STRESS RESPONDING IN PERIADOLESCENT RATS EXPOSED TO CAT ODOUR AND LONG-TERM OUTCOMES FOR STRESS-RELATED ASPECTS OF THE ADULT PHENOTYPE

by

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DEDICATION

This thesis is dedicated to my beautiful daughter, Charlotte Meridian Hawkins, without whom it would never have been completed.

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ABSTRACT

Prior work has shown important effects of the early life environment on development of adult stress response systems in both rats and humans. The present thesis is based on experiments that attempt to explore: 1) adolescent stress responding at hormonal and behavioural levels, and 2) the effects of repeated adolescent stressor exposure on adult stress responding (hormonal and behavioural) and levels of dopamine receptors expressed in prefrontal cortex, using both male and female rats. Defensive behaviours exhibited during stressor exposure and post-stress levels of circulating corticosterone were quantified as behavioural and hormonal measures of stress responding, respectively. In the first study, responses were compared among groups of adolescent rats exposed repeatedly to one of two different types of cat odour stressor stimuli (J-cloth coated in hair/dander or cat collar previously worn by a cat) or control stimuli, and long-term outcomes were examined in adulthood. Adolescent rats showed behavioural responses to both stressor stimuli, but behavioural inhibition was more consistent using repeated cat collar exposure, and this treatment resulted in long-term increases in anxiety-like behaviour in adulthood, whereas a stress-induced adolescent corticosterone elevation was observed only in the group that received exposure to the J-cloth stimuli. In the second study, adolescent and adult rats were compared directly using repeated exposure to the cat collar stressor or control stimuli. Adolescents were found to be more sensitive to the effects of the stressor stimuli, relative to adults. Finally, in the third study, repeated exposure to the J-cloth stressor or control stimuli was used, and stressor-exposed females showed elevated baseline corticosterone levels prior to the final exposure. Furthermore, stressor-exposed males and females showed lower levels of the D2 dopamine receptor in infralimbic and dorsopeduncular cortices of the prefrontal cortex in adulthood. In addition, these studies together provide evidence that sex differences in corticosterone levels emerge during the adolescent period. It may be concluded that adolescence should be considered a sensitive developmental timeframe for stress response programming.

LIST OF ABBREVIATIONS

ACTH—adrenocorticotrophic hormone

ADOL-C—Control treatment involving exposure of adolescent rats to clean stimuli

ADOL-collar—Experimental treatment involving exposure of adolescent rats to worn cat collar stimuli

ADOL-control—Control treatment involving exposure of adolescent rats to clean collar stimuli

ADOL-J-cloth—Experimental treatment involving exposure of adolescent rats to cat hair/dander stimuli

ADOL-S—Experimental treatment involving exposure of adolescent rats to cat odour stressor stimuli

ANOVA—analysis of variance

CO—control odour

Cort—corticosterone

CRH—corticotrophin-releasing hormone

DA—dopamine

D1DR—D1 dopamine receptor

D2DR—D2 dopamine receptor

EH—early handling

GABA—gamma-aminobutyric acid

GR—glucocorticoid receptor

HB—hide box

HPA—hypothalamic-pituitary-adrenal

mPFC—medial prefrontal cortex

MR—mineralocorticoid receptors

MRI—magnetic resonance imaging

NH—not handled

NMDA—N-methyl-D-aspartate

OF—open field

OF1—novel exposure to the open field

OF2—re-exposure to the open field one day after initial exposure

OF3-J-cloth—exposure to a cat hair/dander stimulus in the open field

OF4-collar—exposure to a worn cat collar stimulus in the open field

PFC—prefrontal cortex

PND—postnatal day

PO—predator odour

PPO—periadolescent predator odour

PT—predator stress test

PVN—paraventricular nucleus of the hypothalamus

SPSS—Statistical Package for Social Sciences

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Overview

Appropriate stress responding is a necessary part of everyday life for survival of all organisms. It may manifest as any of a number of behavioural strategies that are guided by interacting neural and physiological subsystems and that are orchestrated by an individual to deal with a disruption to homeostasis. Because each species inhabits a defined ecological niche, members of a species are likely to encounter certain challenges that are common in their environment. Thus, species have evolved characteristic coping strategies that have proven to be successful in overcoming the challenges they face. Furthermore, stress response strategies change across development, as individuals become independent and expand their territories. Individuals may come to favour particular strategies that have worked to combat stressors within their lifetimes.

A primary purpose of the experiments presented herein was to examine behavioural and physiological defensive responses of adolescent male and female rats to an important stressor challenge, exposure to cues of predation threat. A secondary purpose was to examine long-term effects of adolescent stressor exposure on adult neural, physiological, and behavioural measures related to defense strategies. These experiments were designed to model the kinds of effects adolescent stressor exposure may have in humans and other mammalian species.

1.2. Hypothalamic-Pituitary-Adrenal Stress Responding

The hypothalamic-pituitary-adrenal (HPA) axis is but one of a number of systems in the body's repertoire to maintain homeostasis in response to stressor exposure. However, as the key regulator of glucocorticoid secretion, it is considered very important. Limbic and prefrontal brain regions (e.g. amygdala, hippocampus, infralimbic and prelimbic cortices) have important influences over HPA activity, and they appear to exert this influence through relays with neurons in hypothalamic nuclei and other subcortical sites, rather than via direct projections to the paraventricular nucleus of the hypothalamus (PVN; Herman, Ostrander, Mueller, & Figueiredo, 2005), which is the startpoint of HPA activation. Hypothalamic nuclei devoted to sensory processing send projections to the PVN, which go on to activate the pituitary gland via corticotrophin-releasing hormone (CRH) when stressors are detected. CRH is recognized by CRH receptors in the pituitary, which produces adrenocorticotropic hormone (ACTH) and releases it into the blood circulation. ACTH is recognized by receptors in the adrenal gland, and glucocorticoid secretion from the adrenal cortex is the culmination of HPA axis activation and plays a major role in ensuring a state of restoration following challenges (Herman, Figueiredo, Mueller, Ulrich-Lai, Ostrander, Choi, & Cullinan, 2003), including playing an active role in organizing behavioural strategies during environmental perturbations (Wingfield, 2003).

It has been proposed that cell function, mental performance, and general health are dependent on the integrated orchestration of glucocorticoids acting on two intracellular receptor types: mineralocorticoid receptors (MR) and glucocorticoid receptors (GR; De Kloet, 2004). The major glucocorticoids, cortisol and corticosterone

(cort), bind with much higher affinity to MR than to GR (Reul & de Kloet, 1985), which are co-localized in several structures including the hippocampus, amygdala, and also prefrontal cortex (PFC; Helm, Han, & Gallagher, 2002; Herman et al., 2005). Densities of GR are typically higher than densities of MR; thus, the higher affinity MR quickly become saturated following a stress-induced increase in cort levels, and GR binding is then increased (Reul & de Kloet, 1985). When ligand-bound, either steroid receptor type influences gene transcription through interactions with DNA. MR are involved in stabilizing neuronal networks that mediate immediate responses to stress regulated by CRH type 1 receptors, whereas GR promote behavioural adaptations to stress and play a major role in negative feedback regulation of the HPA axis (De Kloet, 2004; Helm et al., 2002; Reul & de Kloet, 1985; Sapolsky, Meaney, & McEwen, 1985). Athough it is now well accepted that cort plays a major role in regulating behavioural responses to stress, the mechanism(s) by which this occurs cannot be described simply as a consequence of the interaction of cort with its receptors. Indeed, studies attempting to relate cort levels to stress response behaviours have shown varied results (Dal-Zotto, Marti, & Armario, 2000; Kalynchuk, Gregus, Boudreau, & Perrot-Sinal, 2004; Mashoodh, Wright, Hebert, & Perrot-Sinal, 2008; Masini, Sauer, & Campeau, 2005; Mathews, Wilton, Styles, & McCormick, 2008b; Morrow, Elsworth, & