” like phenotype for later responses. Withdrawal from drugs of abuse also causes an increase in burying behavior that is associated with CRF and norepinephrine (Aston-Jones and Harris, 1993; Basso et al., 1999; Harris et al., 2001)

Forced Swim Test

The Forced Swim test was originally developed by Porsolt et al in 1977 as a screen for novel anti-depressant medications (Porsolt et al., 1977; Porsolt et al., 1978). Rats, when placed in a cylinder of water initially exhibit escape behaviors including climbing (describe), swimming and diving. These behaviors alternate with periods of immobility or floating. Porsolt and co-workers interpreted periods of immobility as reflective of “behavioral despair”. Upon re-exposure to the cylinder on the following day the rats quickly adopt the immobile posture, spending approximately 75% of the time immobile (Porsolt et al., 1977). Application of known anti-depressants between swim exposures was shown to decrease immobility on the second day while clinically non-active drugs had no effect (Porsolt et al., 1977). The implication was that this was a valid test for antidepressant activity and therefore the neural basis of the behavior might be relevant to depression. Initial reports suggested that the procedure was ineffective in detecting the antidepressant potential of SSRI’s. However, astute analysis of the behavior, including discrimination of climbing vs. swimming, and certain modifications of the procedure improved detection of this class of drugs (Lucki, 1997). Analysis of distinct forms of escape behavior during the swim stress revealed that selective serotonin and selective norepinephrine uptake inhibitors decrease immobility via increases in climbing and swimming, respectively (Detke et al., 1995).

Much of the criticism of the Forced Swim Test has surrounded the initial characterization of the test as a model of depression associated learned helplessness. Some believe that the test doesn't create the dynamic change in physiology and behavior required to be considered a model of a depressive state (Cryan et al., 2005). Others question the fact that the same drugs which acutely cause such a dramatic decrease in immobility require chronic administration in humans (up to 8 weeks) before full clinical efficacy is achieved, whereas their efficacy in the model is observed with subchronic administration. Therefore some would argue that, while an effective screen for anti-depressant action, the forced swim test has limited utility as a model for depressive behavior or for therapeutic mechanism. While admittedly not a complete model for the human condition, several lines of evidence contradict those arguments. First, in humans, stress has been shown to be a causal factor in the development of depression, which is reversed with anti-depressant treatment. Likewise, various rodent stressors have been shown to promote immobility in the forced swim test, including uncontrollable shock (Weiss et al., 1981) and social defeat (Rygula et al., 2005; Becker et al., 2008), which is prevented with administration of anti-depressants. Secondly, Detke and coworkers demonstrated that chronic administration of desipramine and fluoxetine, respective norepinephrine and serotonin reuptake inhibitors, decrease immobility and increase climbing and swimming at doses which are ineffectual in acute treatments- mirroring the chronic course of treatment required by humans (Detke et al., 1995). For a more in-depth discussion see Cryan et al, 2005.

Locus Coeruleus as a mediator of stress

Anatomy and function

The locus coeruleus (LC) consists of a small group of neurons in the dorsal medulla near the lateral sides of the 4th ventricle. Even though it's a small region, the LC sends highly collateralized axons into cortex, most of the forebrain and midbrain, as well as the spinal cord. Because of this is a primary source of norepinephrine (also known as noradrenaline) for much of the brain, including the sole source for the cortex (Swanson and Hartman; Foote et al., 1983). Because of its extensive innervation, LC activity subserves a variety of functions including general arousal, attention, sensory gating, and cognitive flexibility (Aston-Jones and Bloom, 1981b; Usher et al., 1999; Berridge and Waterhouse, 2003; Lapid and Morilak, 2006). LC neurons are electrotonically coupled and fire both tonically and phasically (Aston-Jones and Bloom, 1981a; Ishimatsu and Williams, 1996). Through alterations in its firing modality, the LC is able to exert its specific effects, whether it is arousal or modulation of the salience of sensory stimuli (Foote et al., 1980; Aston-Jones and Bloom, 1981b). Studies in which LC neurons were recorded in monkeys performing an oddball discrimination task suggested that when subjects are focused on stimuli that are necessary in performing the task, LC neurons are phasically active. In contrast, when LC tonic discharge rate increases above some optimal level, the cells are not phasically responsive to sensory stimuli and this is associated with a behavioral of hyper arousal, scanning attention and going off-task (Aston-Jones and Cohen, 2005).

The primary inputs into the core of the LC are sensory in nature. It receives multimodal sensory input from the nucleus paragigantocellularis and visual input from the prepositus hypoglossi (Aston-Jones et al., 1986). The dendrites of the LC extend into the surrounding area, however, and there receive input from limbic regions like the

amygdala and BNST (Aston-Jones et al., 1986). The LC receives both excitatory and inhibitory inputs (via interneuron connections) from the frontal cortex (Sara and Herve-Minvielle, 1995; Jodo et al., 1998) which may serve as the descending input that carries relevant information about task and reward status (Aston-Jones and Cohen, 2005).

Stress mediator

Like activation of the hypothalamic-pituitary-adrenal axis, activation of the LC-norepinephrine system is a critical component of the central stress response, serving to increase arousal and shift the mode of attention from focused to scanning. LC activation has been shown to occur in response to a variety of stressors and stress-related behaviors including restraint, tailshock, defensive burying, and forced swim (Abercrombie et al., 1988; Duncan et al., 1993; Bondi et al., 2007). CRF is a likely mediator of LC activation during stress. Initially identified and characterized by Vale and colleagues in 1981 as the hypothalamic neuropeptide that initiates peripheral release of adrenocorticotrophic hormone (ACTH), CRF is also a critical neurotransmitter in the coordinated response to stress within many regions of the brain, including the LC (Vale et al., 1981; Chappell et al., 1986; Owens and Nemeroff, 1991; Valentino et al., 1993). LC neurons receive CRF innervation from Barrington's nucleus, the bed nucleus of the stria terminalis, the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala (Swanson and Hartman, 1975; Valentino et al., 1992; Asan, 1998; Bockstaele et al., 1998; Reyes et al., 2005).

CRF release onto LC neurons has profound effects on LC activation and firing modality. Stress-induced release of CRF increases the spontaneous LC discharge and

inhibits sensory evoked phasic discharges (Valentino and Foote, 1987, 1988; Valentino et al., 1991). This shift in firing modality has been associated with scanning, labile attention (Aston-Jones and Cohen, 2005). CRF-induced increases in tonic firing rate raises norepinephrine levels in the cortex and deregulates cortical electroencephalography activity, reminiscent of a highly aroused state (Curtis et al., 1997). In the behaving animal, CRF in the LC has been shown to increase behavioral responses to stressors in a form reminiscent of increased anxiety or arousal (Butler et al., 1990; Swiergiel et al., 1992). Given the interaction between stress, CRF, and NE, it is also likely that stress-induced CRF activation of LC neurons mediates other stress behaviors which are also NE dependent, including defensive burying, grooming, and reduced exploration (Morilak et al., 2005; Howard et al., 2008).

In addition to activating the LC system, stress engages inhibitory endogenous opioid systems that oppose the effects of CRF and may serve as a functional brake on the excitatory effects of stress on the LC system (Valentino and Wehby, 1988b; Curtis et al., 2001; Valentino and Van Bockstaele, 2001; Kreibich et al., 2008). Receptors for each of the 3 major opioid systems are expressed in the LC; however, they each inhibit LC function in a specific manner (Valentino and Wehby, 1988b; Tjounakaris et al., 2003; Reyes et al., 2007). Morphine acting at the μ -opioid receptor has been shown to primarily decrease spontaneous activity in both anesthetized and unanesthetized rats while having limited effect on evoked activity, effectually increasing the signal to noise ratio of the cell (Valentino and Wehby, 1988b). However, Zhu and Zhou have shown that morphine also interacts with excitatory amino acid innervation to induce synchronous oscillations in LC activity (Zhu and Zhou, 2005). This may indicate multiple levels of fine tuning available

in response to stress. In contrast to the effects of morphine, Kreibich, et al recently found that κ -opioid agonists have no effect on spontaneous LC firing rate, but they robustly attenuate phasic activity induced by sensory stimulation probably through presynaptic actions on glutamatergic axon terminals innervating LC neurons (Kreibich et al., 2008). Interestingly, κ -opioid agonists also attenuate stress-induced LC activation, which suggests that they inhibit pre-synaptic release of both excitatory amino acids and CRF onto LC neurons.

Goals of Thesis Research

The primary goals of this research were to determine if and when agonistic social stress alters the behavioral profile of adolescent rats in tests related to affective disorders and to investigate the possible neurological mechanisms involved. These studies were undertaken because research in humans indicates that adolescence is a time of increased risk for stress-induced affective disorders. As previous investigations of adolescent social stress have been limited in their scope, both in terms of the ages analyzed and the measures taken, the current thesis seeks to systematically examine these questions and provide a broader understanding of how social stress interacts with adolescent development.

To model adolescent social stress, an adaptation of the resident-intruder stress is used. This social stressor has been well validated in adult rats as an effective means of inducing long-lasting depressive-like phenotypes in rodent behavioral tests. As rat social behavior develops and changes throughout adolescence, it was hypothesized that resident-intruder stress would alter behaviors in a manner dependent on the intruder's

stage of social development. Performance in the open field test, the defensive burying test, and in response to swim stress is used as the behavioral endpoint. The open field test is thought to measure the state of anxiety primarily through innate behavioral avoidance of an aversive open space. In contrast, the defensive burying test is thought to measure the state of fear reactivity or agitation via active-coping with an identifiable threat. Because of their distinct natures, behaviors in the open field and defensive burying tests are not always concordant and therefore may be reflective of distinct elements of the rodent fear circuitry. Finally, performance in the swim stress provides an indication of depressive-like behavioral traits induced by social stress.

As each of these behaviors is modulated by elements of the norepinephrine system, *in vivo* single unit electrophysiology was performed on locus coeruleus neurons to determine a plausible neuronal mechanism for behavioral change. It was hypothesized that activity within the LC would reflect any stress-induced alteration in behavior. Because CRF is a primary mediator of stress-induced LC activity, it was further hypothesized that changes in the LC would be associated with the CRF system.

Stress can have devastating consequences on the health and well-being of children and adolescents. These studies were designed to help elucidate the conditions and mechanisms whereby stress precipitates psychopathology. Additionally, the results of these studies could help to direct the treatment of adolescent stress-disorders in a more focused manner by highlighting the potentially distinct effects of stress during adolescence vs. adulthood on the CRF-LC-NE system.

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**CHAPTER TWO: EARLY ADOLESCENCE AS A CRITICAL WINDOW
DURING WHICH SOCIAL STRESS DISTINCTLY ALTERS BEHAVIOR AND
BRAIN NORADRENERGIC ACTIVITY**

Abstract

Many neural programs that shape behavior become established during adolescence and adverse events that impact on these programs can have enduring consequences for mental health. Here we show that repeated social stress exposure differentially impacts behavior and brain physiology during critical windows throughout adolescent development. Early-adolescent (p28-p35, EA), mid-adolescent (p42-p49, MA), and adult (p63-p70) rats were subjected to 7 days of social stress using the resident-intruder paradigm and tested in behavioral models of affective disorders 24-72h later. In EA rats selectively, social stress increased active coping behaviors in the defensive burying and forced swim tests. Because the locus coeruleus (LC)-norepinephrine system has been implicated in these active behaviors, LC neuronal activity was quantified. Socially stressed EA rats had elevated spontaneous LC discharge rates and diminished phasic responses to sensory stimuli compared to controls, similar to the effects produced by the stress-related neuropeptide, corticotropin-releasing factor (CRF). Moreover, microinjection of a CRF antagonist into the LC selectively inhibited neurons of stressed EA rats, suggesting that exposure to social stress during early adolescence induces tonic CRF release onto LC neurons, shifting the mode of discharge to a high tonic state that may promote active coping. Interestingly, opposing behavioral and neuronal consequences were seen in adults as well as in socially stressed EA rats that were tested as adults. The results suggest that the interaction of social stress with common neural substrates at different developmental stages has distinct effects on coping strategies that may be expressed as different psychological dysfunctions.

Introduction

Stress can shape future behavior and is a precipitating factor for affective disorders such as anxiety and depression and for substance abuse (Gilbert and Allan, 1998; Goeders, 2003; McEwen, 2003; Binder et al., 2008). Stress has complex interactions with development such that individuals may be especially vulnerable to stressors during specific developmental periods (Heim and Nemeroff, 2001). In humans and in animal models, the prenatal and neonatal periods are critical windows in development during which stress can have enduring effects on behavior and endocrine function (Plotsky and Meaney, 1993; Liu et al., 2000; Talge et al., 2007; Cottrell and Seckl, 2009; Murgatroyd et al., 2009; Buss et al., 2010). Similarly, adolescence is a critical window in stress susceptibility as this is a time of substantial cerebral development and reorganization as well as altered hypothalamic-pituitary-adrenal function (Spear, 2000; Teicher et al., 2003; Turner and Lloyd, 2004; Romeo and McEwen, 2006; Andersen and Teicher, 2008; Danese et al., 2009; McCormick and Mathews, 2009; McCormick et al., 2010).

Social stress is a salient and effective stressor that has particular relevance to adolescents (Gladstone et al., 2006; Herrenkohl et al., 2009; Sebastian et al., 2010). In humans and animals, adolescence is characterized by an increase in child-parent conflict, search for autonomy, and a shift in social interaction from primarily familial to peer relationships (Panksepp, 1981; Spear, 2000). With an increased importance of social signals and activity there comes an increased potential for adverse social stimuli to elicit a stress response. An ethologically relevant model that has been used to study social stress in rats is the resident-intruder stressor (Miczek, 1979). In this model, a rat

(intruder) is placed in the home cage of a larger, aggressive rat (resident) and subject to repeated threatening encounters. Exposure of adult rats to this social stressor produces anhedonia, promotes depressive-like behaviors, alters the function of the hypothalamic-pituitary-adrenal axis and increases the propensity to self-administer psychomotor stimulants (Heinrichs et al., 1992; Tidey and Miczek, 1997; Buwalda et al., 2005; Rygula et al., 2005; Wood et al., 2010). In contrast to the numerous studies of adult social stress, few studies have examined the effects of social stress exposure during adolescence. In hamsters, adolescent social stress altered patterns of aggression in a context specific manner (Ferris, 2003; Wommack and Delville, 2003). The few studies that examine adolescent social stress in rats suggest an increase in adult social anxiety (Vidal et al., 2007; Watt et al., 2009).

The present study was designed to compare the behavioral and endocrine effects of social stress in rats during early adolescence, mid adolescence, and adulthood. Additionally, the effects of restraint were assessed on some of the behaviors and on endocrine function to determine whether the observed effects were selective to social stress. Because some of the behavioral changes observed during these initial experiments have been linked to activation of the locus coeruleus (LC)-norepinephrine system, subsequent experiments were performed, in which the age-dependent effects of social stress on LC discharge characteristics were compared in stressed and control rats (Detke et al., 1995; Bondi et al., 2007).

Methods

Animals

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were either stressed or subject to control manipulation during early-adolescence (p28-p35, EA), mid-adolescence (p42-p49, MA) or adulthood (p63-p70). They were delivered 6 days previous to the stressor to allow for acclimatization to the facility. Long-Evans retired breeder rats (Taconic Farms, Germantown, NY and Charles River, Wilmington, DE >500g, single-housed) were used as resident animals in the resident-intruder experiments. Animals were initially housed 2-3 per cage in standard 26 cm by 46 cm polypropylene cages (Allentown Inc, Allentown, NJ) in 12-hour light/dark (lights on at 6:00), climate controlled room with *ad lib* access to food and water. On the first day of the experiment, all animals were transferred to new cages and individually housed. Care and use of animals was approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia.

Stress

For social stress, an adaptation of the resident-intruder stress was used (Miczek, 1979). Rats were randomly assigned to control or stress groups. For 7 consecutive days, the experimental animals (intruders) were weighed and then placed in the home-cage of a novel Long-Evans retired breeder rat (resident) that had been screened for proper aggressive behavior based on our previous work (Bhatnagar and Vining, 2003; Bhatnagar et al., 2006). Residents that failed to consistently attack or that were overly aggressive and caused injury to intruders were eliminated from participation. Each session began with a period of investigation and free interaction until one of three criteria was met: 1) the intruder exhibited submissive defeat posture (frozen supine position for 2 sec) 2) the

intruder was attacked 5 times or 3) 15 min had elapsed. Upon reaching one of these criteria, the animals were separated by a wire mesh barrier (1 cm weave), allowing continued olfactory and visual contact for the duration of the 30 minute session. Controls were weighed daily then returned to their home cage. For an additional comparison, a separate group of EA rats were placed into the home cage of a novel, age-matched conspecific daily for 7 consecutive days. The rats were allowed to interact for 15 min before being separated by the wire mesh barrier as described above for an additional 15 min prior to being returned to home cages. They were compared to control animals that were weighed daily and returned to their home cage. Adult intruders used for electrophysiology and endocrine experiments were subject to a slightly modified resident-intruder paradigm than described above. Similar to our previous study (Wood et al., 2010), they were single housed upon arrival to the facility, were not subject to the 5 attack limit and their controls were placed in novel cages behind a wire mesh barrier for 30 min daily.

For restraint stress, rats were placed in a flexible rodent restrainer for 120 min (Decapicone™; Braintree Scientific, Braintree, MA). This was secured with tape around the base of the tail, leaving enough room for the animal to defecate. Controls were weighed daily and returned to their home cages.

Plasma Corticosterone

In a subset of rats, blood (150-200 μ l) was collected via tail vein nick into either heparanized capillary tubes (Kimble-Chase, Vineland NJ) or eppendorf tubes containing EDTA to prevent coagulation. Blood was obtained immediately before and after the

resident-intruder sessions on days 1, 4, and 7. The rat was briefly restrained in an absorbent pad while the tail was nicked then was allowed free range of movement during the bleed (<2 min). Blood was also collected from restraint stressed rats and matched controls corresponding to times 0, 30, and 120 min of the restraint on days 1 and 7 to assess habituation (Jaferi and Bhatnagar, 2006). Whole blood was centrifuged at 3000 rpm for 15 min and the plasma was kept at -20°C until the assay. Corticosterone was measured using a kit from MP Biomedicals (Orangeburg, NY). The minimum level of detection was approximately $0.7\ \mu\text{g}/\text{dl}$. Significance was determined using a 3-way ANOVA across day, stress, and time. Pair-wise comparisons were done using post-hoc ANOVA's and t-test's.

Behavioral Endpoints

Open field behavior was recorded on the day following the last stressor. Rats were placed in a 70 cm x 70 cm black Plexiglas open field arena for 5 min and activity was videotaped and acquired using Roxio video acquisition software (Santa Clara, CA) and analyzed offline using Noldus Ethovision behavioral analysis software (Wageningen, Netherlands). The time spent in each of 4 concentric zones and the total distance traveled were quantified and compared between control and stress conditions using a Student's *t*-test with significance at $p < 0.05$.

Defensive burying behavior was measured 48 h after the last social stress session. Rats were placed in a cage filled with 5 cm of bedding. An electrified probe consisting of two 18 gauge copper wires wrapped around a plastic rod was inserted 6 cm into a hole drilled in the cage and positioned at 7 cm above the cage bottom. A 1.7 mA shock was

delivered when the rat touched the probe and completed of the circuit. Once the rat was visibly shocked, it remained in the cage for 15 min. The test was run under dim light conditions with ambient hall light and a red light to aid in video capture. Cages were cleaned with 10% bleach then wiped dry before being refilled with new bedding for each use. The probe was brushed lightly with sandpaper to remove dust and debris and the current was checked between trials using a multimeter. Burying behavior was defined as spraying or pushing of bedding material towards the probe with the forepaws or snout. Data was analyzed offline for latency to begin burying and bury duration and compared using a 2-way ANOVA with stress and age as the main factors. Pair-wise comparisons were made using a post-hoc t-test.

The response to a 15 min swim stress was examined 72 h after the last social stress session as previously described (Wood et al., 2010). The test was videotaped and analyzed offline at 5 sec intervals for incidences of swimming, climbing and immobility behaviors by an observer blind to the experimental condition as previously described (Detke et al., 1995). A subset of these rats had an additional 5 min swim session 24 h later.

Electrophysiology

Single unit LC activity was recorded in the isoflurane-anesthetized state 24 hours following the last social stress (or control) session for most subjects. MA animals used in the electrophysiology experiments were aged between 42-49 days on the first day of the resident-intruder stress due to a delivery error and electrophysiological experiments in this group occurred 1-9 days following the last stress in these animals. Note, even with

the delivery error, the stress occurred during adolescence. Electrophysiological studies were done in rats that had not undergone behavioral testing with the exception of rats stressed in early adolescence and tested in adulthood. This latter group was exposed to the defensive burying test as adults (p70) to assess the endurance of the behavioral impact of social stress and 1 week later LC neuronal activity was recorded. Note that because electrophysiological recordings could only be performed on 2 rats daily, paired control and stressed rats from the same age group were run on the same days. Because of this, comparisons were between treatment groups of the same ages rather than between ages.

The methods for surgery and recording were as previously described (Curtis et al., 1997). LC spontaneous activity was recorded for at least 3 min. This was followed by a recording of LC sensory-evoked activity during a trial of sciatic nerve stimulation (50 stimuli, 3.0 mA, 0.5 ms duration, 0.2 Hz). Spontaneous and sensory-evoked activity were recorded from 1-6 neurons per subject. The effect of the CRF antagonist, DPheCRF₍₁₂₋₄₁₎, was tested in some subjects. For these studies, double-barrel micropipettes were used to record neuronal activity and simultaneously microinfuse DPheCRF₍₁₂₋₄₁₎ (3 ng in 30 nl) by applying small pulses of pressure to the calibrated infusion pipette (15–25 psi, 10–30 ms in duration, Picospritzer; General Valve) (Kreibich et al., 2008). LC activity was recorded for at least 3 min after the infusion. DPheCRF₁₂₋₄₁ was only administered once to an individual rat.

Recording sites were marked by the iontophoresis of Pontamine sky blue from the recording electrode (15 μ A for 15 min). Brains were dissected out and 30 μ m frozen sections were cut and stained with neutral red for localization of the recording site. Data

were analyzed only from those neurons histologically identified as being within the nucleus LC.

For quantification, LC activity during sciatic nerve stimulation trials was recorded as peri-stimulus time histograms (PSTHs) and analyzed as described previously (Valentino and Foote, 1987; Kreibich et al., 2008). Synchronizing pulses initiated 2s sweeps beginning 500 ms prior to the stimulus and the cumulative number of spikes in each 8 ms bin (250 bins total) was plotted. Thus discharge activity was recorded for 500 ms before and 1.5 s after the stimulus. The 500 ms previous to each stimulus, representing tonic or unstimulated discharge, was compared between groups. Evoked discharge was defined as that which occurred after the stimulus and exceeded the mean tonic discharge rate by 2 standard deviations. The signal-to-noise ratio was the ratio of evoked-to-tonic discharge rate. Spontaneous, tonic, and evoked discharge rates and the signal-to-noise ratio were compared using a 2-way ANOVA with age and stress as factors. As an additional analysis, a mean PSTH was generated for each experimental group by averaging the number of spikes in each 8 ms bin for every cell in the group. Components of the mean PSTHs were compared between groups using repeated measures ANOVA with stress as the main factor and time as the repeated measure. For this analysis, the post-stimulus inhibitory component was defined as the period following the evoked component when the rate was 2 standard deviations below the mean tonic rate.

Drugs

DPheCRF₍₁₂₋₄₁₎ (Jean Rivier, The Salk Institute, San Diego, CA) was dissolved in water at a concentration of 1 mg/ml and 10 µl aliquots were then concentrated using a speed-vac and kept at -20°C until the day of the experiment. Aliquots were then dissolved in artificial cerebrospinal fluid (10 µg/30 µl).

Results

The expression of the defeat posture in response to social stress develops during adolescence.

As previously described (Grant and Mackintosh, 1962; Miczek, 1979; Fernandez-Espejo and Mir, 1990), residents responded to adult intruders with an initial period of investigation followed by a show of aggression involving biting and pouncing attacks to the back, rump, or shoulders. Adult intruders usually took a characteristic supine posture that signaled defeat or subordination and the decision to physically separate the intruder from the resident was most often based on this criterion for this age group (Fig. 1). The placement of EA intruders into the cage also elicited a show of aggression from the resident characterized by biting and pouncing attacks directed at the back, head, and rump of the intruder. Often the resident would pounce on the intruder and repeatedly drive it into the bedding using rapid thrusting movements with the forepaws. In contrast to adult rats, EA rats rarely exhibited the supine posture and more often would freeze in a crouched position in response to aggressive attacks. Following each attack, the EA intruders would often remain motionless for a short period of time before reengaging social contact with the resident. Thus, EA intruders were most often separated from the residents because they met the criterion of sustained 5 attacks or the 15 min limit (Fig. 1).

Compared to EA rats, the expression of the defeat posture was more established in MA and adult rats, in an age-dependent manner (Fig. 1A). A one-way ANOVA indicated an effect of age and subsequent Newman-Keuls Multiple Comparison Tests indicate significant differences between all groups. EA rats reached the 5 attack limit without exhibiting defeat posture more often than both MA and adult intruders (Fig. 1B). Whereas the EA and MA intruders occasionally were separated from the residents based on the 15 min time limit, adult intruders were never separated based on this criterion (Fig. 1C). The total number of attacks sustained was also significantly greater for EA intruders compared to MA or adult animals (Fig. 1D).

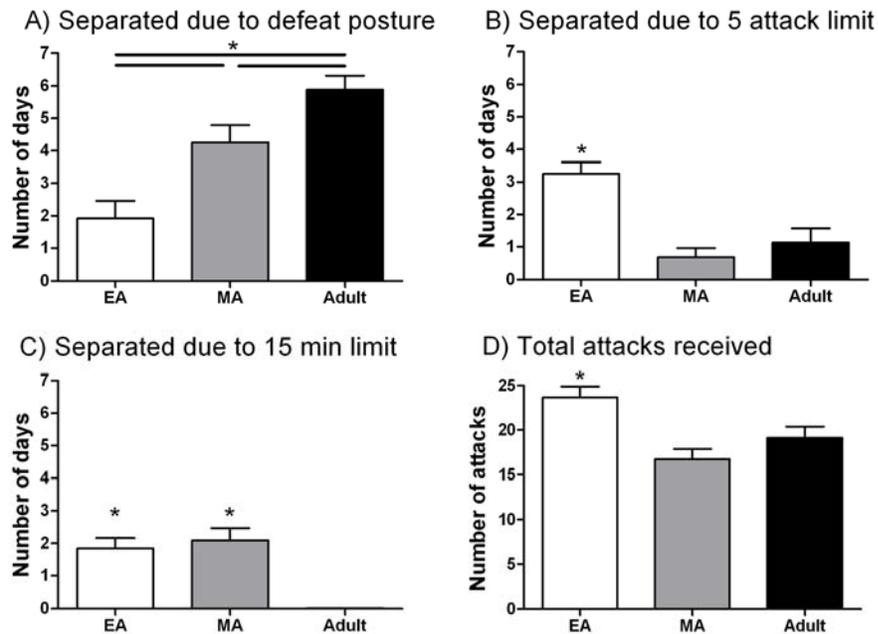


Figure 1. Expression of defeat behavior in response to resident-intruder attacks is dependent on age. A) Average number of days intruder animals were separated from the resident because they exhibited defeat postures (maximum is 7). A one-way ANOVA revealed a significant effect of age ($F_{(2,31)} = 13.1$, $p < 0.001$). B) Average number of days the intruders were separated from the resident because they received five attacks without exhibiting defeat posture. Main effect of age $F_{(2,31)} = 16.94$, $p < 0.001$. C) Average number

of days the intruders were separated from the resident after 15 min without exhibiting defeat posture or receiving 5 attacks. A one-way ANOVA revealed a main effect of age $F_{(2,31)} = 10.41$, $p < 0.001$). D) Total number of attacks received by age. A one-way ANOVA revealed a main effect of age ($F_{(2,31)} = 9.44$, $p < 0.001$). Bars are the average of 12, 12, and 8 animals for the EA, MA, and adult age groups respectively. Vertical lines represent S.E.M. *All pair-wise $p < 0.05$ Student Newman-Keuls.

Social stress elevates plasma corticosterone independent of age.

Social stress elevated plasma corticosterone in all groups compared to baseline. On the first day of the manipulation, plasma corticosterone levels at the 30 min time point were elevated in all rats including controls and stressed rats of each age group. However, by the seventh day of manipulation, the plasma corticosterone levels in control rats had completely habituated to baseline levels whereas the corticosterone levels in stressed rats remained elevated, although there was a degree of habituation in the stressed adolescent groups (Table 1).

Table 1. Corticosterone response to 7 days of resident intruder stress

Age	Time	Control ($\mu\text{g/dL} \pm \text{SEM}$)			Social Stress ($\mu\text{g/dL} \pm \text{SEM}$)		
		Day 1	Day 4	Day 7	Day 1	Day 4	Day 7
EA	0	6.1 \pm 1.6	5.0 \pm 1.4	6.5 \pm 1.0	7.1 \pm 1.9	5.2 \pm 1.3	6.4 \pm 1.9
	30	38.6 \pm 3.0	19.2 \pm 3.5	7.9 \pm 1.6 #	41.8 \pm 4.5	27.6 \pm 3.7	19.7 \pm 3.7 *.#
MA	0	5.7 \pm 1.3	2.4 \pm 0.6	1.0 \pm 0.1	3.2 \pm 1.1	2.5 \pm 0.7	4.0 \pm 1.4
	30	31.8 \pm 6.0	7.5 \pm 2.2	4.7 \pm 0.6 #	51.1 \pm 5.1 *	40.5 \pm 6.0 *	29.9 \pm 6.4 *.#
Adult	0	0.9 \pm 0.2	1.6 \pm 0.5	4.3 \pm 1.7	1.1 \pm 0.1	2.2 \pm 0.5	4.3 \pm 1.4
	30	22.2 \pm 5.1	19.9 \pm 4.9	6.2 \pm 2.5 #	51.9 \pm 4.3 *	42.3 \pm 5.4 *	42.6 \pm 3.7 *

(*) denotes significant difference from corresponding control ($p < 0.05$ Student's t-test)

(#) denotes significant difference from day 1 ($p < 0.05$ Student's t-test)

EA Main effects: Time ($F_{1,75} = 159.8$; $p < 0.001$), Stress ($F_{1,75} = 6.7$; $p < 0.05$), Day ($F_{2,24} = 24.4$; $p < 0.001$)

Interactions: Time by Day ($F_{2,75} = 23.1$; $p < 0.001$), and Time by Stress ($F_{1,75} = 5.6$; $p < 0.05$)

Post-hoc ANOVA Day 1: Time ($F_{1,24} = 119.8$; $p < 0.001$)

Post-hoc ANOVA Day 7: Time ($F_{1,25} = 9.06$; $p < 0.006$), Stress ($F_{1,25} = 5.6$; $p < 0.05$), Time by Stress ($F_{1,25} = 5.8$; $p < 0.05$)

MA Main effects: Time ($F_{1,79} = 204.4$; $p < 0.001$), Stress ($F_{1,79} = 36.6$; $p < 0.001$), Day ($F_{2,79} = 13.2$; $p < 0.001$)

Interactions: Time by Day ($F_{2,79} = 9.2$; $p < 0.001$), and Time by Stress ($F_{1,79} = 35.6$; $p < 0.001$)

Adult Main effects: Time ($F_{1,117} = 180.3$; $p < 0.001$), Stress ($F_{1,117} = 46.8$; $p < 0.001$)

Interactions: Time by Day ($F_{2,117} = 4.3$; $p < 0.05$), and Time by Stress ($F_{1,117} = 44.7$; $p < 0.001$)

Social stress has divergent effects on anxiogenic and depression-related behaviors that are dependent on the age of exposure.

Repeated social stress had no effect on open field activity at any age (Table 1: supplementary data). In contrast, exposure to social stress had differential, age-dependent effects on defensive burying behavior. Exposure to social stress during EA increased defensive burying behavior as indicated by an increased duration of burying and a decreased latency to begin burying (Fig. 2A, B). This effect was absent in MA rats. Notably, exposure to social stress in adulthood had an opposing effect to decrease defensive burying as indicated by a decreased burying duration (Fig. 2A).

To determine whether the ability of social stress to increase active defensive behaviors endured into adulthood, rats were exposed to social stress as early adolescents and then were allowed to grow to adulthood, at which time they were tested for defensive burying behavior (PND 70). Interestingly, the behavior of these rats in the defensive burying test resembled those that had been stressed as adults in that the bury duration was decreased compared to matched controls (Fig. 2C).

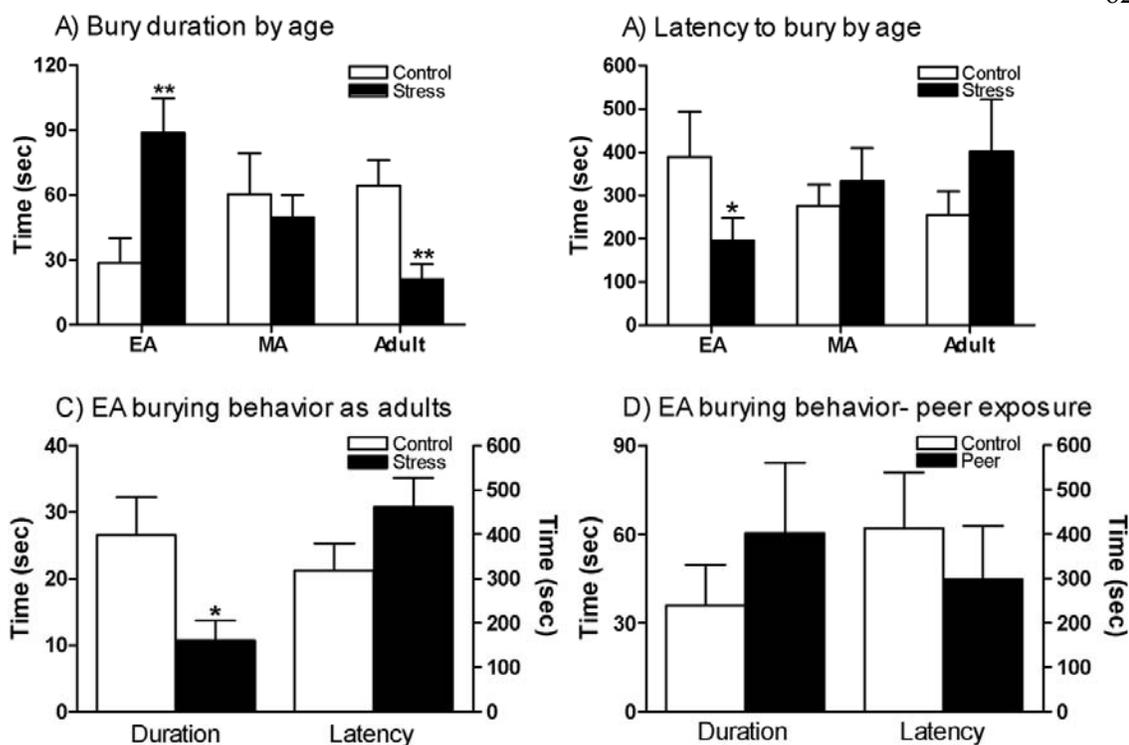


Figure 2. Effects of social stress or exposure to age-matched conspecific on defensive burying behavior A) Bars indicate the average burying duration of EA (n=11 control, n=12 stressed), MA (n=12 control, n=12 stressed) or adult (n=8 control, n=7 stressed) rats. A two-way ANOVA reveals an Age by Stress interaction ($F_{(2,56)} = 6.53, p < 0.01$). B) Bars indicate the average latency to begin burying for the same animals as in A. C) Bars indicate the average burying duration (left) or latency (right) of EA rats that were stressed as adolescents and tested as adults (n=20 control, n=18 stressed). Welch ANOVA for unequal variance ($F_{(1,40)} = 4.94, p = 0.03$). Six controls and 6 stressed rats had been previously tested in the defensive burying task as adolescents. Of these, 1 control and 2 stressed animals were removed as adults because they began burying before an obvious shock occurred. D) Bars indicate the average burying duration (left) or latency (right) of EA rats exposed to an age-matched peer (n=5 control, n=6 peer exposed). Vertical lines represent S.E.M. *p<0.05 **p<0.01 post-hoc Student's t-test

As a second measure of coping behavior, the behavior of rats during swim stress was measured. Exposure to social stress also increased total climbing behavior of EA rats during an initial exposure to swim stress (Table 2). A subset of these animals was then

retested 24 h later in a 5 min swim and the EA intruders again showed a trend towards an increase in climbing. The effects of social stress in EA rats on swim stress behavior were limited to climbing, as there were no significant effects on the incidence of either swimming or immobility behavior. Exposure of MA or adult rats to social stress had no effect behavior in response to swim stress.

Table 2. Swim behavior following social stress

Day	Age	Climbing		Swimming		Immobility	
		Control	Stress	Control	Stress	Control	Stress
Day 1	EA (12,11)	8.3 ± 1.6	13.9 ± 1.8 *	23.8 ± 5.0	29.6 ± 6.3	147.9 ± 6.3	136.5 ± 7.9
	MA (12,12)	11.8 ± 1.5	13.8 ± 1.7	23.3 ± 3.6	28.0 ± 3.8	144.8 ± 3.6	138.3 ± 4.4
	Adult (8,8)	15.3 ± 1.2	15.8 ± 1.8	49.1 ± 2.7	47.3 ± 3.6	115.6 ± 3.1	117.0 ± 4.3
	EA peer (6,6)	16.5 ± 2.7	22.7 ± 3.6	42.7 ± 3.5	40.7 ± 5.0	120.8 ± 2.5	116.7 ± 7.0
Day 2	EA (6,6)	0.7 ± 0.2	3.7 ± 1.6 §	6.2 ± 2.0	9.3 ± 2.0	53.2 ± 2.1	47.0 ± 3.5
	MA (6,6)	5.2 ± 2.2	11.0 ± 4.4	13.7 ± 2.0	9.3 ± 1.5	41.2 ± 2.7	39.7 ± 3.2
	Adult (8,8)	6.4 ± 2.1	6.6 ± 1.1	19.0 ± 1.6	17.3 ± 2.6	34.6 ± 2.7	36.1 ± 2.7
	EA peer (6,6)	5.8 ± 2.8	7.5 ± 2.8	9.2 ± 1.7	9.2 ± 1.9	45 ± 4	43.3 ± 4

n = (control,stress) *p<0.05 ttest vs control, §p=0.10 ttest vs control

The effects of social stress on EA behavior are specific to an adverse social event.

Because the early adolescent period corresponds to the height of social play behavior (Panksepp, 1981), the effects of exposure of EA rats to a novel age-matched peer were assessed. When EA rats were placed into the cage of an age-matched peer there was a short period of investigation after which the rats would engage in rough and tumble play. This included chasing, nuzzling of the head and nape, and light pouncing. Dominant roles would reverse rapidly and often during the session such that the “attacker” became the “attacked.” It is important to note that these interactions never resulted in either rat adopting the classic defeat posture. In contrast to social stress, EA exposure to an age-matched peer did not increase burying behavior in the defensive burying test (Fig. 2D). There were no statistically significant changes in either burying duration or latency in the experimental group compared to controls. Additionally, there were no group differences

in climbing behavior when peer-exposed EA rats were subjected to swim stress (Table 2). Interestingly, repeated exposure to age-matched peers tended to increase time spent in the inner zones of the open field (Table 3: supplementary data).

Restraint stress has age-dependent effects on plasma corticosterone but does not alter defensive burying behavior.

To determine whether the effects of social stress during adolescence generalized to other stressors, the effects of repeated restraint stress on plasma corticosterone and on defensive burying behavior were compared across ages. Like social stress, restraint stress increased plasma corticosterone in all age groups (Fig. 3). Similar to what has been reported by others, the effect of restraint stress on plasma corticosterone was age-dependent (McCormick et al., 2007; Doremus-Fitzwater et al., 2009). In MA and adult rats, restraint increased corticosterone levels for the duration of the 120 min stressor on Day 1 (Fig. 3 B, C). By Day 7, however, restraint corticosterone levels were significantly decreased at 120 min such that, in the adult animals, they were not different from baseline, consistent with previous reports (Bhatnagar et al., 2002; Jaferi and Bhatnagar, 2006). In contrast to MA or adult rats, EA rats maintained a high level of corticosterone release on Day 7 for the duration of the 120 min stressor (Fig. 3A).

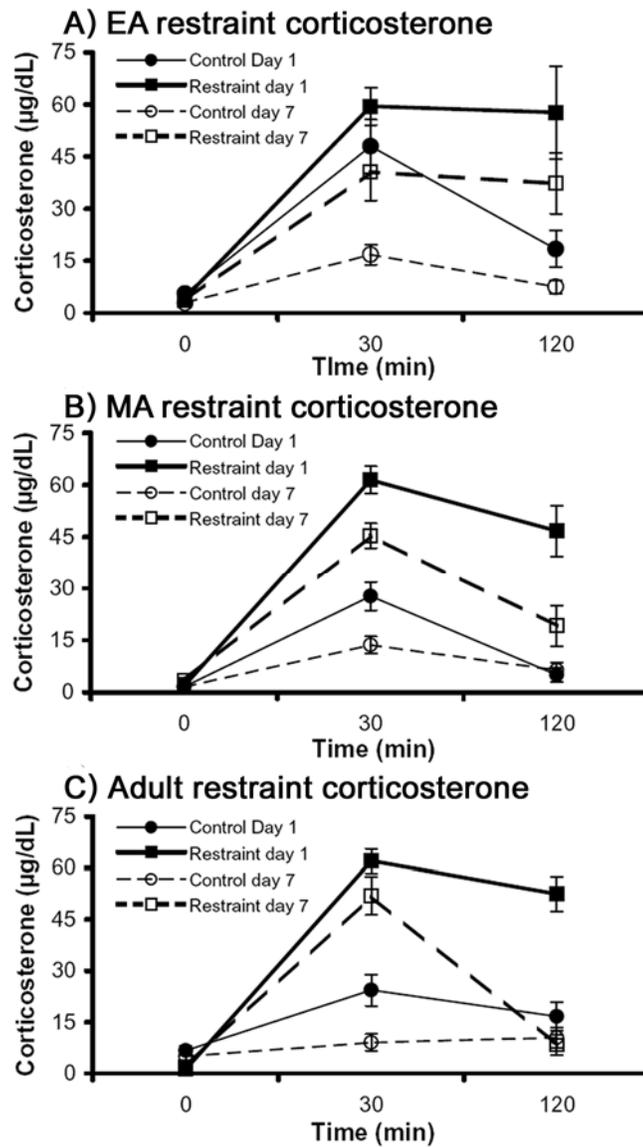


Figure 3. Effects of restraint stress on plasma corticosterone. A) Time course of plasma corticosterone for EA rats exposed to restraint stress on day 1 (solid squares and line) and day 7 (open squares, broken line) or controls on day 1 (solid circles and line) and day 7 (open circles, broken line). Main effect of Time ($F_{2,73} = 43.9$; $p < 0.001$), Stress ($F_{1,73} = 26.9$; $p < 0.001$), Day ($F_{1,73} = 17.8$; $p < 0.001$), Time by Day ($F_{2,73} = 4.3$; $p < 0.05$), and Time by Stress ($F_{2,73} = 9.3$; $p < 0.001$). B) Time-course of plasma corticosterone for MA rats exposed to restraint stress or control. Main effect of Time ($F_{2,80} = 111.1$; $p < 0.001$), Stress ($F_{1,80} = 107.6$; $p < 0.05$), Day ($F_{1,80} = 21.7$; $p < 0.001$), Time by Day ($F_{2,80} = 6.7$; $p < 0.01$), Time by Stress ($F_{2,80} = 25.7$; $p < 0.001$), Day by Stress ($F_{2,80} = 6.2$; $p < 0.05$) and Day by Stress by Time ($F_{2,80} = 5.8$; $p < 0.01$). C)

Time-course of plasma corticosterone for adult rats exposed to restraint stress or control. Main effect of Time ($F_{2,82} = 23.8$; $p < 0.001$), Stress ($F_{1,82} = 114.2$; $p < 0.05$), Time by Day ($F_{2,82} = 55.1$; $p < 0.001$), Time by Stress ($F_{2,82} = 13.1$; $p < 0.001$), and Day by Stress by Time ($F_{2,82} = 25.5$; $p < 0.001$). Each point is the mean of 6-8 determinations. Vertical lines represent S.E.M.

In spite of the finding that restraint stress increased plasma corticosterone in all age groups to values that were comparable to or greater than levels produced by social stress, this did not affect defensive burying behavior of any group. Both the latency to bury and burying duration were comparable in rats exposed to restraint stress compared to controls (Fig. 4)

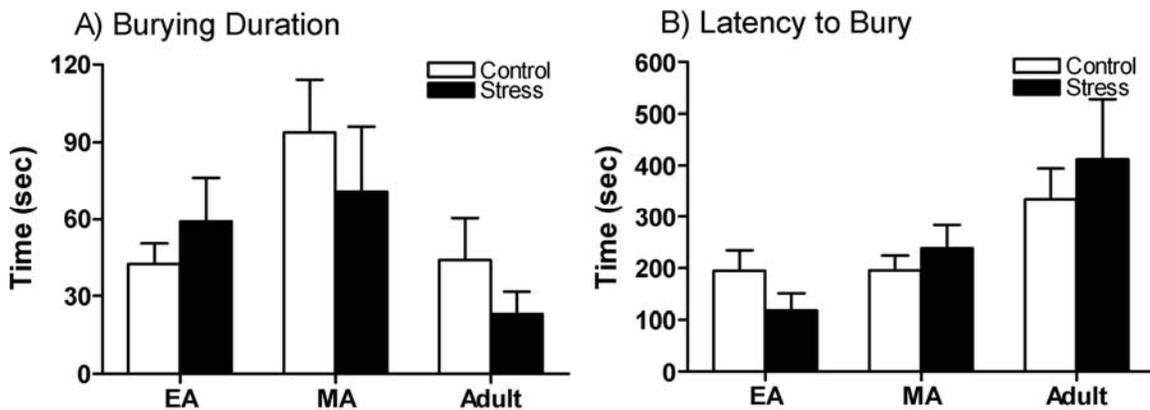


Figure 4. Effects of restraint stress on burying behavior A) Bars represent the mean burying duration for EA (n=7 control, n=8 restraint), MA (n=8 control, n=8 restraint) and adult (n=8 control, n=8 restraint) rats. Vertical lines represent S.E.M. A two-way ANOVA shows no significant effects of Stress ($F_{1,41} = 0.4$; $p = 0.52$) or Stress by Age interactions ($F_{2,41} = 0.7$; $p = 0.45$) B) Bars represent the mean latency to bury for the same EA, MA and adult rats as in A. Vertical lines represent S.E.M. A two-way ANOVA shows no significant effects of Stress ($F_{1,41} = 0.1$; $p = 0.77$) or Stress by Age interactions ($F_{2,41} = 0.8$; $p = 0.44$)

Social stress alters the physiology of LC neurons.

Because the LC-norepinephrine system has been implicated in the active coping behaviors that were elevated in EA rats exposed to social stress (defensive burying and climbing), LC neuronal activity was compared in control and socially stressed rats at different age groups (Detke et al., 1995; Bondi et al., 2007; Howard et al., 2008). Figure 5 compares the mean LC spontaneous discharge rates of stressed and control rats of different age groups as well as the tonic and evoked components of the LC sensory response and the signal-to-noise ratio of the sensory response.

Exposure to resident-intruder stress selectively elevated the spontaneous LC discharge rate in EA rats (Fig. 5A), whereas a comparison of LC sensory-evoked discharge revealed differential effects depending on the age at which stress occurred. Stress exposure during early adolescence tended to increase tonic or unstimulated discharge ($p = 0.07$), consistent with an increase in spontaneous discharge rate (Fig. 5B). At the same time, sensory-evoked discharge was decreased in EA rats and this had the effect of decreasing the signal-to-noise ratio (Fig. 5C, D). Exposure to social stress during adulthood had no effect on spontaneous or tonic LC discharge rate but, opposite to EA exposure, increased sensory-evoked LC discharge rate and this tended ($p < 0.07$) to increase the signal-to-noise ratio (Fig. 5C). Interestingly, the profile of LC neuronal activity of adult rats that were subjected to social stress in early adolescence resembled that of rats that were subjected as adults. In this group LC spontaneous and tonic activity were unaffected by stress exposure but sensory-evoked activity and the signal-to-noise ratio of the sensory response was increased. Exposure to social stress during mid-adolescence had no effect on LC activity.

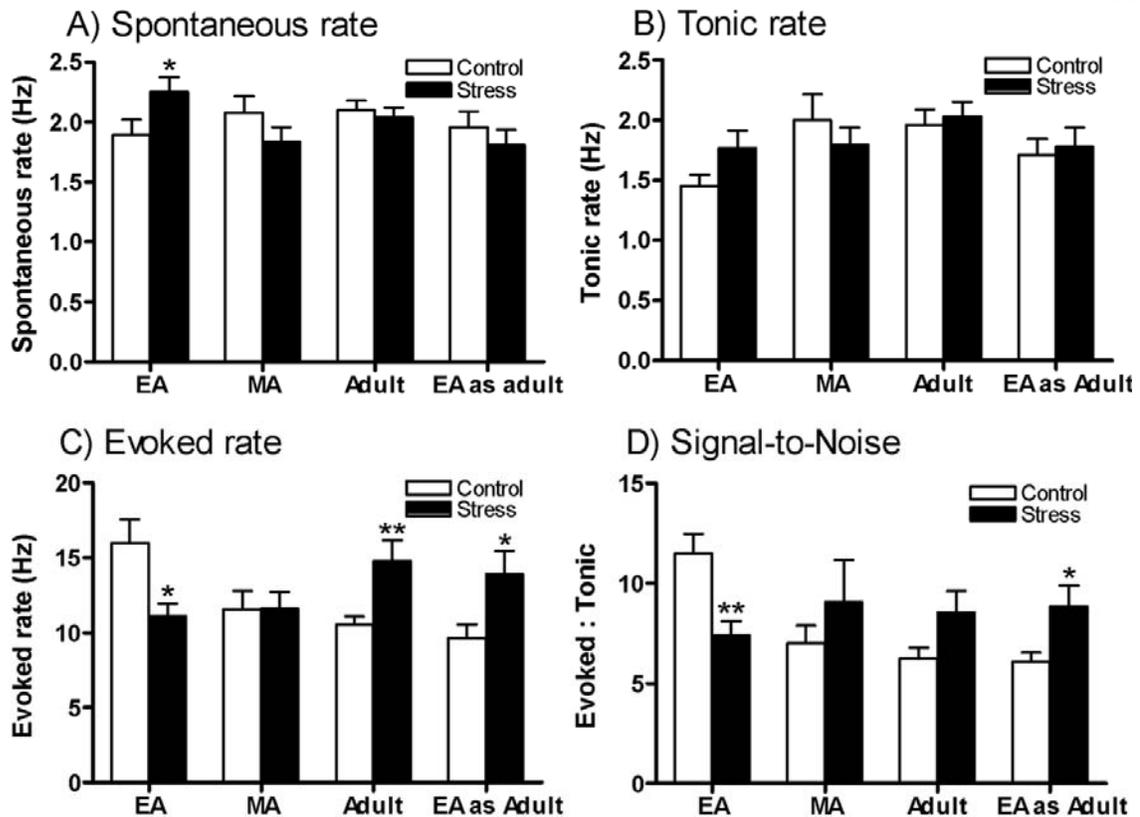


Figure 5 Age-dependent effects of social stress on LC discharge characteristics. A) Bars indicate the mean LC spontaneous discharge rate determined in EA control rats (103 cells, 32 rats), EA stressed rats (103 cells, 29 rats), MA control rats (41 cells, 8 rats), MA stressed rats (43 cells, 8 rats), adult control rats (110 cells, 42 rats), adult stressed rats 105 cells, 33 rats), EA as adult control rats (36 cells, 7 rats), and EA as adult stressed rats (38 cells, 7 rats). A two-way ANOVA between EA, MA, and adult rats indicates a significant interaction between Stress and Age ($F_{(2,499)} = 3.02$ $p < 0.05$). Post-hoc analysis shows a significant difference between EA control and stressed rats ($p < 0.05$ Student's t-test) at the level of EA. There was no effect of Stress across ages, however ($F_{(2,499)} = 0.0$ $p < 0.86$) B) Bars indicate the mean tonic LC firing rate during trials of sciatic nerve stimulation for EA control rats (42 cells, 25 rats), EA stressed rats (37 cells, 23 rats), MA control rats (28 cells, 8 rats), MA stressed rats (30 cells, 8 rats), adult control rats (50 cells, 31 rats), adult stressed rats (51 cells, 41 rats), EA as adult control rats (25 cells, 7 rats), and EA as adult stressed rats (24 cells, 7 rats). Similar to the spontaneous firing, EA stressed rats tended to have a higher tonic rate ($p = 0.07$ Student's t-test) but there were no effects of Stress ($F_{(1,214)} = 0.3$ $p < 0.61$) nor any interaction with Age ($F_{(2,214)} = 1.58$ $p < 0.21$). C) Bars indicate the mean evoked LC firing rate during trials

of sciatic nerve stimulation for EA control rats (40 cells, 25 rats), EA stressed rats (33 cells, 21 rats), MA control rats (26 cells, 8 rats), MA stressed rats (29 cells, 8 rats), adult control rats (41 cells, 26 rats), adult stressed rats (44 cells, 36 rats), EA as adult control rats (23 cells, 7 rats), and EA as adult stressed rats (23 cells, 7 rats). A two-way ANOVA between EA, MA, and adult rats indicates a significant interaction between Stress and Age ($F_{(2,207)} = 7.72$, $p < 0.001$) with post hoc analysis showing a significant difference between control and stress in EA and adult rats ($p < 0.05$ Student's t-test). There was no general effect of Stress ($F_{(2,207)} = 0.0$, $p < 0.86$). A Student's t-test also indicates a significant difference between control and stress in the EA as adult group ($p < 0.05$). D) Bars indicate the mean signal-to-noise ratio of the LC sensory response for EA control rats (40 cells, 25 rats), EA stressed rats (33 cells, 21 rats), MA control rats (26 cells, 8 rats), MA stressed rats (29 cells, 8 rats), adult control rats (41 cells, 26 rats), adult stressed rats (44 cells, 36 rats), EA as adult control rats (23 cells, 7 rats), and EA as adult stressed rats (23 cells, 7 rats). A Student's t-test also indicates a significant difference between control and stress in the EA as adult group ($p < 0.05$). A two-way ANOVA between EA, MA, and adult rats reveals an Age by Stress interaction ($F_{(2,206)} = 5.24$; $p < 0.01$) with post-hoc tests indicating significant differences between EA control and stress rats ($p < 0.01$ Student's t-test). There were no general effects of Stress ($F_{(1,206)} = 0.0$; $p < 0.94$). * $p < 0.05$, ** $p < 0.01$ t test vs. control. Vertical lines represent S.E.M.

Figure 6A shows the averaged PSTH generated from all LC neurons of control and stressed rats for EA and adult groups. Components of the histograms containing the evoked response (Fig. 6B) and post-stimulus inhibition (Fig. 6C) were isolated and compared using repeated measures ANOVAs. The excitatory component was blunted in stressed EA rats and there was a faster recovery from post-stimulus inhibition. Consistent with the results shown in Figure 5, analysis of the mean excitatory component of the PSTH in adult animals demonstrated an opposing effect of social stress on LC evoked discharge in adult compared to EA rats, with stress resulting in an enhanced sensory response. During the inhibitory phase in adult animals (0.616 ms to 0.912 ms), stress

tended to increase inhibition, although this effect was not statistically significant.

Similar effects of social stress on the average PSTH components were noted in EA rats tested as adults (data not shown).

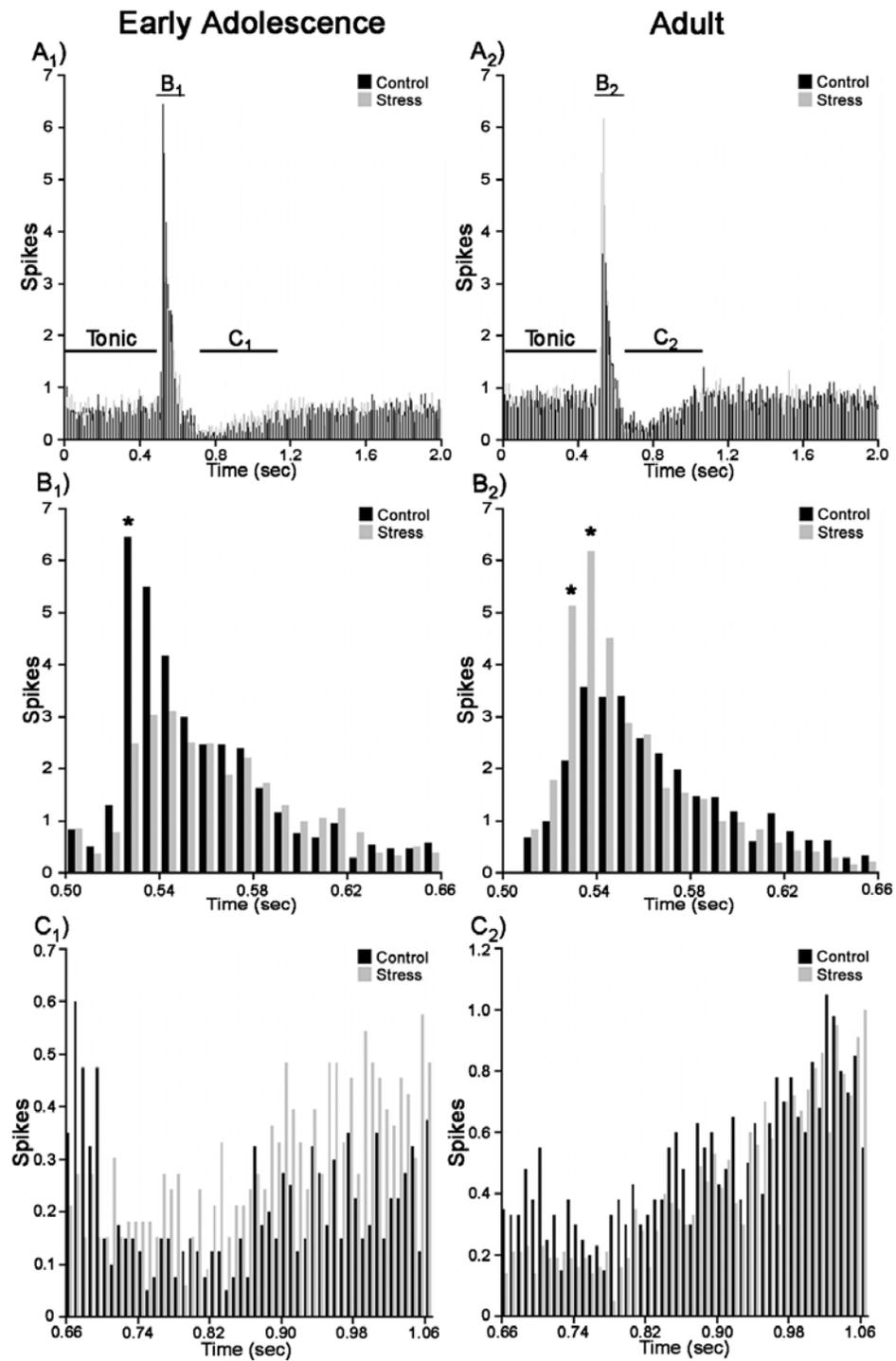


Figure 6. Age-dependent effects of social stress on LC discharge characteristics as compared using an averaged peri-stimulus time histograms (PSTH). Panels A₁ and A₂ represent the entire average PSTH of all cells from EA and Adult rats respectively. Bars indicate average spikes / bin (8 ms) in Control (black) or Stress (gray) rats. In panels B₁-B₂ and C₁-C₂ the components of the PSTH are broken down and compared between experimental groups. B₁-B₂) Average evoked phase (from 520 ms to 624 ms) plus several bins on either side for reference. A repeated-measures ANOVA between social stress and control over time indicated main effects of Time ($F_{(12,852)} = 10.2$; $p < 0.0001$), Stress ($F_{(1,852)} = 4.3$; $p < 0.05$), and a Time by Stress interaction ($F_{(12,852)} = 2.5$; $p < 0.01$) in EA animals (B₁). Bonferroni pair-wise analysis by bin indicates a significant difference between control and stress in the bin corresponding to 520 ms ($*p < 0.05$). Adult animals (B₂) had opposing main effects of Time ($F_{(12,972)} = 21.0$; $p < 0.0001$), Stress ($F_{(1,972)} = 4.82$; $p < 0.05$), and a Time by Stress interaction ($F_{(12,972)} = 4.3$; $p < 0.0001$). Bonferroni pair-wise analysis by bin also indicates a significant difference between control and stress in the bins corresponding to 520 ms and 528 ms ($*p < 0.05$). C₁-C₂) Average post-stimulus inhibitory phase (from 656 ms to 1048 ms) plus several bins for reference. In adolescent animals (C₁) a two-way repeated measures ANOVA indicated a main effect of time ($F_{(49,3479)} = 2.2$; $p < 0.0001$), a trend to an effect of stress ($F_{(1,3479)} = 3.9$; $p = 0.0509$) and a significant time by stress interaction ($F_{(49,33479)} = 1.56$; $p < 0.05$). In adult animals (C₂) a two-way repeated measures ANOVA indicates only an effect of Time ($F_{(38,3078)} = 4.0$; $p < 0.0001$) on inhibition with a trend towards an effect of Stress ($F_{(1,3078)} = 3.3$; $p = 0.07$) and no stress by time interactions.

Role of CRF in the neuronal changes produced by EA exposure to social stress

The increase in LC spontaneous activity and decrease in sensory-evoked activity in EA rats exposed to social stress is reminiscent of the effects of CRF or of stressors that release CRF in the LC (Valentino and Foote 1987, 1988; Valentino and Wehby, 1988). To determine whether CRF release in the LC is involved in the effects of social stress on neuronal activity, LC firing rate was recorded before and after microinfusion of the CRF antagonist, DPheCRF₍₁₂₋₄₁₎, into the LC of stressed EA rats and controls. DPheCRF₁₂₋₄₁

decreased LC spontaneous discharge rates of EA exposed to social stress but not controls (Fig. 7).

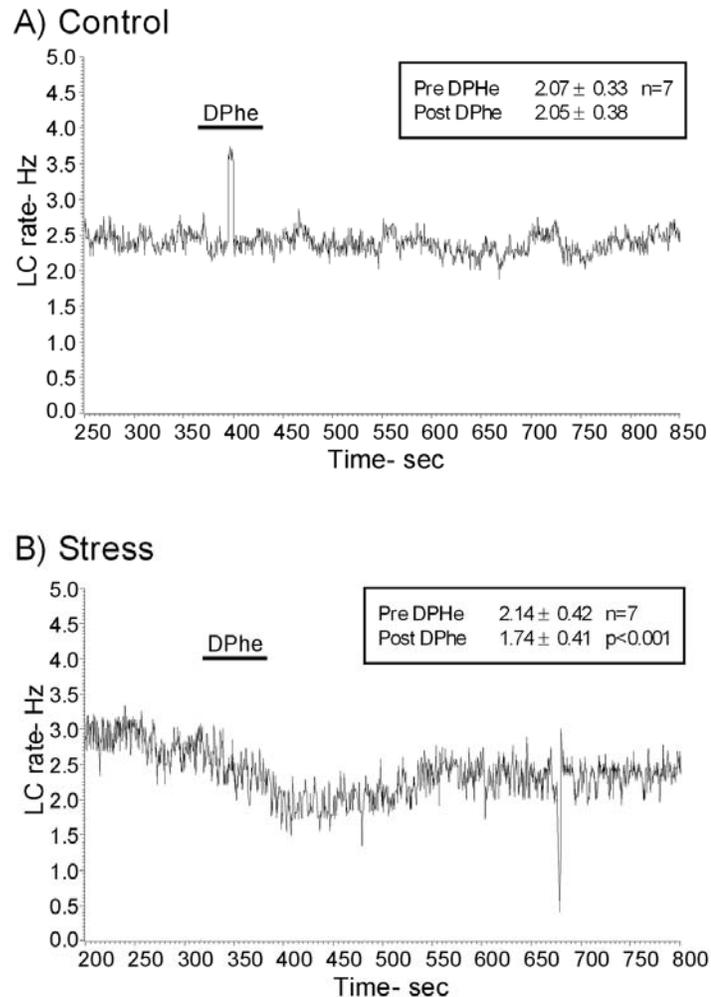


Figure 7. Representative rate meter records from a single LC neuron in a control and stressed EA rat before and after DPheCRF₁₂₋₄₁ (10 ng/30 nl) microinfusion into the LC. The mean discharge rates for 7 cells in each group before and after DPheCRF₁₂₋₄₁ administration are indicated in the upper right corner of the graphs. The CRF antagonist decrease LC spontaneous discharge rates of stressed but not control rats, p<0.001 Student's paired t-test.

Discussion

This study demonstrated that social stress interacts with adolescent development to determine the pattern of behavioral and neuronal responses to stress. The expression of the defeat posture developed through early adolescence to adulthood. Temporally correlated to this, the effects of social stress on coping shifted from facilitation to inhibition of active responses. Early adolescence was shown to be a critical developmental window during which social stress promoted active-coping behaviors. Consistent with this increase in active coping, early adolescent social stress shifted the discharge mode of LC neurons to a higher tonic state that has been associated with hyperarousal and behavioral flexibility (Aston-Jones and Cohen, 2005) and this was mediated in part by CRF release. The impact of early adolescent social stress on behavior and LC activity endured into adulthood, although it was differentially expressed as a decrease in active behavior and a shift towards increased phasic LC activity, identical to socially stressed adults. In contrast to early adolescence and adulthood, mid-adolescence was a time of transition or resilience. Finally, the effects of social stress did not generalize to another stressor or a non-threatening social interaction. Together, the findings suggest that social stress at different ages engenders contrasting neuronal and behavioral consequences, perhaps by affecting common neural substrates that are at different stages of development. This may be expressed as one type of psychopathology in childhood (e.g., hyperactivity, conduct disorder, increased aggression) and another in adulthood (e.g., depression).

Relationship to previous studies

Aversive social interactions, such as the resident-intruder stress, model a common stressor encountered by adolescent humans (Björkqvist, 2001; Huhman, 2006). Although there are many reports on the impact of resident-intruder stress in adult rats, studies using adolescent animals are relatively rare. These have shown that adolescent rats exposed to this stressor exhibit decreased social interaction and increased behavioral inhibition when tested as adults (Vidal et al., 2007; Watt et al., 2009). The present study significantly expands on previous work by systematically analyzing the behavioral and neuronal effects of agonistic social stress from the pubertal transition to adulthood in rats.

Developmentally distinct behavioral effects of social stress

The adoption of a subordinate posture in response to agonistic social stress developed during adolescence. EA rats more typically froze when confronted by an aggressive resident rather than assume the subordinate posture that was the consistent response of adults. Notably, plasma corticosterone levels in EA rats were elevated by exposure to social stress and most EA rats exhibited the defeat posture at least once during the seven stress exposures. This indicates that the deficiency in expressing defeat posture was not due to a perception that the experience was non-stressful or an inability to express the motor pattern. Rather, incomplete development of circuitry linking brain regions involved in the perception of the social stimulus with those underlying the motor response for defeat could account for the decreased incidence of defeat behavior.

The present study is the first to analyze the effects of adolescent agonistic social stress on coping behaviors in the defensive burying test and in response to swim stress. Defensive burying is used to model anxiety because anxiogenic and anxiolytic drugs

increase and decrease the incidence of burying in this test, respectively (Treit et al., 1981; Treit, 1990; De Boer and Koolhaas, 2003). In contrast to most anxiety models in which inhibited behavior is the anxiogenic endpoint, defensive burying is an active response to a fear-inducing stimulus (De Boer and Koolhaas, 2003). Likewise, behavioral responses to swim stress, have been used to screen for antidepressant efficacy and so this has been considered to model aspects of depression (Porsolt et al., 1978). However, in the present study, behavioral responses to a single exposure to swim stress were used as an additional test to compare active (climbing, swimming) and passive (immobility) coping behavior between groups. The most striking behavioral finding of this study was that EA social stress increased active coping in both tests. The increase in active behavior was not likely related to an “anxiogenic” effect, as climbing in response to swim stress is not considered an anxiogenic response and there were no group differences in open field behavior. In rats, burying attenuates the impact of stress by decreasing plasma corticosterone; therefore, this active coping phenotype could be considered a positive adaptation (Korte et al., 1992; De Boer and Koolhaas, 2003). On the other hand, this heightened reactivity to stress could potentially translate to a dysfunctional psychological state expressed as hyperactivity, increased aggression, or conduct disorder in humans. Consistent with this, social stress in early adolescents has been reported to increase the risk for hyperactivity, conduct disorder and violence in adolescents (Pelcovitz et al., 1994; Kaplan et al., 1998; Duke et al., 2010).

Social stress in adolescence had an enduring ability to affect coping strategy in adulthood. However, similar to the effect of social stress on adult rats, the consequence of EA social stress in adulthood was expressed as behavioral inhibition, in the form of

decreased burying in the defensive burying test. The results suggest that social stress at different ages converges on common neural substrates and the contrasting responses are a result of developmental changes in these substrates.

Social stress and LC activity

The LC-norepinephrine system is a potential substrate for the effects of social stress reported here because it is activated by stressors and has been implicated in both defensive burying behavior and climbing behavior. For example, defensive burying is associated with increased plasma and brain norepinephrine levels (Korte et al., 1992; Bondi et al., 2007). Increases in forebrain norepinephrine facilitate defensive burying, whereas noradrenergic receptor antagonists and selective LC lesion inhibit it (Bondi et al., 2007; Howard et al., 2008). Similarly, climbing in response to swim stress is enhanced by agents that increase extracellular levels of norepinephrine (Detke et al., 1995).

Norepinephrine projections arising from the LC form a vast network that innervates the entire neuraxis. Through this broad projection system, different patterns of LC discharge activity modulate states of arousal, attention, and cognitive flexibility (Berridge and Waterhouse, 2003; Bouret and Sara, 2004; Aston-Jones and Cohen, 2005). Stressors or exposure of LC neurons to CRF shift the mode of LC discharge from phasic to a high tonic state that is associated with increased arousal, blunted responses to discrete sensory stimuli and behavioral flexibility (Valentino and Foote, 1987, 1988; Valentino and Wehby, 1988a; Aston-Jones and Cohen, 2005). In the present study, social stress in early adolescent rats shifted the mode of LC discharge towards a higher tonic

state in which responses to discrete sensory stimuli were diminished compared to controls. The ability of the CRF antagonist to selectively inhibit LC neurons of stressed EA rats suggests that this effect was mediated by tonic CRF release within the LC. Given that CRF elicits LC-dependent burying behavior, CRF antagonists attenuate defensive burying, and that CRF microinfusion into the LC increases active responses to swim stress, tonic CRF release in the LC could mediate the observed increases in active responses (Butler et al., 1990; Korte et al., 1994; Howard et al., 2008). Because administration of CRF antagonists during behavioral testing would attenuate burying behavior in both control and stressed animals, this conclusion could not be directly tested. However, given the aforementioned data and literature, it is likely that social stress in early adolescence causes an increase in active coping behavior via tonic CRF release in the LC, which increases the spontaneous activity of these neurons.

