STRESS DURING EARLY-ADOLESCENCE REDUCES RATS’ AGGRESSION AND INCREASES THEIR SEROTONIN FIBRE DENSITY IN ADULTHOOD

by

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Abstract

Stressful experiences during youth can lead to a maladaptive behaviour profile in adulthood, including an increase in anxiety and aggression-related behaviours in humans. Rats exposed to the intermittent physical stress (IPS) paradigm in early-adolescence (PD 22 – 34) have lasting increases in anxiety-related behaviour. Greater anxiety-related behaviours have been shown to be associated with greater aggression-related behaviours. Therefore, I hypothesized that stress during early-adolescence would also lead to increases in aggression. In addition, greater anxiety and aggression have been associated with altered serotonergic function in the prefrontal cortex and ventral hippocampus. However, it is unclear whether a similar mechanism accounts for the enduring impact of stress during adolescence on those responses. In this experiment, I examined if IPS during early-adolescence increased anxiety-related behaviour in the elevated plus-maze and shock-probe burying test, as well as increased aggressive behaviour in the resident intruder test. In addition, I determined if there were changes in serotonin fibre density in the prefrontal cortex and ventral hippocampus. Male Long Evans rats (N = 24) were randomly assigned to either the early-adolescent stress or no-stress control groups. Rats were exposed to IPS stress (involving foot-shock, water immersion and elevated platform exposure) during early-adolescence and tested in the elevated plus-maze, shock-probe burying test and resident intruder test in adulthood. At the end of behavioural testing, brain tissue was examined for serotonin fibre density in the regions of interest using immunohistochemistry. Animals exposed to early-adolescent stress did not display greater levels of anxiety; however, they did display lower levels of aggression and an increase in serotonin fibre density in the prefrontal cortex. These results support that early-adolescence is a period of vulnerability of emotional development and raise the possibility that the impact of stress in adolescence on aggression in later life depends on when the stressors were experienced.
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Chapter 1

Introduction

1.1 Stress and Psychopathology

Clinical research suggests that events in the early-life of an individual shape their mental, physical and emotional health in adulthood. Environmental conditions play a large part in guiding individual development toward a behavioural profile that is adaptive for their environment (Veenema, 2009). Stress during adolescence is thought to alter the trajectory of neurodevelopmental processes that occur during this period (Crews, He, & Hodge, 2007). Experiencing a stressful environment during development increases the likelihood of developing psychopathologies, such as anxiety and PTSD, as well as increasing the risk of engaging in substance abuse and aggressive behaviour as adults (Birmaher et al., 1996; Bulik, Prescott & Kendler, 2001; Pechtel & Pizzagalli, 2011; Thornberry, Ireland, & Smith, 2001). The direct and indirect cost of emotion related disorders (anxiety and depression) in Canada is $50 billion annually (Lim & Dewa, 2008). This fact, in addition to the great distress that these disorders can bring on individuals, families and communities highlights the negative impact these disorders have on Canadian society. It is imperative that we understand the mechanisms whereby adversity in adolescence increases the risk for emotion related disorders in later life. Such research may lead to improved treatment and prevention of these negative outcomes.

1.2 Animal Models of Early-Adolescent Adversity

Animal research provides an opportunity to examine complex behaviours and their underlying neurobiological mechanisms that cannot be directly tested in human research due to ethical limitations. In addition, animal models can be effective at investigating human mental
illness as they often confirm findings from correlational research in humans (Bremner & Vermetten, 2001; De Bellis & Thomas, 2003). There are a number of animal models used to examine the lasting impact of stress. Such models can differ in: type of stress (physical, social), timing of stress (pre-natal, juvenile, adolescent, adult, geriatric), and model animal (rodent, non-human primate) (Gutman & Nemeroff, 2002; Sanchez, Ladd & Plotsky, 2001; Tirelli, Laviola, and Adriani, 2003). In rats, adolescence is divided into three stages: early-adolescence (postnatal day; PD [postnatal day] 21-34), mid-adolescence (PD 35 – 44) and late-adolescence (PD 45 – 59) (Tirelli, Laviola, and Adriani, 2003). Common physical stressors include exposure to painful stimuli, fatigue, physical restraint and food restriction (Bremner & Vermetten, 2001). This research suggests that individuals in early-adolescence (pre-puberty PD 21-34) are particularly vulnerable to the enduring impact of stress on anxiety-related behaviour. For example, Wilkin, Waters, McCormick and Menard (2012) developed an animal model to examine the effects of physical stress in early- and mid-adolescence. They found that early-adolescent (PD 21 – 34) exposure to physical stressors increased rats’ anxiety and depression-related defensive behaviour in adulthood whereas mid-adolescent (PD 35 – 47) exposure to physical stressors decreased rats’ anxiety and depression-related behaviour in adulthood. Other researchers have found similar increases in anxiety- and depression like behaviours in adult male rats with a prior history of stress in early-adolescence (Avital, & Richter-Levin, 2005; Brydges et al., 2014; Horovitz, Tsoory, Yovell, & Richter-Levin, 2014; Tsoory, Cohen & Richter-Levin, 2007; Tsoory, & Richter-Levin, 2006).

1.3 Anxiety and Aggression

Clinical research suggests that early-life stress in the form of maltreatment or bullying can lead to other emotion-regulation related problems (Lee & Hoaken, 2007; Bulik, Prescott &
One of the most strongly associated outcomes is maladaptive aggression, initially presenting as childhood bullying behaviour that can progress to aggression-related criminal behaviour in adulthood (Maxfield & Widom, 1996; Repetti, Taylor & Seeman, 2002; Sanchez, Ladd & Plotsky, 2001; Widom, 1989; Widom & Maxfield, 2001). Aggression is an expression of behaviours that can result in harm towards oneself, another individual or the environment (Berkowitz, 1993). There are various modes of expressing aggression including physical and verbal avenues. In addition, these expressions may have an emotional or mental origin (Berkowitz, 1993). A given aggressive behaviour may have many purposes depending on the context, including: an expression of anger/frustration, assertion of dominance, intimidation, achievement of a goal, claim over resource, competition with a conspecific, or a response to fear and pain (Blanchard, Wall, and Blanchard, 2003).

In animal models, aggression is most commonly studied by assessing conspecific aggression – that is aggression directed at a member of the same species. The resident intruder test is one of the most common ways to study rodent aggression. Male rats become territorial residents of their home cage and this behaviour is enhanced by housing the male with a female for a period of 1 week (Koolhaas et al., 2013). If a male intruder rat is placed in a male resident rat’s cage the resident rat will exhibit defensive behaviours to protect his territory. This behaviour is natural and adaptive in most cases, however, it can become maladaptive if it either increases or decreases extremely (Lorenz, 2002; Thor & Carr, 1979; Veenema, 2009).

Behavioural, anatomical, and developmental research indicate an association between anxiety and aggression. Mice that were naturally the most dominant and aggressive when interacting with conspecifics, showed lower open-arm exploration in the elevated plus-maze test of anxiety compared to mice that were subordinates (Ferrari, Palanza, Parmigiani & Rodgers,
1998). This association also extends to other tests of anxiety. Rats that display the most burying in the shock-probe burying test also display the most territorial aggression in the resident intruder test (Sgoifo, De Boer, Haller and Koolhaas, 1996; Sluyter, Korte, Bohus and Oortmerssen 1996). Anatomical evidence also supports the relationship between anxiety and aggression as they share overlapping neural circuits (Delville, De Vries & Ferris, 2000; Ernst & Fudge, 2009; Ricci, Morrison & Melloni, 2012). This suggests that a similar neurobiological mechanism might mediate stress-related changes in anxiety and aggression. In further support, anxiolytic drugs, such as benzodiazepines and serotonin agonists, have been shown to alter both aggression and anxiety in rats (Miczek, Weerts, Vivian, & Barros, 1995; Muehlenkamp, Lucion, & Vogel, 1995; Rodgers & Waters, 1985; Van Praag, 1996). However, these changes are not always straightforward as different doses have been shown to impact anxiety and aggression behaviour profiles in dissimilar ways (Rodgers & Waters, 1985). This indicates that we need to investigate the relationship between stress, anxiety and aggression more closely.

Animal studies demonstrate that increases in aggression can follow developmental stress. Several rodent studies show that exposure to various stressors (including social defeat, social isolation, and physical stress) at early periods of development increase adult aggressive behaviour (Bibancos, Jardim, Aneas, & Chiavegatto, 2007; Ferris, 2000; Veenema, 2009; Wommack, Taravosh-Lahn, David, & Delville, 2003). However, it is unclear if physical stress during early-adolescence leads to increased aggression in adulthood in rats, as prior studies used hamster models. Hamsters are solitary animals, unlike rats that live in colonies (Veenema, 2009). This difference in social structure may mean that rat models will be more reflective of human behaviour. In addition to this, no studies have restricted the stressors to the early-adolescent period and examined the effects on aggression. Wilkin et al. (2012) did find increases in anxiety
as a result of stress restricted to early-adolescence however they did not investigate if aggression was also affected.

Given the positive correlation between behaviours in the shock-probe burying test and the resident intruder test (Sgoifo, De Boer, Haller and Koolhaas, 1996; Sluyter, Korte, Bohus and Oortmerssen 1996) and that early-adolescent stress increased adult levels of shock-probe burying (Wilkin et al., 2012), I predicted that male rats exposed to IPS during early-adolescence would display increases in their aggression-related responses in adulthood.

### 1.4 Neurobiological Mechanisms of the Stress-Psychopathology Relationship

The serotonergic system has been implicated as a potential player in the regulation of anxiety and aggression (Ricci, Morrison & Melloni, 2012; Delville, De Vries & Ferris, 2000). Serotonin or 5-hydroxy-tryptophan (5-HT) is produced by cells in the raphe nuclei which project serotonin containing fibres to limbic and cortical structures in the brain (Hornung, 2003).

Current research involving behavioural and pharmacological manipulations of serotonin and aggression show that these variables correlate with each other. Behavioural data suggests that serotonin levels are associated with trait aggression and that cerebrospinal serotonin levels acutely increase following aggressive behaviours (van der Vegt et al., 2003; van der Vegt, Lieuwes, Cremers, de Boer & Koolhaas, 2003). Pharmacological inhibition of serotonin disrupts normal aggressive behaviour (van der Vegt et al., 2003). Given that serotonin is dysregulated in the adult presentation of maladaptive levels of anxiety and/or aggression, it seems possible that the developmental trajectory of the serotonin system is vulnerable to disruption from adolescent stress. In fact, several studies found that stress in adolescence led to long-lasting increases in both aggression and serotonergic activity in the amygdala, hypothalamus, and hippocampus.
(Delville, De Vries & Ferris, 2000; Ferris, 2000; Ricci, Morrison & Melloni, 2012). Ferris (2000) found that adult hamsters, previously exposed to social defeat stress in adolescence, demonstrated a higher number of serotonin terminals in the anterior hypothalamus, along with an increased aggressive response to smaller intruders. Ricci et al. (2012) found that anabolic/androgenic steroid exposure in adolescence was associated with higher levels of aggression and high levels of serotonergic innervation in various brain regions (anterior hypothalamus, medial and central amygdala) in adulthood. Similarly, Deville et al. (2000) found greater serotonin levels in the dentate gyrus along with increases in aggressive behaviour in adult golden hamsters with a prior history of stress in adolescence. However, all of this research was conducted with hamsters, which are solitary animals. It remains unclear if the same changes would occur in more social animals such as rats.

Interestingly, the opposite relationship has also been demonstrated; that is, lower serotonin activity being associated with higher aggression (Veenema, 2009). Several studies demonstrate this relationship: lower aggression after SSRI (selective serotonin reuptake inhibitor) administration in rats (Sánchez & Meier, 1997), serotonin levels decreased in the prefrontal cortex during aggressive behaviour in rats (van Erp & Miczek, 2000), and several other studies that demonstrate an association between low serotonin levels and high aggression in primate and rat models of aggression (see review: Ferrari, Palanza, Parmigiani, de Almeida, & Miczek, 2005). The prefrontal cortex appears important for complex behaviours involved in aggression (Devinsky, Morrell, & Vogt, 1995). The prefrontal cortex is known to develop throughout adolescence and is altered structurally (e.g., changes in glutamate receptors) and functionally (e.g., changes in working memory) by physical stress in early-adolescence (Yuen et
al., 2009). Researchers postulated that low serotonin innervation in the prefrontal cortex may lead to increased aggressive behaviour (Siever, 2008).

Few studies have examined serotonin’s potential involvement in the lasting impact of stress during adolescence on anxiety-related behaviour in adulthood. Watt et al. (2009) found that following social defeat in adolescence, adult levels of serotonin in the rat were increased in the ventral dentate gyrus (part of ventral hippocampus) and this was associated with increased anxiety-related behaviour. However, in that study, social defeat stress was administered in mid-adolescence and there were no evident changes in serotonin levels in the prefrontal cortex. Thus, it remains unclear whether similar or different outcomes on the serotonin system would occur if stress was restricted to early-adolescence. This is exemplified by prior evidence that the outcomes of adolescent adversity depend on when the adversity is experienced (Wilken et al., 2012).

Thus, one of the goals of this study was to examine whether stress during early-adolescence alters serotonergic innervation profiles, and whether this maps on to altered anxiety- and aggression-related behavioural profiles in adulthood. We can examine the innervation of the serotonin system using immunohistochemistry, a powerful technique that uses antibodies to mark a target protein.

To examine the innervation profiles of serotonergic fibres, researchers can label serotonergic axons with antibodies for the serotonin re-uptake transporter (SERT) protein. SERT is a \( \text{Na}^+/\text{Cl}^- \) dependent membrane protein involved in the re-uptake of serotonin into the pre-synaptic cell after it has been released into the synaptic cleft (Schloss and Williams, 1998). The SERT molecule is inserted all along the axon and at axon terminals, making it an ideal target.
protein for immunohistochemical investigation of serotonergic innervation profiles. Nielsen, Brask, Knudsen & Aznar (2006) found that the SERT protein is a more valid indicator for visualizing serotonergic fibres than using serotonin itself because the SERT molecule is not subject to transient metabolic fluctuation. Using a system of concentric circles virtually overlaid on immunohistochemically processed brain slices, the number of axons in a given area can be used to determine length density which measures the density of serotonergic innervation in a given area (Papesh, & Hurley, 2012). Serotonergic fibre innervation profiles are altered in animals that have experienced stress. Hamsters stressed in adolescence show greater levels of adult aggression, anxiety and serotonergic innervation in several areas of the brain including: hypothalamus, central amygdala and medial amygdala (Delville, De Vries & Ferris, 2000; Ricci, Morrison & Melloni, 2012). Other areas that have been implicated in anxiety and aggression include: the hippocampus, the septum and the prefrontal cortex (Delville et al, 2000; Noristani, Olabarria, Verkhratsky, & Rodríguez, 2010; Ricci et al., 2012; Watt et al., 2009). Evidence of increased serotonergic fibre density in similar areas was found in female macaques that had greater stress resilience (Bethea et al., 2014). However, it is unclear if a similar change in serotonergic fibre innervation density would occur in rats in response to early-adolescent stress.

In this study, I aimed to determine if early-adolescent stress would lead to increased anxiety-related behaviour in the elevated plus-maze and shock-probe burying test (as found in Wilkin et al., 2012) as well as an increase in aggression-related behaviour in the resident intruder test. In addition, I aimed to determine if early-adolescent stress would lead to a decrease in serotonergic fibre density found in the prefrontal cortex and hippocampus.
1.5 Thesis Objectives

1. Replicate previous findings suggesting that early-adolescent stress leads to greater anxiety-related behaviour in the elevated plus-maze and shock-probe burying test (as found in Wilkin et al., 2012).

2. Determine if stress during early-adolescence also increases aggression-related behaviour in the resident intruder test.

3. Determine if these behavioural changes are accompanied by corresponding changes in the serotonergic system; specifically, whether there is a decrease in the serotonin fibre density in the prefrontal cortex and an increase in serotonin fibre density in the ventral hippocampus.
Chapter 2

Method

2.1 Subjects

Twenty-four male, Long-Evans rats were generated from an in-house breeding program (Department of Psychology, Queen’s University). The pups were weaned at PD 21 and housed individually in 26 cm x 48 cm x 20 cm Plexiglas cages with wood chip bedding (Beta Chips, NEPCO; Warensburg, NY). Rats were maintained on a 12 hour light/dark cycle with lights on at 07:00 and lights off 19:00. Rats had access to food (Lab diet, PMI Nutrition International; Brentwood, MD) and water, *ad libitum*. All methods were approved by Queen’s University Animal Care Committee and are in keeping with the guidelines of the Canadian Council of Animal Care.

2.2 Intermittent physical stress paradigm

On the day of weaning (PD 21), rats were randomly divided into stress and no-stress control groups. A maximum of 2 rats per litter were assigned to each group. Rats in the stress group were exposed to the intermittent physical stress paradigm (described below) in the early-adolescent period (PD 22 – 34). Stress exposures occurred between 9:00 am to 5:00 pm, with no more than one stress session administered per day. To control for handling effects, control animals were briefly handled for 2 – 4 minutes on each of the stressor application days that the stress group experienced. At the end of the stress regimen, the rats were left undisturbed for 27 days, except for feeding and cleaning of cages. Behavioural testing began on PD 60 (adulthood) for all rats. Bodyweight was measured at weaning (PD 21), start of stress paradigm (PD 22), end
of stress paradigm (PD 34), after each behavioural test (PD 61, PD 68, and PD 90) and at endpoint (PD 104) to ensure there were no differences in growth between groups.

The intermittent physical stress paradigm is composed of three stressors: the elevated platform stressor, foot-shock stressor and water immersion stressor; which are each applied twice (for a total of 6 stress exposure days) throughout the 12 day period. Only one stressor was applied on any given stress exposure day. The procedure for each stressor is described below.

**Elevated platform stressor.** The elevated platform is a validated method of inducing stress in rats, which taps into rats’ natural fear of open spaces (Degroot et al., 2004). Rats were brought from their home cage to a testing room containing the elevated platform. The apparatus consisted of a 10 cm x 10 cm x 100 cm plexiglas tower with a flat 15 cm x 15 cm platform placed on top. This tower was placed inside of a plexiglas open field (48 cm x 100 cm x 100 cm) lined with 4 cm thick microfoam. The open-field apparatus prevented the rats from leaving the testing area and also prevented the microfoam from moving out of place. Rats were placed individually on the platform for a total time of 30 minutes. If a rat fell or jumped off of the platform, the timer was paused until the rat was returned to the platform. The platform was cleaned with disinfectant (70% ethanol (ETOH)), rinsed with water, and dried between sessions.

**Foot-shock stressor.** Rats were brought one at a time from their home cage to a testing room with operant chambers (29 cm x 22 cm x 20 cm; Med Associates Inc., St. Albans, VT) equipped with grid floor. The chambers were pre-programed to apply 3 foot-shocks (0.6 mA lasting 3 seconds) randomly over a 5 minute period. Only one rat was placed in each chamber. At the end of the stressing, rats were returned to their home cage and taken back to the colony.
room. After each session the boxes were cleaned with disinfectant (Swish Quato 44, Swish Canada).

*Water immersion stressor.* Rats were transported from their colony into the stressing room in their home cage. The apparatus contained 4 parts: the restraint tube, immersion tank, stabilizer plate and ventilated tube cover. Each individual rat was placed into a ventilated cylindrical restraint tube (black polyvinyl chloride, PVC; 7 cm x 20 cm). These tubes allow the rat to stand or sit but not locomote forward or backward. Each tube was fitted into the immersion tank and was held upright by the Plexiglas stabilizing plate (35 cm x 50 cm). The stabilizing plate was inserted in the immersion tank, approximately 15 cm from the bottom of the tank. A clear Plexiglas ventilated tube cover (2.5 cm x 10 cm x 35 cm) covered 3 restraint tubes to ensure animals did not leave the restraint tube. There were four tube covers, each covering 3 restraint tubes. Animals were monitored continuously for 45 minutes. At the end of the stress session, the animals were removed and dried using a terrycloth towel and then left under a heat lamp in the home cage for 15 minutes before being returned to the colony room. After each exposure, the immersion tank, restraint tubes, stabilizer plate and ventilated tube covers were all washed with water and disinfectant (Swish Quato 44, Swish Canada).

### 2.2 Behavioural Testing

Three different behavioural tests were used: the elevated plus-maze, shock-probe burying test, and the resident intruder test (described further, below). Test trials for the elevated plus-maze, shock-probe burying test and resident intruder test were recorded by a digital camcorder (Sony 8 DCR-TVR315 NTSC) using the MovieStar 5 recording program. Coding of...
the data was conducted using the Noldus Observer 5.0 (Wageningen, Netherlands). The tester and the coder were blind to the rats’ stress condition during testing and coding, respectively.

*Elevated plus-maze.* The elevated plus-maze consisted of two opposing open arms (50 cm x 10 cm) and two opposing closed arms (50 cm x 10 cm x 40 cm) made of urethane-sealed wood, which were elevated 50 cm above the floor. The rat was placed in the centre of the maze, facing one of the closed arms and was observed for 5 minutes. Rats normally spend more time in closed arms and avoid open arms (Pellow et al., 1985). Open-arm exploration is increased by anxiolytic drugs and decreased by anxiogenic drugs, which supports its use as an index of anxiety (Pellow et al., 1985). A coder stood quietly at the far end of the test room and observed the rats visually in each trial, as well as later on tape, and recorded the number of open and closed arm entries and time spent in each arm. An entry was recorded when all 4 paws were in an arm of the maze. In this test, an increase in anxiety-like response is indicated by a decrease in the proportion of the number of open-arm entries (open-arm entries/open + closed arm entries) and or a decrease in the proportion of time spent in the open-arms (open-arm time/open + closed arm time) (Pellow et al., 1985). After each trial, the maze was cleaned using disinfectant (70% ethanol (ETOH)), rinsed with water, and dried between each trial.

*Shock-probe burying test.* The shock-probe burying test is a validated animal model of anxiety measuring a rat’s natural defensive responses to a localized pain stimulus. After receiving a contact-induced shock from the electrified probe, rats typically display burying behaviour, that is, using their front paws to push bedding material towards and over the probe (Treit, 1990). Rats were placed in a 40 cm x 30 cm x 40 cm Plexiglas test chamber filled with wood bedding chips up to 5 cm in depth (Beta Chips, NEPCO; Warrensburg N.Y.). The rats were placed in the chamber without the electrified probe for 15 minutes for the four consecutive
days before the test day to habituate them to the chamber. Testing took place between the hours of 12:00 and 17:00. On the test day, an electrified probe wrapped in copper wire (0.5 cm x 0.5cm x 6 cm) is placed into the chamber through a hole in the centre of the wall, 8 cm from the floor. The probe is attached to a 2000 V shock source, with a 2.25 mA intensity of shock. Whenever the rat touches the electrified probe, the rat receives a brief contact-induced shock. The 15 minute trial begins once the rat receives its first shock from the probe. Rats normally attempt to “bury” the shock-probe by using alternating movements of their front limbs and snout to push wood chips from the floor towards and over the probe. The duration of time spent burying is used as the index of anxiety. Increased time spent burying is considered to be indicative of a higher anxiety-like response. This has been validated by experiments where anxiolytic drugs decrease time spent burying (indicating less anxiety) and anxiogenic drugs increase time spent burying (indicating greater anxiety) (Treit, 1990). The coder observed rats on recorded tapes and coded the following behaviours: duration of time burying (i.e., using the front two paws in rapid forward thrusting motions to push bedding material towards the probe); duration of time immobile (i.e., sitting and/or lying on the test chamber floor with no movement except that needed for respiration); total number of shocks; and shock reactivity (immediate reaction to the shock). Shock intensity varies based on the duration and degree of contact and therefore rats’ shock reactivity is coded using a 4 point scale, with the least reactive response at the bottom and highest reactive response at the top: (1) head flinch, (2) whole body flinch, (3) whole body flinch with locomotion (walking) away from the probe, (4) whole body flinch or jump and rapid movement (running) away from the probe. The chamber was cleaned of fecal boli after each test and the bedding smoothed to a uniform thickness. The chamber was replenished with clean bedding at the end of each test day and soiled bedding was removed and replaced as required.
**Resident intruder test.** The resident intruder test is a validated model of territorial aggression (Koolhaas et al., 2013) Animals undergoing resident intruder testing were moved to another dedicated colony room (which housed only male resident rats initially without and later with a female cage-mate). Female rats (150 g – 200 g) were acquired from Charles River, and triple-housed for one week, in a separate colony, to habituate them to the facility. After habituation, each female rat was housed with an experimental (resident) male rat in a large cage (28 cm x 48 cm x 20 cm) in the resident colony for one week prior to testing. Paired housing with a female has been shown to increase territoriality in male rats, hereinafter referred to as residents (Koolhaus et al., 2013). In addition, bedding was not changed in the residents’ home-cage for one week prior to testing to ensure that the residents established the home cage territory as their own. This ensures optimal expression of aggressive behaviour, as residents may not express territorial aggression if the cage smells like a novel location (Koolhaus et al., 2013). Eighteen naïve adult male stimulus rats (350 g – 375 g) to be used as intruders were acquired from Charles River and given one week to adjust to the facility. The intruders were housed in a general colony, separate from the resident colony.

On each of four days consecutive prior to testing, the residents and their female cage-mates were brought into the testing room for 15 minute so that the resident could acclimatize to the room. On the fifth day, a resident rat and its female cage-mate were taken to the testing room. The female was then immediately removed and placed in a holding cage in another room. A male intruder rat was then placed in the resident male’s home-cage with the resident male being present. Intruder rats were between 30 g to 100 g lighter than the resident male to promote aggression on the part of the resident male (Koolhaus et al., 2013). The intruder-resident pairings
are created such that the resident would outweigh their opponent by at least 30 g. Testing lasted for 20 minutes. A second testing session was conducted 24 hours later with a new intruder.

Observed behaviours were divided into three composite measures: aggressive interaction, social interaction and non-social activity, as reported in Koolhaus et al. (2013). In addition, the frequency of biting behaviour was also examined, as it can be an indicator of maladaptive aggression (Koolhaus et al., 2013).

Aggressive interaction is composed of the following behaviours: keep down (defined as a dominant position where the resident rat is on top of the intruder rat holding the intruder down while the intruder is on its back with all four paws in the air; also considered to be a “defeat” of the submissive rat), aggressive upright posture (defined as the resident male standing fully upright on his hind legs, accompanied by the intruder doing the same, with both rats placing their forepaws on the other’s shoulders), lateral threat (defined as a posture in which the resident turns its body perpendicular to the intruder, curves its back upwards and moves in a sideways fashion, towards the intruder), clinch attack (occurs when both rats are holding onto each other and are vigorously rolling), and chase (defined as the resident vigorously running toward the intruder who is running away).

Social interaction is composed of three behaviours: social grooming (resident grooming the intruder) social exploration (defined as sniffing and investigation of any part of the body of the intruder except the areas that are surrounding the genitals) and anogenital sniffing (sniffing of the genital region and area at the base of the tail). Anogenital sniffing behaviour is examined separately from social exploration because it has been shown to be involved in the establishment
and maintenance of social hierarchies, and can be a precursor to aggressive behaviour (Koolhaus et al., 2013; Wesson, 2013).

Non-social activity is composed of the following behaviours: rearing (the resident animal rises onto hind legs to explore), digging/burying (defined as moving of bedding towards or away from itself using its paws), and exploration (defined as movement around the cage that is not near the intruder).

Biting behaviour is categorized based on the target into vulnerable targets (the throat, belly, and paws) and non-vulnerable targets (targeting the nape of the neck, and flank) (Koolhaus et al., 2013). A high frequency of bites targeting vulnerable areas can be considered to be a sign of maladaptive display of violent aggression (Koolhaus et al., 2013).

Once the twenty minute test was complete, the intruder was removed and returned to its home-cage in the home colony. The female companion was returned to the resident’s cage and the home-cage was returned to the colony. Both resident and intruder were examined for injury, which, if noted, was immediately reported and treated by the veterinary technician.

2.4 Immunohistochemistry of Serotonin Fibres

Tissue collection. Animals were perfused pericardially with 4% paraformaldehyde in 0.1M phosphate buffered saline with a pH of 7.4. Brains were extracted and placed into 4% paraformaldehyde in 0.1 M phosphate buffered saline with pH of 7.4 for 24 hours. Brains were transferred to a 15% sucrose solution, followed by a 30% sucrose solution for 24 hours each. Brains were stored in a fridge at 4 °C during this time. This process was conducted to reduce crystallization damage that can occur during the freezing process. Brains were then flash frozen using methyl butane at -80 °C. Brains were stored until ready for use. Brains were sliced on a
Leica 1800 (Heidelberg, Germany) cryostat at -20 °C into consecutive coronal sections (40 μm). Slices were placed into antifreeze solution in 1.5ml Eppendorf tubes and stored at -20 °C. Slices were subdivided and grouped into 5 groups based on area. A total of 64 slices (1 in 4 series, 16 slices per tube) were taken from the prefrontal cortex (from bregma 3.70 mm to 1.70 mm) and 60 slices (1 in 6 series, 10 slices per tube) from the ventral hippocampus (from bregma -4.16 mm to -6.72 mm).

*Immunohistochemistry.* In each group, the brain tissue from one animal from each litter was randomly selected for immuno-histochemical processing. This was done to allow me to process tissue from all of the litters in a single run. This protocol was adapted from Papesh and Hurley (2013). Slices from the prefrontal cortex and ventral hippocampus were processed to visualize SERT in the various regions of interest (ROI). Slices were washed with a phosphate buffered saline (PBS) for 5 minutes to remove any unwanted chemical residue that may be present. This was repeated 5 times to ensure that the tissue was clean before starting. Tissue was placed in 0.5% hydrogen peroxide for 30 minutes. Slices were placed in blocking solution (1% bovine serum albumin + 5% normal goat serum + PBS-Tx (PBS-triton-X)) for 60 minutes. Then they were incubated in 1:5 avidin: PBS-Tx solution for 20 minutes and then 1:5 biotin: PBS-Tx solution for 20 minutes (avidin/bovine blocking kit, Vector Labs). They were then washed again in PBS as above. Slices underwent primary antibody incubation, at 4 °C on a rotator plate for 48 hours. The concentration of primary antibody in PBS+1% normal goat serum was 1:5000 (SERT antibody; immunostar #24330, Hudson, WI). One series was incubated without primary antibody as the primary control.

After 48 hours, slices underwent five 5-minute washes in PBS-Tx. They then underwent the secondary antibody incubation for 1 hour. The concentration of biotinylated goat anti-rabbit
antibody +2% normal goat serum in PBS-Tx was 1:500. A few slices that received the primary antibody, but not the secondary antibody were set aside to be used as the secondary control. Following another set of five 5-minute washes in PBS-Tx, the secondary antibody was amplified with the ABCComplex (Vectastain Elite ABC kit, Vector Labs, Burlingame, CA) in PBS for 45 minutes. Following a 15-minute wash in PBS-Tx, the slices were then incubated in DAB solution containing nickel (DAB kit, Vector Labs). Slices were then rinsed in distilled water, plated on chromium gel coated slides, and cover-slipped using DPX Mounting medium (Sigma-Aldrich, St. Louis, MO). Slices from both individuals in stress and control groups were processed at the same time to eliminate any run-to-run variance.

Quantification of serotonin fibre innervation. Serotonergic fiber length density (SFLD) was estimated using the LvRef method (length density reference) (Papesh & Hurley, 2012) using StereoInvestigator software (MBF Bioscience, Williston, VT) at 40x magnification on a Nikon Eclipse 80i light microscope. StereoInvestigator generates and places a virtual grid of spheres randomly such that there is an average of 20 space ball probes within the region of interest. Space ball probes are virtual hemispheres that were superimposed over the centre of a tissue slice (balls have radius of 10 μm). There is a buffer zone of 2.5 um at the top and lower edge of the section and edge of the sphere. A minimum of 2 slices was acquired for each area of interest and the average serotonin fibre length density per area of interest was calculated for each subject.

Regions of interest. ROI were selected from two brain regions: the prefrontal cortex (from bregma 3.70 mm to 1.70 mm) and the ventral hippocampus (from bregma -4.16 mm to -6.72 mm). In the prefrontal cortex, the ROI’s were: medial optic area (found from bregma 3.70 mm to 3.20 mm), infralimbic cortex (found from bregma 3.20 mm to 1.70 mm), prelimbic cortex (found from 3.20 mm to 1.70mm) and cingulate cortex (found from 3.70 mm to 1.70mm). In the
ventral hippocampus the ROI’s were: CA1 (found from bregma -4.80 mm to -5.60 mm), CA3 (found from bregma -3.80 mm to -5.60 mm), dentate gyrus (found from bregma -4.30 mm to -5.60 mm) and subiculum (found from bregma -5.20 mm to -5.60 mm).

2.5 Statistical Analysis

Behavioral data analysis. To reduce any litter effects, data from littermates in the same group were collapsed into a composite (average) score and counted as one. This resulted in the total sample size of 24 (control, n = 12 and stress, n = 12) to become the following number of unique litters per group: control n = 8 and stress, n = 8. The composite litter scores were used in the behavioural data analysis. Outliers were assessed using: visual inspection of quartiles with a box and whisker plot. Scores were more closely examined if they fell outside of the 2nd and 3rd quartile. This was followed by Grubs test to determine if they were statistical outliers.

Bodyweight data were analyzed using a mixed measures ANOVA, with Time as the within-subjects variable and Treatment (stress vs. control) as the between subjects factor.

Data from the elevated plus-maze and shock-probe burying test were analyzed using one-way ANOVAs to test for effects of Group (stress vs. controls) on the rats’ behavior in adulthood.

Data from the resident intruder test were analyzed using a mixed measures ANOVA, with Test Day (Day 1 vs Day 2) used as the within-subjects variable and Treatment (stress vs. control) as the between subjects factor. This was performed for each of the composites of behaviours: aggressive interactions, social interactions and non-social activity. To further analyze significant main effects or interactions, we used tests of simple effects and pairwise comparisons using the Least Significant Difference (LSD) with a significance level set at p < .05. To further understand which behaviours accounted for any main effects detected in the analysis of behaviour.
composites, one-way ANOVAs were performed on the components of behaviour composites. The frequency of biting behaviour was also analysed using a one-way ANOVA.

**Serotonergic fibre length density data analysis.** Only one animal per litter per group was used in this analysis. The serotonin fibre length density is calculated using the total number of intersections between the SERT-positive fibres and the space balls using the following equation:

\[ L_v = 2 \frac{Q}{N_A}, \]

where \( L_v \) is the length of the fibres per unit volume, \( Q \) is the total number of times that fibre intersects with the virtual probe, \( N \) is the number of probes and \( A \) represents the surface area of the probe (Calhoun & Mouton, 2000). The constant “2” is used to account for the fact that hemispheres are used instead of spheres. Hemispheres have been shown to reduce bias that is introduced when using a spherical probe (Mouton, Gokhale, Ward and West, 2002). This equation is used to calculate the density within one region of interest for each slice. These were then used in calculations to get an estimate for the region of interest overall.

The serotonergic fibre lengths were calculated for each ROI in each slice, which were then averaged together (across the two sampled slices) based on anatomical location in the rat-atlas for each animal. The mean for each ROI was compared between the control and treatment groups using a 1-way ANOVA. In the prefrontal cortex, the largest amount of high quality tissue was produced from bregma 3.20 mm to 2.20 mm. Therefore only this tissue was used in analysis.
Chapter 3

Results

3.1 Body Weight

To examine if there were any effects of treatment on growth rates, rats were weighed seven times throughout the experiment, once at weaning, once before and after the 12-day stress regimen, once before each behavioural test was administered and one right before euthanasia. As expected, a 2 x 7 mixed-model ANOVA revealed that was a significant increase in weight over time, $F(6, 132) = 2220.16, p < .01$. There were no significant group by weight sampling interactions $F(6, 132) = .84, p = .54$ or any between group differences, $F(1, 22) = .p = .57$.

3.2 Elevated Plus-Maze

No outliers were found using visual inspection of quartiles with a box and whisker plot or Grubb’s test of outliers (Grubbs, 1950), therefore, the number of unique litters for the groups were not adjusted. This resulted in a sample size of 8 unique litters per group (controls n = 8 and stress n = 8).

Using a 1-way ANOVA, I determined that animals exposed to early-adolescent stress showed no differences in the two measures of open-arm exploration relative to rats in the control group: % of open-arm entries, $F(1, 14) = .46, p = .512, \eta^2 < .03$ and % of open-arm time, $F(1, 14) = .98, p = .34, \eta^2 = .07$ (see Figures 1 and 2). There were no significant between group differences in the number of closed arm entries, $F(1, 14) = 1.22, p = .29, \eta^2 = .08$ or total arm entries, $F(1, 16) = 1.63, p = .22, \eta^2 = .10$ (see Table 1).
**Figure 1. Mean % of Open-Arm Entries (±SEM).** % of open-arm entries by handled controls (n = 8) and early-adolescent intermittent physical stress animals (n = 8) on the elevated plus-maze was not significantly different, $F(1, 14) = .46, p = 0.51, \eta^2 < .03$.

**Figure 2. Mean % of Open-Arm Time (±SEM).** Mean % of open-arm time by controls (n = 8) and early-adolescent intermittent physical stress (n = 8) animals on the elevated plus-maze was not significantly different, $F(1, 14) = .98, p = 0.34, \eta^2 = .07$. 
Table 1

Mean Frequency (± SEM) of Behaviours Displayed in the Elevated Plus-maze Test

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Controls (n = 8)</th>
<th>Stress (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Closed Entries</td>
<td>9.81 (0.98)</td>
<td>8.25 (1.03)</td>
</tr>
<tr>
<td>Total Entries</td>
<td>11.31 (1.24)</td>
<td>9.13 (1.18)</td>
</tr>
</tbody>
</table>
3.3 Shock-Probe Burying Test

No animals were outliers or removed from analysis, therefore, the sample sizes for the groups were not adjusted. This resulted in a sample size of 8 unique litters per group (controls n = 8, stress n = 8).

As determined by a one way ANOVA, the handled control group and early-adolescent stress group did not differ in on any measures in the burying test: total duration of time spent burying, $F(1, 14) = .05$, $p = 0.83$, $\eta^2 = .01$; time spent immobile, $F (1, 16) = .03$, $p = 0.86$, $\eta^2 = .01$; mean shock reactivity, $F (1, 16) = 2.19$, $p = 0.16$, $\eta^2 = .14$ and total number of shocks, $F (1, 14) = .35$, $p = .56$, $\eta^2 = .03$ (see Figure 3 and Table 2).
Figure 3. Mean Duration of Burying (±SEM). Mean burying by controls (n = 8) and early-adolescent intermittent physical stress animals (n = 8) in the shock-probe burying test was not significantly different, $F(1, 14 = .05, \ p = 0.83, \ \eta^2 = .01)$
Table 2

*Mean (±SEM) of Behaviours Displayed in the Shock-Probe Burying Test*

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Controls (n = 8)</th>
<th>Stress (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of immobility (s)</td>
<td>6.51 (1.95)</td>
<td>6.00 (1.60)</td>
</tr>
<tr>
<td>Mean shock reactivity</td>
<td>2.00 (0.17)</td>
<td>2.41 (.18)</td>
</tr>
<tr>
<td>Mean total number of shocks</td>
<td>3.75 (.50)</td>
<td>3.25 (.56)</td>
</tr>
</tbody>
</table>
3.4 Resident Intruder Test

Two animals (both were single animals from a unique litter) were removed from analysis because their data were incomplete due to technical error. This resulted in a sample size of 7 unique litters per group (controls n = 7, stress n =7).

Three independent mixed-model ANOVAs were conducted to examine the data from the three behavioural composites: aggression interactions, social investigations and non-social activity, as measured across both test exposures. Aggression interactions included keep down, upright posture, lateral threat, chase, and clinch attack. The social investigations composite was made up of anogenital sniffing, social grooming and social exploration. Non-social activity was comprised of rearing, digging or burying and non-social exploration (defined as movement around the cage that is not directed towards the intruder). In addition, biting behaviour was examined and included bites at vulnerable targets (belly, throat and paws) and non-vulnerable areas (back of neck and flank).

3.4.1 Aggressive Interactions

As can be seen in Figures 4 and 5, animals exposed to early-adolescent stress displayed lower levels of aggressive interactions than did rats in the handled control group. This pattern was confirmed as significant by the main effect of Treatment on the frequency of aggressive interactions, \( F(1,12) = 5.13 \ p = .04, \ \eta^2 = .30 \) and a non-significant trend on the duration of time spent on aggressive interactions, \( F(1,12) = 3.35 \ p = .09, \ \eta^2 = .22 \). There were no main effects of Test Day on either frequency, \( F(1,12) = .03, \ p = .87, \ \eta^2 = .03 \), or duration, \( F(1,12) = .02, \ p = .89, \ \eta^2 = .01 \) and there were no significant Treatment X Test Day
interactions in: frequency, $F(1,12) = .45 \ p = .51, \ \eta^2 = .01$; or duration, $F(1,12) = .47, \ p = .51, \ \eta^2 = .04$.

Aggressive interactions were collapsed across Test Day and examined by their individual components to examine at the main effect of Treatment. When collapsed across Test Day, animals exposed to early-adolescent stress specifically displayed a significantly lower level of keep down behaviour than the rats in the control group, when compared by frequency, $F(1,12) = 14.05, \ p < .01, \ \eta^2 = .54$ and duration, $(1,12) = .18.20, \ p < .01, \ \eta^2 = .60$. All other aggression interactions were not found to be significantly different (all p’s > 0.05; please refer to Table 3). In addition, the frequency of biting behaviours were compared between animals that experienced early-adolescent stress and control animals and no significant differences were found (all p’s >.05; please refer to Table 4).
Figure 4. Mean Frequency of Aggressive Interactions (±SEM). Mean frequency of aggressive behaviours by controls (n = 7) and early-adolescent intermittent physical stress animals (n = 7) in the resident intruder test. There was no significant interaction or within subjects effects of Test Day on frequency scores (p > .05). There was a significant main effect of Treatment between stress and control animals on frequency, $F(1,12) = 5.13$, $p = .04$, $\eta^2 = .30$. 
Figure 5. Mean Duration of Aggressive Interactions (s) (±SEM). Mean duration of aggressive behaviour by controls (n = 7) and early-adolescent intermittent physical stress animals (n = 7) in the resident intruder test. There was no significant interaction or within subjects effects of Test Day (p > .05). There was a non-significant trend if comparing by Treatment between stress and control animals, $F(1,12) = 3.35, p = .09, \eta^2 = .22$. 
Table 3

Mean (± SEM) Frequency and Duration (sec) of Aggressive Behaviours Displayed in the Resident Intruder Test

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Frequency</th>
<th>Duration (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Stress</td>
</tr>
<tr>
<td>Keep Down</td>
<td>6.82 (1.85)</td>
<td>2.46 (0.85)**</td>
</tr>
<tr>
<td>Lateral Threat</td>
<td>15.79 (3.30)</td>
<td>13.43 (2.87)</td>
</tr>
<tr>
<td>Clinch Attack</td>
<td>3.82 (1.42)</td>
<td>2.07 (0.78)</td>
</tr>
<tr>
<td>Upright Posture</td>
<td>12.68 (3.33)</td>
<td>8.07 (2.43)</td>
</tr>
<tr>
<td>Chase</td>
<td>0.36 (0.22)</td>
<td>0.43 (0.36)</td>
</tr>
</tbody>
</table>

Note. *p < .05, **p < .01

Table 4

Mean (± SEM) Frequency of Biting Behaviours in Resident Intruder Test

<table>
<thead>
<tr>
<th>Targets</th>
<th>Controls (n = 8)</th>
<th>Stress (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Vulnerable</td>
<td>2.25 (0.94)</td>
<td>1.76 (0.76)</td>
</tr>
<tr>
<td>Vulnerable</td>
<td>0.29 (0.18)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
3.4.2 Social Interaction

When examining social interaction, there was no significant Treatment X Test Day interaction in frequency, $F(1,12) = 1.95$, $p = .19$, $\eta^2 = .14$ or duration, $F(1,12) = .55$, $p = .47$, $\eta^2 = .04$. There was no main effect of Treatment between stress and control animals in number, $F(1,12) = .87$, $p = .37$, $\eta^2 = .07$ or duration, $F(1,12) = .48$, $p = .50$, $\eta^2 = .04$. There was no significant within-subjects effect of Test Day, in number, $F(1,12) = .02$, $p = .89$, $\eta^2 = .01$ and duration, $F(1,12) = 1.03$, $p = .33$, $\eta^2 = .08$.

3.4.3 Non-Social activity

When examining non-social activity, there was no significant Treatment X Test Day interaction in number, $F(1,12) = 1.58$, $p = .23$, $\eta^2 = .12$ or duration $F(1,12) = .87$, $p = .37$, $\eta^2 = .07$. There was no main effect Treatment between stress and control animals in number, $F(1,12) = .01$, $p = .94$, $\eta^2 = .01$ or duration, $F(1,12) = 1.43$, $p = .26$, $\eta^2 = .11$. There was no significant within-subjects effect of Test Day, in number, $F(1,12) = 1.34$, $p = .27$, $\eta^2 = .10$ and duration, $F(1,12) = .17$, $p = .69$, $\eta^2 = .01$. 
Figure 6. Mean Frequency of Social Interactions (±SEM). Mean frequency of social interaction by controls (n = 7) and early-adolescent intermittent physical stress animals (n = 7) in the resident intruder test. There was no significant interaction, within subject effect of Test Day or main effect of Treatment on frequency scores (p > .05).
Figure 7. Mean Duration of Social Interactions (s) (±SEM). Mean duration of social interaction by controls (n = 7) and early-adolescent intermittent physical stress animals (n = 7) in the resident intruder test. There was no significant interaction, within subject effect of Test Day or main effect of Treatment (p > .05).
Figure 8. Mean Frequency of Non-Social Activity (±SEM). Mean frequency of non-social activity by controls (n = 7) and early-adolescent intermittent physical stress animals (n = 7) in the resident intruder test. There was no significant interaction, within subject effect of Test Day or main effect of Treatment on frequency scores (p > .05).
Figure 9. Mean Duration of Non-Social Activity (s) (±SEM). Mean duration of non-social activity by controls (n = 7) and early-adolescent intermittent physical stress animals (n = 7) in the resident intruder test. There was no significant interaction, within subject effect of Test Day or main effect of Treatment (p > .05).
3.5 Serotonin Fibre Length Density

3.5.1 Prefrontal Cortex

Data was not available for one animal due to poor quality of tissue. No outliers were found using visual inspection of quartiles with a box and whisker plot or Grubb’s test of outliers (Grubbs, 1950). This resulted in sample size of n = 6 for controls and n = 7 for stress. Each animal produced between 2-4 slices ranging from bregma 3.20 mm to 2.70 mm.

Animals exposed to early-adolescent stress demonstrate significantly higher serotonin fibre length density than control animals in the cingulate region of the prefrontal cortex, $F(1,11) = 4.90, p = .049$. All other regions of interest (infralimbic and prelimbic) examined did not show significantly different serotonin fibre length densities between control and stress animals, ($all\ p's > .05$) (see Figure 10; see appendix for images).
Figure 10. Mean Serotonin Fibre Length Density in Prefrontal Cortex (mm/mm³; ±SEM). Mean serotonin fibre length density of controls (n = 6) and early-adolescent intermittent physical stress animals (n = 7. Stress animals demonstrate significantly higher serotonin fibre length density than control animals in the cingulate region of the prefrontal cortex, $F(1,11) = 4.90$, $p = .049$. (IL = Infralimbic cortex, PreL = Prelimbic cortex, Cg = Cingulate Cortex).
3.5.1 Ventral Hippocampus

Data was not available for five animals due to poor quality of tissue. No outliers were found using visual inspection of quartiles with a box and whisker plot or Grubb’s test of outliers (Grubbs, 1950). This resulted in a sample size $n = 6$ for controls and $n = 4$ for stress. Each animal produced between 2 – 5 slices ranging from bregma -3.80 mm to -5.60 mm.

As can be seen in Figure 11., early adolescent stress rats demonstrate greater serotonin fibre density in CA1 and CA3. This is confirmed by a non-significant trend in CA1, $F(1,9) = 4.19$, $p = .075$, and in CA3, $F(1,9) = 3.54$, $p = .097$. All other regions of interest (dentate gyrus and subiculum) examined did not show any significantly different serotonin fibre length densities between control and stress animals, (all $p's > .05$).
Figure 11. Mean Serotonin Fibre Length Density in Ventral Hippocampus (mm/mm$^3$; ±SEM).
Mean serotonin fibre length density of controls (n = 6) and early-adolescent intermittent physical stress animals (n = 4). DG = dentate gyrus; SUB = subiculum. Stress animals demonstrate a non-significant trend of greater serotonin fibre length density than control animals in CA1, $F(1,8) = 4.19, p = .075$, and in CA3, $F(1,8) = 3.54, p = .097$. (CA1 = CA1 region of Hippocampus, CA3 = CA3 region of Hippocampus, DG = Dentate Gyrus, SUB = Subicum).

Serotonin Fibre Length Density (mm/mm$^3$)

Regions of Interest

- CA1
- CA3
- DG
- SUB

Control
Stress
Chapter 4

Discussion

I hypothesized that adult rats exposed to early-adolescent stress would have a higher display of anxiety-related behaviours (indexed by less open-arm exploration in the elevated plus-maze as well as greater burying behaviour in the shock-probe burying test) and higher display of aggression-related behaviours (higher number and duration of aggressive behaviours). In addition to these behavioural changes, I also predicted that animals exposed to early-adolescent stress would display lower serotonergic fibre length density than control animals in the prefrontal cortex and ventral hippocampus. Unlike my predictions, I found no significant difference between stress and control groups in anxiety-related behaviours. I found that the stress group displayed a significantly lower level of aggression-related behaviours, a significantly greater serotonergic fibre density in the anterior cingulate cortex of the prefrontal cortex and non-significant trend indicating a greater serotonergic fibre density in areas CA1 and CA3 of the ventral hippocampus. These findings suggest that stress during early-adolescence leads to long-lasting changes in social behaviour and that these changes might partly be due to alterations in serotonergic innervation of the anterior cingulate cortex.

4.1 Behavioral Measures

Testing in both the elevated plus-maze and shock-probe burying test did not reveal any significant statistical differences between animals with a history of early-adolescent stress and control animals. This was unexpected, given that previous research found increases in anxiety related behaviour following physical stress in early-adolescence (Brydges et al., 2012; Pohl et al., 2007; Tsoory, Cohen & Richter-Levin, 2007; Tsoory, & Richter-Levin, 2006; Wilkin et al., 2012). In addition to this, significantly lower aggression-related behaviour was displayed by
animals exposed to early-adolescent stress in the resident intruder test. This was also surprising, given that my initial prediction was that animals exposed to early-adolescent stress would display greater aggression-related behaviour. I predicted an increase because of the previously established positive correlation between shock-probe burying behaviour and aggressive behaviour in the resident intruder test (Sgoifo, De Boer, Haller and Koolhaas, 1996; Sluyter, Korte, Bohus and Oortmerssen 1996).

4.1.2 Elevated Plus-Maze

It is not clear what would account for my null findings in the elevated plus-maze test, as I used the same stress paradigms, testing paradigms, and testing facility as described in Wilkin et al., 2012. I had expected to find lower open-arm exploration in rats exposed to stress in early-adolescence, based on numerous studies that support this prediction (Brydges et al., 2012; Tsoory, Cohen & Richter-Levin, 2007; Tsoory, & Richter-Levin, 2006; Wilkin et al., 2012). It should be noted that these other studies used a variety of different stressors including swim stress, restraint stress and predator scent, which strengthens the argument that stress (of various kinds) during early-adolescence can lead to reductions in open-arm exploration in adulthood.

One potential account for my null effects in the elevated plus-maze test is the sample size was small, which might have resulted in lower power. An underpowered test would increase the chance of a type 2 error, reducing the chance that any difference between the groups would be detected. Alternatively, the baseline levels of open-arm exploration I obtained were unusually low. Specifically, the percentages of open-arm exploration in control animals were 11% open-arm entries and 8% open-arm time. This may have led to a floor effect, which could have obscured differences between groups. Most experiments from the Menard lab report baseline
levels of open-arm exploration of 20%, almost double what was found in this experiment. To better understand if there was a floor effect, I examined the scores from animals with a history of early-adolescent stress as a percentage of the control animals’ scores. I found that animals with a history of early-adolescent stress displayed 35% lower percentage of open-arm entries than controls and 57% lower percentage of open-arm time than controls, which is consistent with the direction of my prediction.

4.1.2 Shock-Probe Burying Test

In the shock-probe burying test, I did not find any significant differences between stress and control groups. Wilkin et al. (2012) previously found that adult male rats with a prior history of early-adolescent stress buried significantly more than controls. Similar to what I observed in the elevated plus-maze test, the control rats in my study displayed relatively high baseline levels of burying behaviour (mean = 234.51 seconds, SEM = 37.59 seconds). This is much higher than the baseline levels of burying reported by Wilkin et al. (2012) (mean = 155.80 seconds, SEM = 53.67 seconds). In fact, most studies in the literature report baseline control levels of burying between 50 to 150 seconds (Menard & Treit, 1996; Pesold & Treit, 1992; Treit, Robinson, Rotzinger & Pesold, 1993). Interestingly, all of those studies were conducted by female experimenters. Sorge et al. (2014) demonstrated that male experimenters can evoke a greater stress response than female experimenters. Although speculative, it could be possible that control rats from my experiment were relatively more stressed than control rats in Wilkin et al. (2012), simply because of a sex-dependent experimenter effect. At any rate, my controls were likely more stressed than controls in other studies, as indexed by their relatively higher levels of burying behavior. The relatively high baselines I observed might have masked the lasting effects of prior stress during early-adolescence on anxiety-related behaviors. Alternatively, it might be
that the effects of stress during early-adolescence on burying behaviour in adulthood are less robust than previously assumed.

4.1.3 Resident Intruder Test

In contrast to the null findings in tests of anxiety, I observed lower frequency and duration of aggressive behaviours in animals with a history of early-adolescent stress compared to control animals. In particular, animals exposed to early-adolescent stress displayed a lower frequency and duration of keep down behaviour. This finding was unexpected in that the changes are in the opposite direction to my initial prediction.

Aggressive behaviour that is within a certain range is adaptive (Hawley & Vaughn, 2003; Koolhaas et al. 2013). Most studies have focused on increases in aggressive behaviour where the aggressor starts to exhibit maladaptive violent aggression, characterized by a lack of signalling threat behaviours before attacks (Toth, Mikics, Tulogdi, Aliczki, & Haller, 2011), biting vulnerable areas, and directing aggression at inappropriate recipients (i.e., female rats, unconscious stimulus rats) (Koolhaas et al., 2013). Adaptive aggression behaviour profiles normally include displays of aggression towards appropriate conspecifics to protect or acquire resources and opposing control mechanisms to prevent violent behaviour which reduces the likelihood of injury (Koolhaas et al., 2013). In the current study, I did not observe any evidence to suggest a shift into violence but rather a decrease in adaptive aggression.

Reduced levels of adaptive aggressive behaviour can have direct implications for a rodent’s ability to control its’ social environment and ultimately could prove to be a net maladaptive pattern of behaviour (Koolhaas et al., 2013). Adaptive aggression is a means for resident rodents to socially communicate to intruding conspecifics that they are entering the
resident animal’s territory (Blanchard, Wall and Blanchard, 2003). These behaviours include bites targeting non-vulnerable areas, the use of threat behaviours to signal a proceeding attack and the expression of aggressive behaviours in valid contexts towards appropriate recipients (intruding male conspecifics) (Koolhaas et al., 2013). In the natural environment, when a resident rat optimally uses territorial aggression, it minimizes the risk of harm to itself and the intruder, while maintaining control of its home territory. Control over the territory raises the odds that the resident/dominant male will have exclusive access to food and potential mates that are located in the area (Blanchard, Wall & Blanchard, 2003; Koolhaus et al., 2013; Lorenz, 2002; Thor & Carr, 1979). In contrast, unaggressive males will have reduced access to females, in turn lowering the males’ likelihood of reproductive success (Koolhaas et al., 2013; Thor & Carr, 1979). In a similar vein, reduced access to food resources could compromise a submissive animal’s survival rate. This could in turn make them less able to compete with dominant males that have full access to resources. Residents that lose their interactions with opponents are affected for future interactions and can continue to develop into a submissive behaviour profile and possibly become subordinate to an intruder (Van de Poll, De Jonge, Van Oyen, Van Pelt, 1982). Therefore, an adaptive context for the expression of aggression is one in which the resident has resources to protect (i.e., access to territory with food and/or mates) and low chance of injury and (smaller opponent) has high chance of success (smaller opponent). Under the current experiment’s ethological context, lower levels of aggression could be regarded as a maladaptive pattern of behaviour (Thor & Carr, 1979).

In this study, I used a version of the resident intruder test that favoured displays of aggression and more specifically, behaviours signalling dominance from the resident rat. Therefore, lower levels of aggressive behaviours, especially the keep down behaviour, may not
be adaptive. A context favouring aggression was established by housing the resident male with a female and, by pitting residents against smaller intruders at testing. These conditions normally facilitate aggression in the resident rats (Koolhaas et al., 2013; Thor & Carr, 1979). Nonetheless, resident animals with a history of early-adolescent stress were not prone to demonstrating territorial aggressive behaviour, despite the fact that their opponent was smaller than them. Animals exposed to stress in early-adolescence demonstrated levels of aggression that could be suboptimal for the conditions, suggesting that the lower aggression displayed by these animals was maladaptive.

The keep down behaviour is also known as “on top” or “full attack posture” (Blanchard and Blanchard, 1977) and is involved in the establishment of dominance in a male-male conspecific social confrontation (Baenninger, 1966; Grant & Chance, 1958). In the current experiment, animals exposed to early-adolescent stress displayed the keep down behaviour less often. This suggests that residents displaying reduced dominant behaviours may be acting maladaptively, as the outcome of the first conflict has been shown to shape future interactions (Lehner, Rutte and Taborsky, 2011). Researchers have observed that victorious rats in a conspecific interaction were more likely to win subsequent conflicts, whereas rats that lost were more likely to lose in future conflicts (Seward, 1944; Van de Poll, De Jonge, Van Oyen, & Van Pelt, 1982). This has also been demonstrated in animals that were assigned to a winning or losing condition (Lehner et al., 2011), emphasizing the importance of the first few interactions with a conspecific. Given that the context of the resident intruder test in the current study favoured successful territorial aggression, it would be adaptive to demonstrate aggressive dominance behaviours, which would favour success in the future as well. Animals that were exposed to early-adolescent stress demonstrated lower levels of keep down behaviour compared to control
animals. Residents that partially exhibit dominance may open up the possibility to be challenged again. This could lead to longer, more strenuous bouts in the future, with a higher risk of becoming a subordinate. Subordinates generally have lower reproductive and survival success. Negative outcomes include: a higher incidence of stress induced ulcers (Mine et al., 1981); hippocampal atrophy (Magariños, McEwen, Flügge, & Fuchs, 1996); increased anxiety-related and depression-related behaviours (Blanchard, Sakai, McEwen, Weiss, & Blanchard, 1993); and shortened lifespans (Blanchard et al., 1993).

4.2 Anxiety and Aggression

I had previously predicted that there would be an increase in aggression after exposure to early-adolescent stress, as previous studies had associated higher anxiety (decreased open-arm exploration and increased burying behaviour) with higher aggression (Ferrari, Palanza, Parmigiani and Rodgers, 1998; Sgoifo, De Boer, Haller and Koolhaas, 1996; Sluyter, Korte, Bohus and Oortmerssen 1996). The current experiment is inconsistent with this pattern, given that I found no significant changes in anxiety and a reduction in aggression. However, the current results do suggest that the anxiety-related behaviours and aggression may be linked in a more complex manner than commonly thought. A review by Rodgers and Waters (1985) suggests that the relationship between aggression and anxiety is not straightforward. They found that while these behaviours share overlapping neural circuitry, various doses of anxiolytic drugs differentially changed behaviours associated with aggression and anxiety, respectively (Rodgers and Waters, 1985). For example, Miczek (1974) found that low doses of the benzodiazepine receptor agonist, chlordiazepoxide, (2.5 mg/kg and 5.0 mg/kg) increased aggression-like behaviour and high doses (20 mg/kg) decreased aggression-like behaviour. In contrast, Crawley and Goodwin (1980) demonstrated that chlordiazepoxide has anxiolytic effects at both low (2.5
mg/kg) and high doses (20 mg/kg). Other researchers have demonstrated reductions in aggression behaviours independent of any changes in anxiety behaviours when disrupting receptors or proteins specific to serotonin function (Mosienko et al., 2012; Pagani et al., 2015). The current study shows a similar pattern following administration of early-adolescent stress, suggesting that early-adolescent stress may affect components of the serotonergic system that are related to aggression but not anxiety.

4.3 The Relationship between Early-Adolescent Stress and Aggression

Models of early-life stress have previously demonstrated that exposure to social defeat stress during adolescence leads to increases in aggression during adulthood (Ferris, 2000; Veenema, 2009; Wommack, Taravosh-Lahn, David, & Delville, 2003). These studies do not match my findings, however, there may be several differences in methodology contributing to this including: species (these previous studies used hamsters, rather than rats), age during stress exposure (the timing of stress application in these studies included the mid-adolescent period, rather than being restricted to early-adolescence) and stressor type (these studies used social defeat, rather than physical stressors). Combined with the findings from other studies, the current findings might suggest that the lasting effects of intermittent physical stress in adolescence on aggression might differ from the effects of social defeat stress. Interestingly, Kabbaj, Isgor, Watson, and Akil (2002) demonstrated that the effects of stress during adolescence on tests of amphetamine sensitization differed depending on if the stressors were physical or social. Alternatively, it might be the case that stress restricted to early-adolescence and stress during mid-adolescence yield opposing outcomes (decreases and increases, respectively) on aggression-related responses in adulthood. Although additional experiments are needed to test that possibility, such a pattern has been observed when comparing the effects of stress during early-
vs. mid-adolescence on rats’ open-arm exploration in the elevated plus-maze (Wilkin et al., 2012). That is, adult rats with a prior history of stress during early-adolescence displayed decreases in open-arm exploration whereas rats with a history of stress in mid-adolescence displayed increases in open-arm exploration (Wilkin et al., 2012). With regards to aggression, timing of stress could be important for adult outcomes.

4.4 Serotonergic Fibre Length Density

Animals with a history of early-adolescent stress had significantly greater serotonergic fibre density in the anterior cingulate of the prefrontal cortex and a non-significant trend suggesting greater serotonergic fibre density in CA1 and CA3 of the ventral hippocampus. They also demonstrated lower aggressive behaviour than controls; suggesting that denser serotonin innervation is related to lower aggression. While these data do not directly support my hypothesis, they do support the relationship between aggression-related behaviour and serotonin innervation that my hypothesis had described. I had predicted that there would be greater aggression-related behaviours with lower serotonin fibre density. Instead, I found the reverse relationship, low aggression-related behaviours with high serotonin fibre density. Greater serotonin fibre density is indicative of increased development of the serotonergic system (Papesh, & Hurley, 2012). This suggests that early-adolescent stress increased development of the serotonergic system innervating the anterior cingulate cortex, which may also impact the serotonergic system’s functioning in that area.

When examining aggression and the serotonergic system, a common relationship is an increase in aggression, accompanied by a decrease in serotonergic functioning in the prefrontal cortex (Nelson & Trainor, 2007; Veenema, 2009). This pattern is supported by multiple findings
including: lower aggression after the administration of SSRIs (Sánchez & Meier, 1997), and higher aggressive behaviours associated with low serotonergic activity in primate models and rat models (Ferrari, Palanza, Parmigiani, de Almeida, & Miczek, 2005). The current work concurs with previous findings as I found a similar relationship, that is, animals with a significantly lower level of aggression also had a significantly greater level of serotonergic fibre density in the anterior cingulate cortex (an area of the prefrontal cortex). This provides additional support that changes in the serotonergic system lead to changes in aggressive behaviour. In my study, stress in early-adolescence lead to increases in serotonergic innervation of the anterior cingulate and these changes mapped on to lower levels of aggression.

4.4.1 Anterior Cingulate Cortex

The anterior cingulate cortex has been shown to be involved with the initiation and maintenance of goal directed behaviours in humans (Devinsky, Morrell, & Vogt, 1995). With regards to aggression, the anterior cingulate cortex is believed to suppress aggression when 5-HT$_2$ receptors are activated and researchers postulate that low levels of serotonergic innervation could result in excessive aggression (Siever, 2008). Most research focuses on how a lack of serotoninergic activation could lead to an over expression of aggression, however, the current study demonstrates that exposure to early-adolescent stress could lead to an over-developed serotonergic innervation of the anterior cingulate cortex, which may over-suppress aggression. Pharmacologically raising the activity of serotonin in the prefrontal cortex has been shown to reduce aggression in both rodents (Sánchez, & Meier, 1997) and humans (Coccaro, Berman, & Kavoussi, 1997). The current results are somewhat unique in that they demonstrate how serotonin innervation in the prefrontal cortex is involved in the relationship between early-adolescent stress and lower adult aggression.
Clinical research also suggests that the anterior cingulate cortex is involved in aggressive behaviour. Analysis of data obtained using positron emission tomography (PET) scans showed that individuals with impulsive aggressive disorder had reduced serotonin transporter density (a measure of serotonergic fibre density) and serotonin transporter availability in the anterior cingulate cortex compared to age matched controls (Frankle et al., 2005). In addition, individuals with impulsive aggressive disorder are not sensitive to serotonin releasing agents that normally act on receptors in the anterior cingulate cortex to reduce aggressive behaviour (Siever et al., 1999; New et al., 2002). This suggests that animals exposed to early-adolescent stress may have overly sensitive serotonergic systems in the anterior cingulate cortex, which is contributing to lower aggressive behaviour than control animals. The current study may help understand the anterior cingulate cortex’s role in the initiation and suppression of aggressive behaviour.

Some researchers have demonstrated that aggression can be reduced by disrupting serotonergic activity, and that these same disruptions do not affect anxiety-related behaviours (Mosienko et al., 2012; Pagani et al., 2015). Both of those studies highlight how anxiety-related behaviour and aggression-related behaviour are dissociable. Something similar may be happening to animals in the current experiment with a history of early-adolescent stress. Both Mosienko et al. (2012) and Pagani et al. (2015) used knock-out models targeting the TPH2 protein (a precursor to serotonin) and oxytocin receptors specifically attached to serotonin fibres, respectively. In both cases, reducing normal serotonergic function in the dorsal raphe nucleus reduced aggression without affecting anxiety-related behaviour. The current study demonstrates that physical stress during early-adolescence can also lead to reductions in aggression similar to what was found in Mosienko et al. (2012) and Pagani et al. (2015). In the current study, there is a possibility that both the normal trajectory of oxytocin receptors on serotonin fibres and TPH2
protein production development in the dorsal raphe nucleus are being disrupted. This warrants further study as a possible underlying mechanism driving reductions in aggressive behaviour.

4.5 Play Fighting and the Development of Aggression

Play fighting behaviour emerges and peaks during early-adolescence (PD21 – PD34) and is believed to be a precursor to aggressive behaviours in adulthood (Palagi et al., 2015; Thor & Holloway, 1985; Vanderschuren, Niesink, & Van Ree, 1997). It is believed that disrupting juvenile and adolescent play fighting behaviour could facilitate the development of maladaptive aggression-related behaviours in adulthood (Potegal & Einon, 1989; Von Frijtag, Schot, van den Bos, & Spruijt, 2002).

In the current study, housing animals individually may have disrupted play fighting opportunities. This may have lowered aggression in both groups as the overall observed levels of aggression could be considered relatively low. Koolhaas et al. (2013) assessed levels of aggression in rats and they categorized rats displaying less than 15% of time on aggressive behaviours as “low aggression” animals. Control rats in the current experiment spent an average of 15.94% of time on aggressive behaviours, which places them at the low end of the category of “medium aggression”, which ranges from 15% to 55% of time on aggressive behaviours. One possibility to explain this low display of aggression is that all animals in the current study were in isolation (single) housing. Isolation housing involves placing one rat in a cage so that there is no physical interaction between individuals, however, rats are still able to hear and see other rats that are in the colony. Animals were single housed as a method to control unwanted social stress and therefore, both control animals and animals exposed to early-adolescent stress were socially
isolated from weaning (PD 21). This would disrupt the peak play fighting development period and may have lowered all animals’ aggression in the experiment.

Regardless of the effects of isolation, IPS further reduced levels of aggression, suggesting that the combined effects of losing play fighting opportunities due to isolation housing and early-adolescent stress are greater than isolation housing alone. It may be that the loss of play fighting produces an effect which is exacerbated by early-adolescent stress, impacting their aggressiveness. The loss of play fighting behaviour may be causing impairments in decision making about the situation or partner which could lead animals to inhibit their aggressive responses. Researchers report that the capacity to engage in basic aggressive behaviours themselves are not affected by preventing play fighting behaviour (Thor & Holloway, 1985; Vanderschuren, Niesink, & Van Ree, 1997) but rather the complex socio-cognitive decision making processes that are involved in aggression such as impulse control (Baarendse, Counotte, O'Donnell & Vanderschuren, 2013) and negotiating dominance relationships (Pellis et al., 2006). The current experiment supports this, as all possible aggressive behaviours were observed during testing. The lower levels of aggression that were displayed by the experimental animals may be occurring due to over inhibiting aggressive impulses and having difficulty identifying aggression-related cues in the environment. Additional support for this comes from alterations in serotonin fibre length density in the prefrontal cortex. The prefrontal cortex had greater serotonin fibre density. The prefrontal cortex is believed to be involved in impairments to impulse control and understanding dominance relationships. Both Baarendse et al. (2013) and Pellis et al. (2006) found evidence to suggest that changes in the prefrontal cortex was responsible for maladaptive impulsivity and maladaptive aggression respectively that they observed. Furthermore, Bell et al. (2009) suggested that adult rats lose the ability to modify the roughness of their behaviour and do
not use complex strategies if the medial prefrontal cortex is removed. These studies, along with the results from the current study, suggest that the development of aggression (via play fighting behaviour) and the serotonin system in the prefrontal cortex may be vulnerable in early-adolescence. Furthermore, the current study suggests that the IPS paradigm applied in early-adolescence may have led to long term disruptions in aggression related decision making, which manifested as reductions in adult aggression behaviour.

4.6 Limitations

Animal models of early-life physical stress do not completely reproduce all of the elements that are found in humans exposed to early-life stress. The intermittent, physical stress paradigm that I used was developed to model physical abuse in humans. Abuse is characterized as an immediate, unpredictable, physically noxious event which we modeled by exposing animals to sporadic (randomly delivered) noxious stressors. However, in the human condition, physical abuse is typically delivered by a caregiver or schoolmate. This limits the face validity of my paradigm.

A major limitation of the current study is that individual animal housing procedures can introduce an additional source of stress on experimental animals. Social isolation during adolescence is regarded as a stressor (Fone and Porkess, 2008). However, the outcomes of this approach are often mixed. For example, both increases (Meng, Li, Han, Shao, & Wang, 2010; Workman, Fonken, Gusfa, Kassouf, & Nelson, 2011), decreases (Toth et al., 2011) and no changes (Grippo et al., 2007) in aggression have been observed in isolation-housed rodents relative to group housed controls. These contradictory findings might reflect differences in species, breed, isolation duration, aggression behaviours measured and the type of test utilized.
Notably, Toth and colleagues, reported that isolation-housing from weaning reduced the frequency of offence (a composite of aggressive grooming, lateral threat, offensive upright posture, mounting and chasing), as well as dominant posture (keep down) displayed by adult rats in a resident intruder test (Toth et al., 2011), and their behavioural measures and test procedure are similar to those of the current study.

Notably, control rats in my study were handled for each stress exposure, which has been shown to reverse the effects of social isolation on behavioural outcomes in adulthood (Gentsch, Lichtsteiner, Frischknecht, Feer, & Siegfried, 1988; Holson, Scallet, Ali, & Turner; 1990; Sciolino et al., 2010). An alternative method to avoid social deprivation stress is to house animals in pairs. Unfortunately, this also presents problems as pairs of animals will establish a dominance-hierarchy. The impact of dominance-hierarchies is difficult to control and equate across animals. Research has shown that subordinate rats in pair housing have had lower body weight and higher plasma corticosteroid concentrations than dominant rats and also lower than rats in isolation housing (Raab et al., 1986). More specifically, subordinate rats lost weight over time whereas dominant rats and rats in isolation housing had increases in weight over time (Raab et al., 1986). This suggests that while being a dominant rat would be less stressful, being a subordinate could be more stress inducing than being in isolation housing. Pair housing could unpredictably mitigate or aggravate the effects of stress. In my study, I chose to house all animals individually in both experimental conditions, so that any unwanted third factor due to social deprivation would be consistent across all of the groups. In addition, I wanted to avoid animals getting an unpredictable amount of practice for the resident intruder test through interactions with their cage partner. This said, I cannot rule out the possibility that my findings
reflect an interaction between isolation stress and IPS in early adolescence. Future studies are needed to resolve this possibility.

Another limitation is that sample size in this experiment was low. As discussed previously, this could have contributed to problems replicating previous findings in the elevated plus-maze. Low sample size has been thought to be a common problem in the field of behavioural neuroscience that can contribute to difficulties replicating previous studies due to an inability to detect effects, as well as an overestimation of effect sizes (Button et al., 2013). Small sample sizes result in low power, leading to a reduced likelihood of detecting statistically significant differences. Medium and small effects may be evaluated as not statistically significant and only large effects are detected and published in the literature. This can lead to an overestimation of a statistically significant result’s true effect size (Button et al., 2013). The true effect size may be medium, however if only large effects are detected, then only results with a large effect size are reported and therefore, the true effect may be overestimated (Button et al., 2013). In the current study, my sample sizes were no larger than n = 10, which meant that I would have required a relatively large effect to find any differences between groups. This could have played a part in my inability to replicate the elevated plus maze results found by Wilkin et al. (2012). In addition, this should be considered for other groups attempting to replicate the current study. The current study had low power and found a large effect in the resident intruder test (frequency of aggressive interactions, $\eta^2 = .54$; duration of aggressive interactions, $\eta^2 = .54$) and therefore, the effects found in the current experiment could be overestimations of the true effect sizes.

In this study, I bred animals in-house, to limit stress that animals would experience if transported from a 3rd party breeding facility. This is critical to ensure that animals are only
exposed to stress during the early-adolescent period. A difficulty of using this method is that I do not know how many animals I will be able use as subjects due to the unpredictability of breeding. The nature of using animals for research means there must be a balance between using enough animals and being wasteful. Therefore, in planning stages, I created as many breeding pairs as is required to get an adequate sample, but not too many so that I had an excess number of animals. I followed protocols that were used by other researchers from our lab, and was not as fortunate with the number of animals that were born. For this reason, the sample size is lower than would be ideal.

4.7 Future Directions

This experiment has shown that stress exposure during early adolescence could be disruptive to the development of aggressive behaviours. One gap in our current knowledge is that animals that are isolated are granted less time to engage in social experiences (play behaviour, social investigation, and dominance-subordinate relationships) that may be impacting their ability to perform in tests of aggression. To determine the effects of stress alone, it may be prudent to include another two groups in which animals are housed in pairs while exposed to control or early-adolescent stress conditions. This may allow us to elucidate the compound effects of stress and isolation and also to understand if stress is countered or exacerbated by pair housing. Many studies utilize isolation stress in adolescence, so it would help us to connect studies that utilize physical stressors to that body of work. In addition to this, we could determine differences in the outcomes for animals exposed to early-adolescent physical stress that experience play and animals that do not.
Animals exposed to early-adolescent physical stress in this study were found to be unaggressive, despite the fact that they were pitted against a smaller opponent. An additional question that arises is if these lower levels of aggression in the presence of a larger, more competitive intruder male would create more negative outcomes for the resident rats. Animals that are less aggressive could become a target of aggressive behaviours from competing conspecifics and if defeated, they could exhibit a greater number of submissive behaviours and become subordinates. The current study was not ideal to test this hypothesis as the intruder animals were smaller than the resident animals, which contextually favors the resident as a successful aggressor (Koolhaus et al., 2013). For this reason, the intruders may not have been as aggressive as larger intruders would have been towards the residents. A future experiment could determine if rats exposed to early-adolescent stress would display a greater number of submissive behaviours when their opponent is the same size or larger than themselves. If animals exposed to early-adolescent stress become subordinate to intruding opponents, this would underscore the maladaptive behaviours that we see in the current study. Animals exposed to early-adolescent stress with larger opponents could be more negatively affected than control animals. Early-adolescent stress has been shown to make individuals vulnerable to stressful situations as adults, compounding the effects of stressors at both ages (Horovitz et al., 2014). This may result in a higher rate of residents becoming submissive and suffering the negative effects of being a subordinate. Early-adolescent stress could be pre-disposing animals to display subordinate-typical behaviour profiles as adults. An additional way to examine this would be to compare how easily early-adolescent stress animals become submissive in adaptive contexts (larger, more aggressive and more experienced opponent). If animals exposed to early-adolescent stress display submissive behaviours more often or quickly than control animals, then it suggests
that physical stress during early-adolescence may prime animals to take the subordinates role as adults.

4.8 Summary

In this experiment, I exposed rats to physical stress in early adolescence and found a decrease in their aggression behaviours and an increase in serotonin fibre length density in the cingulate of the prefrontal cortex in adulthood. The current results suggest that early-adolescence is a vulnerable period of emotional development, and in the context of the existing literature, raise the possibility that timing of stress might be an important factor for aggression-related outcomes.

The long-term goal of this work is to be able to model and understand the effects of stress on maladaptive emotion-related behaviours (anxiety and aggression) and the related neurophysiological changes (serotonin fibre density) so that we can use this information for understanding these problems and developing therapies for clinical populations.
References


Appendix

Appendix A

Serotonin fibre density innervation in the Anterior Cingulate Cortex in Control Animals
Appendix B

Serotonin fibre density innervation in the Anterior Cingulate Cortex in Stress Animals