Similar to the effects of adult social stress on behavior, the consequences of adult social stress on LC activity contrasted with those produced in early adolescence. The finding that spontaneous activity was not altered and evoked discharge was elevated compared to controls indicates that the tonic action of CRF produced by social stress in early adolescence is absent in adulthood, perhaps as a result of developmental differences in the system. Potential differences include CRF receptor internalization, decreased CRF drive to the LC, or development of opposing mechanisms such as endogenous opioid influence (Kreibich et al., 2008; Reyes et al., 2008a; Van Bockstaele et al., 2010). The development of these processes from early adolescence through adulthood can account for the lack of effect in mid-adolescence, as it represents a time of transition. The greater sensory-evoked response seen in socially stressed adults and in EA rats tested as adults

suggests that excitatory amino acid afferents to the LC are enhanced, although the functional significance of this with respect to active coping behaviors is less clear.

An important finding of this study is that EA social stress has enduring effects, although the expression of its behavioral and neuronal consequences changes with age to resemble those seen following adult exposure to social stress. This likely reflects the development of adaptive processes similar to those described above for adults that functionally counteract the release or postsynaptic effects of CRF in the LC. It may also indicate that the effect of EA social stress on the CRF system is temporally restricted to adolescence whereas other effects of social stress on the LC endure.

Early adolescent social stress and mental health

In summary, these data suggest that social stress interacts with brain norepinephrine function across development to shape stress coping behaviors. Coping behaviors are associated with the psychological impact of stressors in humans (Rohde et al., 1990; Ravindran et al., 1995). Therefore, the data presented here may provide a mechanism whereby social stress, via alterations in coping behaviors, increases the risk for externalizing disorders, (e.g., conduct disorder, aggression) in adolescence while simultaneously increasing the risk for later stress-related affective disorders in adulthood (Dodge et al., 1990; Pine et al., 2002). Finally, the data reinforce the concept that adolescent behavioral and emotional disorders, while having similar root causes as adult disorders, may be fundamentally different in their expression and neurobiology.

Table 1 (supplementary data) Open Field behavior following resident-intruder stress

Zone	EA		EA (peer)		MA		Adult	
	Control	Stress	Control	Peer	Control	Stress	Control	Stress
1	2.7 ± 1.3	2.3 ± 1.0	0.4 ± 0.2	1.1 ± 0.4	1.1 ± 0.5	1.3 ± 0.6	1.3 ± 0.7	1.1 ± 0.7
2	5.8 ± 2.4	6.8 ± 2.7	2.4 ± 0.6	4.2 ± 0.6 #	8.1 ± 3.3	2.5 ± 0.9	3.6 ± 1.4	3.2 ± 1.4
3	49.4 ± 12.9	44.5 ± 15.2	15.5 ± 0.6	29.2 ± 7.9 #	44.4 ± 10.0	35.2 ± 9.7	38.2 ± 8.3	46.8 ± 6.6
4	242.0 ± 16.2	246.4 ± 18.6	284.1 ± 3.3	265.5 ± 8.2 #	246.4 ± 12.5	260.9 ± 10.5	257.0 ± 9.4	248.8 ± 7.5
Distance (cm)	2106.7 ± 177.3	2416.6 ± 279.4	2268.3 ± 270.8	2519.8 ± 197.5	2017.1 ± 221.8	1484.4 ± 157.4	1817.1 ± 114.7	1586.8 ± 156.3

p<0.08 ttest vs control

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CHAPTER THREE: GENERAL DISCUSSION

Overall Goals of Thesis Research and Summary of Major Findings

The over-arching question of this dissertation is whether the effects of social stress on behavior and physiology across adolescence are dependent on the stage of adolescent development at the time of stress. In humans, adolescence is marked by dramatic physiological, emotional, and cognitive changes that mediate the transition from a sexually immature, physically diminutive, and intellectually limited child to a sexually viable, full-grown adult with the capacity for abstract cognition and intricate emotional relationships. Successful adolescent development into a well-adjusted and functional adult is dependent on the interaction between physiological maturation and the social environment. Exposure to stressful or dangerous social environments during the critical periods of cerebral maturation in childhood and adolescence may alter the trajectory of cognitive and emotional development in these individuals, thereby affecting the risk for psychiatric disorders, like anxiety and depression. As such, the major goal of this dissertation was to investigate the developmentally dependent effects of social stress on behaviors related to affective disorders and to elucidate their neurological basis.

The first aim was to determine if the response to agonistic social stress and its consequential effects on behavior were dependent on the age at stress exposure. The rat resident-intruder paradigm was used as the social stressor and subsequently behaviors were examined in animal models related to anxiety (the open field and defensive burying tests) and depression (swim stress). Additionally, the rat's behavioral response to the social stressor was analyzed. It was determined that the expression of the supine defeat posture, a rodent behavior signaling submission in response to social conflict, developed through early adolescence into adulthood. Concordant with this increase in social

submission, the effects of social stress on the other behaviors were generally shifted from a facilitation of active coping in early adolescence to an inhibition of active coping in adulthood. These behaviors were also dependent on the age at behavioral testing, as animals stressed in early adolescence but tested as adults resembled those stressed as adults in being more inhibited. Social stress had no effect on MA animals, suggesting that this may be a time of resilience or transition. Restrain stress did not change behavior at any age, suggesting that the aforementioned effects are selective to social stress.

The second aim of this dissertation was to elucidate a neurological basis for the behavioral changes induced by social stress. The LC-norepinephrine system was chosen for study because it has been implicated in the behaviors that were found to be altered by social stress. As the LC is an important mediator of the cognitive and behavioral effects of stress and has been implicated in stress-related psychiatric disorders, the study of the developmental effects of stress on this neurotransmitter system is clinically relevant. Indeed, it was determined that agonistic social stress in early adolescence selectively increased the basal firing rate of these cells. LC phasic responses to sensory stimuli were decreased in adolescence but increased in adulthood. These effects were partially mediated by changes in CRF release in the LC and were dependent on both the age at which the stress occurred as well as the age at time of testing.

Animal models of social stress

Much of the stress that children and adolescents face is social in nature. The source can be varied, including family members, parents, friends, or other adults but a common denominator among most social stressors is conflict and a dominant-submissive

relationship. However, in the laboratory, the effects of social stress have been primarily modeled in adult, not adolescent, rodents (Björkqvist, 2001; Huhman, 2006). In the following section, data from the few investigations using agonistic social stress in adolescent rodents will be reviewed. Data from an additional model of social stress, social isolation, will also be presented.

Resident-intruder paradigm (social defeat)

Many of the studies involving the resident-intruder model have used hamsters, a solitary species with aggressive, early developing territorial tendencies. In adult hamsters, defeat by a more aggressive resident results in a marked behavioral depression termed conditioned defeat. In this state, the defeated hamster loses all offensive aggression against intruders into its own territory and in response, increases submissive and avoidant behaviors (Huhman et al., 2003). Endocrinologically, this state is marked by an increase in glucocorticoids that habituates over time and a decrease in testosterone after chronic defeat (Huhman et al., 1991). In contrast to adult hamsters, social defeat in adolescents has been shown to increase aggression and protect against decreases in testosterone caused by later defeats. Like most mammals, golden hamster adolescents will engage in “play fighting” as juveniles and as they mature, play behavior transitions to adult aggressive behavior. In socially subjugated adolescent hamsters, this transition to adult-like aggressive behavior occurs at an earlier age and they become hyper-aggressive towards smaller-sized hamsters (Delville et al., 1998; Ferris, 2003; Wommack et al., 2003).

Even though they are hyper-aggressive towards smaller hamsters, adult hamsters who were subjugated as adolescents continue to exhibit conditioned defeat towards larger intruders, although they are protected from the defeat-induced decrement in testosterone and sexual behaviors seen in hamsters that are only subjugated as adults (Ferris, 2003; Ferris et al., 2005). Thus, in this species, elements of social defeat in adolescence may be adaptive to later adult social defeat, especially with regard to establishing dominance over younger animals and maintaining sexual viability, both of which are lost in adult only defeat. Interestingly, the increased aggressive behavior of the adolescents towards smaller hamsters and the increased submission towards larger ones as adults may parallel the hyperactivity noted in the social stressed EA rats and the behavioral suppression of the social stressed adults.

To date, only two studies have investigated social stress in adolescent rats using the resident-intruder stressor. These studies have shown that adolescent rats exposed to this stressor exhibit, decreased adult social interaction (Vidal et al., 2007) and increased adult behavioral inhibition when placed in the resident's empty cage, as determined by decreased exploration and increased risk assessment behavior (Watt et al., 2009). This increased behavioral inhibition is reminiscent of the decreased burying behavior of adult rats that had been stressed as adolescents in the current study. By analyzing both adolescent and adult behavior at 3 ages following defeat, the current study expands on these previous studies and demonstrates that the effects of defeat are both variable and age-dependent.

Social Isolation

A frequently used model of social stress in adolescents is isolation. This has been examined using many of the same behavioral endpoints that have been used to examine the consequences of social defeat. The effects of social isolation on defensive burying behavior and open field activity are dependent on the age at which the stress occurs. For example, social isolation from p26-p40 decreases burying behavior measured at p40 and this phenotype was maintained until p80, even after resocialization (Arakawa, 2007b; Simpson et al., 2010). However, adult social isolation from p66-p80 increased burying behavior (Arakawa, 2007b). These results contrast with the present findings on social defeat and burying behavior. Because the control rats in the present study were socially isolated, it was important to determine if the noted increase in EA burying duration in the stressed rats was a result of social defeat or due to the fact the experimental animals had more social contact than controls. Therefore, a control group was devised in which the experimental animals were allowed to interact with age-matched peers for 15 min per day. As there were no significant differences between these two groups, it was determined that the increase in burying behavior noted in stressed EA animal was due to the agonistic nature of that social encounter, not the isolation of the controls. Additionally, Arakawa has found that pairing the adult with an older, presumably dominant, animal decreases burying with respect to adults who were placed with a younger, presumably submissive animal (Arakawa, 2007a). This supports both the current finding that adult social defeat decreases burying behavior, as well as the overarching theme that burying behavior is modulated by the nature of the social environment.

Developmental basis for the response to social aggression in rats

In the present study, the strategy of adopting a subordinate posture in response to agonistic social stress increased at each stage throughout adolescence, suggesting that the pathways mediating that response are not fully developed in early adolescence. Little is known regarding the neural pathways responsible for the development of supine defeat posture; however, several potential brain regions or neurotransmitter systems might be associated with differential expression of this behavior across development. Development of these brain regions may also interact with normal adolescent social experience to produce the final, adult-like submissive behaviors

Anatomical substrates

The observed changes in defeat behavior with age may reflect the development of brain areas that mediate active vs. passive coping such as the amygdala, the bed nucleus of the stria terminalis (BNST), and prefrontal cortex (Vivian and Miczek, 1999; Jasnow et al., 2004; Miczek et al., 2004; Robison et al., 2004; Cooper and Huhman, 2007; Panksepp et al., 2007). CRF from the central nucleus of the amygdala (CeA) has been implicated in the acquisition and maintenance of submissive behaviors following social stress in hamsters. As noted in the introduction, learned expression of submissive behaviors following an initial experience of social defeat is dependent on activation of the CeA and the presence of CRF in the BNST (Jasnow et al., 2004; Cooper and Huhman, 2005). In an interesting experiment, Jasnow and colleagues first determined that CRF antagonists administered in the BNST after an initial social defeat blocked later conditioned defeat behaviors in a subsequent conflict. This did not occur with CRF

antagonists administered to the CeA. However, in a unique twist, they lesioned the CeA unilaterally and then administered a CRF antagonist to the contralateral BNST and found that this also blocked conditioned defeat. This implied that previous experience with social defeat mediates later submission via CRF input into the BNST from the CeA. Interestingly, none of these manipulations restored the hamster's natural aggression, they only blocked submission, indicating that territorial aggression and submission may be operations of distinct circuits, not continuums of the same. CRF1 antagonists into the basolateral amygdala of mice also blocked post-defeat submissive postures, suggesting that this region may also play a role in the CeA-BNST mediation of conditioned defeat.

CRF and serotonin circuits between the CeA and dorsal raphe (DR) also have a crucial role in social defeat. Cooper and Huhman have shown in hamsters that a non-specific CRF antagonist administered into the DR, prior to an initial social defeat, decreases submissive behaviors in a later test. This effect seemed specific to the CRF1 receptor because a CRF2 receptor antagonist had no effect when administered prior to the initial defeat, but was active when given in between the initial defeat and the conditioned defeat test (Cooper and Huhman, 2007). Blockade of either receptor in the DR had no effect on the submissive behaviors during the initial training defeat. This, combined with the Jasnow data, suggests that the role of CRF may be one of sensitization- i.e., upon experience in an unwinnable or uncontrollable situation, CRF activation in these regions causes an association between the defeat context (social interaction) with an automatic activation of circuitry which selects for passive coping behavior (submission).

This model fits well with data from swim stress indicating that increased passive coping behavior occurs following an initial CRF release in a prior swim. In this literature,

CRF release in the DR during the initial swim inhibits acute serotonin release, but it also causes an internalization of the CRF1 receptor and subsequent externalization of the CRF2 receptor. The switch from CRF1 to CRF2 is associated with an increased serotonin release and passive coping in the subsequent swim stress (Price and Lucki, 2001; Price et al., 2002; Roche et al., 2003; Waselus et al., 2009). Interestingly, social defeat in adults also increases CRF2 expression in the DR (Debra Bangasser, personal communication). However, in the current study, social stress in EA rats did not increase DR CRF2 expression (data not shown). Hence, CRF influences on DR neurons may not be fully developed in early adolescence and thus the effects of social stress may be buffered. In sum, development of the amygdala, BNST, and dorsal raphe with respect to the influence of CRF, while perhaps not a causal factor for defeat behavior, may bias the neural circuitry to select this behavior in future agonistic encounters.

The PFC is another area that may be involved developmentally distinct social behaviors. The prefrontal cortex is a crucial area for selection of coping behaviors in response to stress (Keay and Bandler, 2001). It is one of the later developing brain regions in terms of synaptic over-expression and pruning as well as axon myelination and therefore may be particularly susceptible to stress (Andersen and Teicher, 2008). As such, adolescent stress has been shown to have immediate and long-lasting detrimental effects on PFC development in rats and humans (Taylor et al., 2006; Andersen et al., 2008; Leussis et al., 2008). Social isolation in early adolescence (p30-p35) decreases markers for synaptic plasticity including spinophilin (19%) and synaptophysin (8%) as well myelin basic protein (49%), a marker for axon myelination (Leussis and Andersen, 2008). This stunting of synaptic plasticity and axonal functioning could alter the

integration of the PFC with other brain regions and thereby limit its ability to function in selecting for behavioral responses to stressors.

Development of functional circuits

Expression of defeat behavior may not be solely dependent on development in isolated brain region. Throughout development, the connections between the amygdala, hippocampus, and frontal cortex all continue to mature in young rats and these connections are important in risk evaluation and the establishment of defensive behaviors (Wiedenmayer, 2009). As such, development and pruning of functional connections between brain areas may play a role in the ontogeny of defeat behavior. Notably, in the present study, most EA rats exhibited the defeat posture at least once during the seven stress exposures, indicating that the deficiency in expressing defeat posture was not due to an inability to express the motor pattern. However, incomplete development of the circuitry linking brain regions involved in the perception of the social stimulus, like the amygdala, with those underlying the motor response for defeat could account for the decreased incidence of defeat behavior in EA animals. Exposure to androgens perinatally and again at the beginning of adolescence may play a role in this connectivity as they seem to pattern the brain for proper social behaviors, such as the formation of dominant-submissive relationships as well as sexual behaviors (Smith et al., 1997; Schulz and Sisk, 2006)

Social experience

Studies have also shown that social experience during adolescence has a crucial role in establishing adult aggressive and submissive behaviors. Play behavior wanes in late puberty when adult-like aggression and submission patterns emerge in both rats and hamsters (Panksepp, 1981; Takahashi and Lore, 1983; Wommack et al., 2004). The quantity and quality of this play behavior predicts later adult social and sexual behavior. Animals who are more aggressive in play as peri-adolescents also tend to be more aggressive in social conflict as adults (Takahashi and Lore, 1983). The current data demonstrating a graded progression in the adoption of defeat posture in the face of social aggression fit with a model in which the final response to social aggression is dependent on the interaction between the rat's physical development and social experience. The ability to express submissive postures in the appropriate context is a highly adaptive function. As an animal ages and begins to compete for resources, the consequences of not withdrawing from a conflict with a larger or more aggressive animal are potentially fatal; therefore, the social skills that allow the animal to withdraw from social conflict serve to prolong life and avoid death or injury. The development of these social skills occurs throughout adolescence and may be dependent on the interaction between brain development and social experience.

Coping in rodent behavioral tests and its translation to humans

Coping in human affective disorders

In animal models, the differences between coping behaviors are fairly easy to discern. Active coping typically involves some sort of action to either escape or confront a threat. Examples of these behaviors include climbing and swimming in the forced swim

test, shuttling in the learned helplessness test, burying in the defensive burying test, or defensive postures in social defeat. Passive coping typically is defined as immobility or freezing behavior. In humans, however, stress coping is more abstract. Often it involves a combination of overt, problem solving actions with mental exercises that change the meaning of an experience or allow for an emotional escape (Folkman and Lazarus, 1980; Scheier et al., 1986; Ursin and Oiff, 1995). The measures of what constitutes active vs. passive coping behaviors vary across study, but a fitting consensus seems to be that seeking social support, re-interpreting the stressor in a more positive light, and problem-solving are considered examples of active coping mechanisms that are protective against the anxiogenic and depressive effects of stress. On the other hand, behaviors like social isolation, mental escapism, and a hopeless perspective are considered passive responses that have been shown increase the risk of affective disorders (Scheier et al., 1986; Rohde et al., 1990; Ravindran et al., 1995; North et al., 2001).

Although humans and laboratory animals exhibit different forms of coping behaviors, the unifying principle among these different behaviors is that active coping is usually associated with engaging the environment or internal emotional state and seeking to “fix” or a remedy a situation. Passive coping avoids dealing with issue and is associated with disengagement and restraint. This generalization also seems to be the basis for interpreting animal behavior in models of coping.

Animal models of coping behavior

In the study of stress and stress-related disorders, behavioral models are developed that allow the investigator to make inferences about how certain drugs, environmental constructs, or physiological conditions would affect humans. These models take advantage of conserved biology and behavior between laboratory animals and humans. In this manner, investigators are able to create behavioral tests which provide insight into questions like the potential efficacy of psychiatric drugs or the neurological effects of stress. Tests which induce stress-coping behaviors are especially useful in this context because the circuitry behind coping behaviors is fairly well conserved (Keay and Bandler, 2001).

Defensive Burying

Originally developed as a screen for compounds with anxiolytic or anxiogenic properties, the defensive burying test is one of the more popular tests for anxiety-like behaviors (Treit et al., 1981; Treit, 1990; De Boer and Koolhaas, 2003). In contrast to most anxiety models in which inhibited behavior is the anxiogenic endpoint, defensive burying is an active response to a fear-inducing stimulus. The interpretation of the behavior in this test is traditionally based on the assumption that increased reactivity to the shock probe, as measured by burying behavior, is associated with a more anxious internal state and decreased burying is associated with a less anxious state. Burying is a species-specific defense behavior and as such, is one of several behaviors that rats use to deal with fear-inducing threats. Therefore, a more accurate interpretation of behavior in the defensive burying test considers changes in burying as shifts in coping mechanisms

that the animal employs to deal with a threat in its environment and not solely a measure of the rat's subjective anxiety (De Boer and Koolhaas, 2003).

In the present study, socially stressed EA rats buried the shock probe more their control counterparts when tested as adolescents. This is interpreted as an increase in active coping behavior. Increased burying may be considered a positive adaptation. This is based on the finding that burying attenuates the shock-induced increase in plasma corticosterone (Korte et al., 1992; De Boer and Koolhaas, 2003). However, increases in active coping behavior could be maladaptive. In humans, a heightened or uncontrolled external reactivity to stress could manifest as a dysfunctional psychological disorder like hyperactivity, increased aggression, or conduct disorder. These are classified as externalizing disorders and they are characterized by an excessive, aggressive, or inappropriate behavioral activity (Achenbach and Edelbrock, 1978). Consistent with this interpretation, increased burying behavior and heightened social aggression have been linked in rats (Sgoifo et al., 1996). When considering this data in combination with the increased aggression noted in subjugated adolescent hamsters, it is tempting to speculate that rodent adolescent social stress could potentially serve as a model for the increased risk of hyperactivity, conduct disorder and violence in socially abused human adolescents (Dodge et al., 1990; Pelcovitz et al., 1994; Kaplan et al., 1998; Duke et al., 2010).

Social stress in adolescence also had enduring effects on coping strategy later in adulthood. Unexpectedly, the effect of social stress in these animals was expressed as behavioral inhibition, in the form of *decreased* burying, similar to rats that had been stressed and tested as adults. Therefore, social stress, regardless of the age at which it occurred, decreased burying behavior in the adult animals. These results may be related

to the emergence of the subordinate posture across adolescence. It is interesting to speculate that the development of the same neural systems which establish a submissive posture in response to social stress would also mediate a shift from active coping in early adolescents to inhibited coping in adults. This model fits with human data indicating that externalizing disorders in adolescence, like violence and conduct disorder, increases the risk for depression later in adulthood (Kim-Cohen et al., 2003).

Swim Stress

Similar to the defensive burying test, the behavioral responses to swim stress have been used to screen for antidepressant efficacy and have been considered to model aspects of depression (Porsolt et al., 1977; Porsolt et al., 1978). At its core, the swim test forces the rat to make a behavioral decision of whether to seek escape opportunities via climbing or swimming, or whether to remain immobile and float, thus conserving energy (Thierry et al., 1984). Climbing and swimming are therefore classified as active coping behaviors whereas immobility is considered a passive behavior (Detke et al., 1995). As a model of stress-induced behavioral depression, the test functions on the basis that a previous experience with swim stress shifts the coping strategy employed in the subsequent swim towards immobility, or passive coping. Both acute and chronic antidepressant treatment can block this shift in coping style, regardless of their mechanism of action, however serotonergic drugs do so via increases in swimming behavior, whereas noradrenergic drugs increase climbing behavior (Detke et al., 1995). In the present study, socially stressed EA rats climbed more during swim stress than controls. This serves as a second measure of active coping, reaffirming that stressed EA

rats have increased active responsiveness to stressors and further implicating the norepinephrine system as the neurological basis for the increased active coping.

Locus Coeruleus: Development and Regulation

As previously described, adolescent development interacts with social stress to alter behavior in the defensive burying test and in swim stress in an age dependent manner. Of specific note, social stress increased burying behavior and climbing behaviors in early adolescence. Previous research has linked those behaviors to increases in brain norepinephrine (Detke et al., 1995; Bondi et al., 2007; Howard et al., 2008). As the locus coeruleus (LC) provides most of the norepinephrine to the brain, it was hypothesized that the LC may be the neural substrate for the actions of social stress on these behaviors.

Characteristics of LC neurons

Norepinephrine projections arising from the LC form a vast network that innervates the entire neuraxis (Swanson and Hartman, 1975). The LC is the sole source of norepinephrine to the cortex and hippocampus as well as a primary source for most of the forebrain and midbrain (Foote et al., 1983). Norepinephrine is released as LC neurons fire in a continuum of two modalities. Spontaneous, or tonic, firing is highly regular and relatively slow (1-2 Hz). Phasic firing occurs in response to sensory stimuli or salient behavior and is characterized by a rapid 2-3 spike burst followed by a period of inhibition (Foote et al., 1980; Aston-Jones and Bloom, 1981a). LC cells are electrotonically connected to each other via gap-junctions (Ishimatsu and Williams, 1996) and this connection aids in generating phasic responses (Usher et al., 1999). As a function of its

firing modalities, and consistent with its anatomical distribution, LC activity modulates states of arousal, attention, and cognitive flexibility via connections to diverse forebrain regions, (Aston-Jones and Bloom, 1981b; Berridge et al., 1993; Berridge and Waterhouse, 2003; Bouret and Sara, 2004; Aston-Jones and Cohen, 2005). Because so few LC neurons (>10,000) innervate the entire neuraxis, the axons are highly collateralized, and although the LC does reflect some topography in its efferent and afferent connections, it is still a relatively homogenous structure (Berridge and Waterhouse, 2003). As described in the following sections, the LC is also under the influence of various regulatory inputs which allow it to respond quickly and variably to the environment.

Ontogeny

Ontogenetically, the LC is one of the earlier brain regions to form. LC cells are born well before the cells in their projection fields and its innervation patterns are mostly adult-like by the second week postnatal (see review by Foote et al., 1983). Because of this, it is thought that LC activity may act as a developmental factor for many of its target fields. Indeed, studies in kittens have shown that LC-NE has a facilitatory role in the plasticity of ocular dominance (Kasamatsu and Pettigrew, 1976). Although its efferent connectivity is well developed by adolescence, the afferent connectivity of the LC continues to mature into adulthood. From p15-p30 the number of afferent LC synapses remains stable. However, during adolescence (p30-p60) there is a 66% increase in synapse number (Lauder and Bloom, 1975). Because the development of its afferent connections is prolonged throughout adolescence, the LC may be susceptible to stress-

induced plasticity during this time. Indeed, as demonstrated in this thesis, stress across development can alter the regulation of the LC-NE system, both in terms of the tonic input it receives as well as its response to sensory stimuli.

Regulation by afferent systems

Glutamate

The primary excitatory input into the locus coeruleus comes from the nucleus paragigantocellularis (PGI) in the ventrolateral medulla, a multi-modal sensory integration area (Andrezik et al., 1981). Tracing studies have shown that the PGI directly innervates the core of the locus coeruleus, while electrophysiological studies have shown that stimulation of this nucleus activates 73% of LC neurons via AMPA receptors (Aston-Jones et al., 1986; Ennis and Aston-Jones, 1988). Because the PGI is the main sensory input into the LC, it is likely the input which relays the sciatic nerve stimulation to the LC and generates the phasic responses that are divergently regulated by stress across development. This divergent regulation could occur either at pre-synaptic terminals through modulation of glutamate release or at post-synaptic receptors through modulation of their sensitivity. As CRF has previously been shown to decrease phasic responses and is tonically released in the stressed EA rats, it is likely that the decrease in phasic responses noted in stressed EA rats is due to the actions of CRF (Valentino and Foote, 1987).

A secondary glutamate input arises from the dorsomedial and prelimbic regions of the medial prefrontal cortex (Jodo et al., 1998). Unlike the PGI input, the afferents from the mPFC terminate in the pericoerulear region (Aston-Jones et al., 1986) and the

response latencies are much longer (30-40 ms vs. 11 ms), indicating that the activation may be indirect (Jodo et al., 1998). Inactivation of the mPFC with lidocaine resulted in an inhibition of LC neurons, suggesting that the mPFC mediates a tonic excitatory influence on the LC (Jodo et al., 1998). The LC also directly innervates the mPFC, therefore the relative balance of activity in these two brain regions may prove crucial in determining the cognitive and behaviorally activating effects of stress. As the mPFC is one of later maturing brain regions, it stands to reason that stress-induced activity or alterations in the LC-NE system during crucial windows in development could bias the activity of this circuit resulting in altered behavioral reactions to later stressors.

CRF

CRF is the primary mediator of the stress-induced activation of LC neurons (Valentino et al., 1983; Valentino and Wehby, 1988a; Valentino and Van Bockstaele, 2008). CRF containing axons originating in the CeA, BNST, PVN, mostly innervate the dendritic zone surrounding the main cluster of LC neurons while those from Barrington's nucleus primarily innervate the core (Valentino et al., 1992; Page et al., 1993; Van Bockstaele et al., 1996; Lechner and Valentino, 1999; Reyes et al., 2005; Reyes et al., 2008b). CRF labeled terminals form primarily excitatory synapses on LC dendrites as well as other terminals, indicating both pre and post-synaptic roles (Valentino et al., 1992). Thus, CRF input is positioned to modulate both tonic firing activity of the cells as well as acute activity from other afferent systems, like glutamate.

Stress, either physical or psychological, causes the release of CRF onto the LC neurons, which subsequently increases their spontaneous discharge rate and decreases

their response to sensory stimuli (Valentino et al., 1983; Valentino et al., 1991; Valentino and Van Bockstaele, 2008). In unanesthetized monkeys and rats, a shift from a phasic to a tonic mode of firing is associated with increased general arousal, blunted responses to discrete sensory stimuli, and behavioral flexibility (Valentino and Foote, 1987, 1988; Valentino and Wehby, 1988a; Aston-Jones and Cohen, 2005).

One of the primary findings of this thesis was that social stress in early adolescence induced a constitutive CRF release onto the LC which caused a shift towards a high tonic / low phasic state. This shift towards a higher tonic state could potentially be an advantageous adaption for the rat in a chronically dangerous environment. Theoretically, it would reduce the salience or importance of any one stimulus and thereby limit the focused attention of that rat, causing it to become more distracted by non-salient stimuli. In an unpredictable and dangerous environment, as modeled by social defeat, it would be advantageous to err on the side of distraction by false alarms instead of being overly focused on a task related stimuli and risk missing a danger signal from the environment. This increased tonic LC discharge could also account for the increase in active coping responses.

The CRF release noted in the stressed EA animals did not persist into adulthood, nor did it occur in adults, suggesting that the effect is developmentally limited to early adolescence. One potential mechanism for this limitation may be related to developmental increases in glucocorticoid regulation. CRF transcription and release are under constitutive glucocorticoid control, as shown by increases in median eminence CRF content and paraventricular CRF mRNA following adrenalectomy (Suda et al., 1983; Sawchenko et al., 1984). In adult rats, adrenalectomy causes a tonic release of CRF

into the LC to increase discharge rate, an effect not seen in intact adult rats (Pavcovich and Valentino, 1997). This suggested that corticosteroids exert an inhibitory influence over CRF release in the LC so that it is not tonically secreted. If the glucocorticoid regulation of CRF release in the LC is not fully developed until adulthood, the resulting CRF release could be partly responsible for the age differences in LC activity following chronic social stress. Consistent with this, adolescents have prolonged HPA responses to stress due to incomplete maturation of glucocorticoid mediated negative feedback systems (Goldman et al., 1973). In contrast, restraint stress has been shown to interact with pubertal androgens across development to decrease the number of CRF positive cells in the central nucleus of the amygdala, an important source of CRF to LC (Gomez et al., 2004). While a decrease in CRF cells is counter to what would be expected based on the data presented in this thesis, social stress and restraint stress were also shown to have very different behavioral and physiological outcomes. The fact that CRF production in the amygdala is plastic during adolescence may be the important factor.

Opioids

The endogenous opioid systems are an important source of LC regulation, both in terms of stress termination as well as in terms of modulation of sensory-evoked stimuli. The three main classes of endogenous opioid receptors, mu, kappa, and delta, are all expressed in the LC, however less is known about the delta receptor's role compared to the others. Mu-receptors are expressed post-synaptically on the LC neurons themselves, while kappa and delta receptors are positioned presynaptically (Tempel and Zukin, 1987;

Bockstaele et al., 1997; Van Bockstaele et al., 2010). Curtis and colleagues have shown that one of the prominent functions of the mu-opioid system in the LC is to functionally counteract, or balance the post-synaptic effects of stress-induced CRF release. In an interesting study, they found that hypotensive stress initially caused an increase in LC firing rate, followed by suppression. If a CRF antagonist was locally applied to the LC during stress, the initial increase in LC discharge rate was blocked, but the post-stress inhibition component was amplified and prolonged. However, if naloxone, a mu-opioid receptor antagonist was applied instead of the CRF antagonist, the post-stress inhibition was selectively blocked and LC neurons took longer to return to baseline levels of firing when the stressor was terminated (Curtis et al., 2001). This counter-CRF effect is especially intriguing in light of the data presented in this thesis that social defeat increases CRF-induced LC activity only in early adolescent rats. Besides glucocorticoid regulation, another plausible mechanism for the loss of CRF influence in adulthood could be latent development of an opposing system, like the mu-opioids. However, this would only oppose the increase in tonic rate associated with CRF release and could not account for the increase in phasic response, suggesting that another system would have to be involved.

In contrast to mu-opioids, kappa agonists have been shown to block the sensory stimulated phasic response in LC neurons as well as the increase in tonic firing rate due to hypotensive stress and opiate withdrawal (Kreibich et al., 2008). Combined with ultra-structural data which show that kappa-receptors are co-expressed with glutamate and CRF on axon terminals, the data suggest that a primary role of the kappa-dynorphin system is to regulate presynaptic neurotransmitter release within the LC (Kreibich et al.,

2008; Reyes et al., 2009). As such, the kappa opioid system may be another alternative means of post stress CRF regulation in the developing rat.

Besides its role in pre-synaptic modulation, recent studies have also shown direct asymmetrical synaptic contacts between dynorphin expressing axons and catecholaminergic neurons in the LC, as well as post-synaptic kappa receptor expression, suggesting that kappa opioids may also have a direct effect on LC neurons (Reyes et al., 2007; Reyes et al., 2009). However, application of kappa agonists to the LC does not directly alter LC firing rate (Kreibich et al., 2008). Given that dynorphin is co-released with CRF and glutamate (Van Bockstaele et al., 2009), this may suggest a post-synaptic role for the kappa system in terms of LC stress and sensory regulation that has yet to be fully defined. Currently, little is known about the development of the kappa system, however, the central nucleus of the amygdala is one of the primary sources of dynorphin to the LC, where it colocalizes with CRF (Van Bockstaele et al., 2009), therefore dynorphin release into the LC may also be under the same developmental regulation as CRF.

Future Directions

The research presented in this dissertation raises some compelling questions that will guide future studies. 1) What underlies the development of the submissive defeat posture, 2) what is the developmental time course of stress-induced changes in LC physiology; 3) how are the electrophysiological changes observed in the current study behaviorally expressed, and 4) how do these findings in rats translate to humans?

Development of the defeat posture

The finding that the expression of the defeat posture signaling subordination is not fully developed until adulthood can be further explored to reveal the developmental aspects of circuitry underlying a program of defensive behaviors in response to social aggression. An initial investigation would use functional neuroanatomy to identify neurons that are activated during the social defeat model. Specifically, one could compare the pattern of the early intermediate gene *c-fos* immunoreactivity immediately after both acute and chronic experience to social aggression in EA and adult rats. Because *c-fos* expression has been associated with neuronal activation, differential expression would not only indicate potential differences in circuit activity between adolescents and adults, but it would also demonstrate differences in adaptation of those circuits across repeated social stressors. A second, related, experiment would use in-situ hybridization to determine the source of the CRF release that impacts on LC neurons in the response to EA social stress. Determining the source of the CRF release would lead to a specific area that is differentially sensitive to social stress across adolescent development and therefore may also play a role in the development of social behavior. Combining retrograde tract tracing with immunohistochemistry for *c-fos* and CRF would identify regions upstream from the LC that are components of this circuit.

Developmental time course of stress-induced changes in LC physiology.

Social stress interacted with development such that in early adolescence the sensory-evoked responses of LC neurons was decreased but in adulthood, they were increased. One of the pressing future directions is to determine what mediates this

transition in LC activity. One potential explanation is that social stress at any age would increase the evoked response of LC neurons; however, in early adolescence, the CRF release that is released as a consequence of social stress acts presynaptically to inhibit the glutamate release responsible for the evoked response to sensory stimuli, essentially counter-acting the would-be increase in evoked response. As the social stress induced increase in CRF tone seems to be limited to early adolescence, this would explain why adult animals that were stressed in early adolescence also have an increased evoked response to sensory stimuli. A potential experiment to test this theory would be to record peri-stimulus time histograms from early adolescent rats exposed to social stress immediately following local application of either artificial cerebrospinal fluid or the CRF antagonist DPhe-CRF. It is hypothesized that CRF antagonism in stressed EA animals would result in an increase in evoked LC activity, similar to that noted in adulthood.

The electrophysiological changes observed and their behavioral consequences

Previous research in behaving animals has shown that LC cells fire phasically in response to a reward-paired sensory cue and that this response is more closely time-locked to the behavioral response required to receive the reward than the presentation of the stimulus itself (Bouret and Sara, 2004; Aston-Jones and Cohen, 2005). This suggests that LC activation may be important for facilitation of behavioral responses to sensory stimuli. However, LC activity has not yet been analyzed in terms of coping behaviors to negative stimuli. Therefore, one of the future directions is to determine how LC activity affects behavior in the defensive burying test and if there is a functional consequence to the stress-induced changes in evoked response to sensory stimuli.

To determine how LC activity affects behavior in the defensive burying test, unanesthetized, in-vivo LC recording during the defensive burying test would be performed using rats previously implanted with a multi-wire array of electrodes. Following one week of social stress, neuronal activity would be monitored during the defensive burying test and would be time-locked with a video recording of behavior and an ammeter attached to the shock generator so that both phasic and spontaneous LC activity could be temporally linked with shock delivery and with later behavior. This link could be manifest in several ways. Data from this study and others suggest that exposure to the shock prod will generally increase LC activity and subsequently increase burying (Bondi et al., 2007; Howard et al., 2008). This increase in LC activity is likely a sustained increase in spontaneous firing, given that animals who have increased basal LC activity also show increased burying, however, this has never been directly tested. With respect to the relationship between the evoked LC response to shock and burying behavior, it is possible that the degree of LC sensory activation upon exposure to the shock-prod dictates the degree of later LC spontaneous activity and therefore burying behavior. As socially stressed adult animals bury less than controls but have greater LC responsiveness to sensory stimuli, it might be expected that an inverse relationship exists between the degree of LC sensory reactivity and increases in later spontaneous activity. Alternatively, the relationship between spontaneous firing and burying behavior may be an inverted-U, such that the increased sensory response of the LC may trigger a larger increase in spontaneous firing than controls, but this increase falls on the right side of the LC activity-burying curve resulting in a decrease in defensive burying. If either of these relationships were established, it would likely indicate an interaction between the initial

sensory-evoked NE release in the forebrain and subsequent descending modulation of LC activity.

Secondly, as phasic LC activity is associated with behavioral responses to stimuli, it is also possible that phasic LC responses could also modulate burying activity. By time-locking burying behavior with LC activity it is possible to create peri-event time histograms describing LC activity immediately prior to initiation of each burying bout. If phasic responses occur and if the evoked response to sensory stimuli seen in stressed animals is indicative of behavioral phasic activity in LC cells, then it could be hypothesized that increases in phasic responding might be inhibitory to later burying behavior. This would also fit with the data from stressed EA rats that have a decreased evoked response to sensory stimuli but increased burying. This data would suggest a role for phasic activity in burying behavior and could fit with a model wherein peri-burying LC activity acts as a “satiety” signal for coping behaviors.

Finally, another alternative hypotheses concerning the link between LC activity and behavior bears description. LC cells fire in regular patterns and preliminary data suggest that social stress in adulthood may deregulate the firing patterns of LC neurons, perhaps indicating that firing pattern is an important element in LC modulation of behavior (data not shown). The deregulation of this firing pattern following social stress may eradicate a “neural code” necessary for expression of burying behavior. In the previously proposed experiment this could be verified using auto- and cross-correlograms of LC neuronal activity to determine the regularity of a neuron’s firing pattern as well as its relationship to the firing patterns of other neurons.

How do these findings translate to humans?

Social stress in adult rats has been shown to be an effective animal model for the role of stress in depressive and anxiety-like behaviors and symptoms in humans. The translational utility of social stress in rats during adolescence has been less well established. Studies in human adolescents have shown that intense social stress increases the risk for violent and emotional disorders in adolescence as well as later affective disorders in adulthood (Dodge et al., 1990; Pine et al., 2002). The experiments in the current study have shown that social stress in early adolescence increases stress reactivity to a threat in the environment however the effects on social behaviors were not directly tested. Therefore, a promising future direction would be to measure elements of social interaction in socially stressed EA rats, including measures like aggression, submission, and social avoidance to better determine if EA social stress in rats models the human experience.

. While much of the research in the stress field has focused on modeling the affective changes that occur in stress-related disorders like depression, another of the primary symptoms of chronic stress is cognitive deficits. Norepinephrine function has been implicated in the deficits in cognitive function in both ADHD and depression (Ferguson et al., 2003; Arnsten and Li, 2005). As the research presented in this dissertation indicates, social stress differentially alters LC-NE activity in adolescents and adult rats. Therefore, experience with social stress may serve as a model for the cognitive deficits associated with ADHD and depression. To test this, a rodent test of cognitive function and attentional processing called the attentional set shifting test (AST) could be used.

The AST is a rodent version of a human test called the Wisconsin Card Sorting test (WCST). In the WCST the subject learns a set of contingencies or rules which predict whether a certain card should be classified by color, symbol, or number. Once the subject learns these rules, they are changed without the subject's knowledge. The subject then has to relearn the new rules, or cognitive set. These abilities are compromised in both depression and ADHD (Degl'Innocenti et al., 1998; Merriam et al., 1999; Tsuchiya et al., 2005; Solanto et al., 2007). Likewise, in the AST, the rat learns that a cue within a specific sensory modality predicts the presence of a reward. The cue is then changed, both within and between sensory modalities and the rat is asked to relearn the new contingency. NE facilitates the cognitive shift required when the rat is forced to learn that a specific sensory modality no longer indicates the presence of a reward (McGaughy et al., 2008). Chronic unpredictable stress impairs performance in the AST and that is reversed with increased acute NE release as well as chronic treatment with NE reuptake inhibitor (Lapiz and Morilak, 2006; Bondi et al., 2008; Newman et al., 2008). Given that a bias towards tonic LC firing is associated with attentional lability and difficulty staying on task in other behavioral tests (Aston-Jones and Cohen, 2005), EA social stress might generally increase the time required to reach consistent success in locating the reward. Conversely, given that increased phasic response to sensory stimuli is associated with selective attention (Aston-Jones and Cohen, 2005), social stress in adulthood may impair the ability the ability to switch from one salient reward cue to another. This ability may be augmented in stressed EA rats, however, because of their presumed increase in basal norepinephrine release.

Conclusion

The data in this thesis support the hypothesis that social stress distinctly alters the behavior and neurophysiology of animals across adolescence and that those effects are dependent both on the stage of adolescent development at the time of stress and at the time of testing. This was evidenced by an age-dependent increase in the expression of submissive defeat postures that temporally related to age-specific shifts in coping behaviors and LC activity. Early adolescence may be a particularly sensitive period in this regard, as several brain regions are still immature and may be especially susceptible to the effects of social stress.

As discussed in this chapter, the LC has a wide influence on cognition and the stress response. It also has multiple regulatory systems that are altered as an age-dependent function of stress. Therefore, these data may have clinical implications in terms of future research and new directions for potential therapies, focusing primarily on CRF and norepinephrine regulation in stress. In that respect, it is noted that there is a great need for basic and clinical research which specifically addresses the differences between juvenile and adult brains, especially with regard to age-specific manifestations of mental illnesses. The use and proper interpretation of animal models is a powerful tool in the investigation of these differences and will aid in the understanding of the neurological basis of social development and affective disorders.

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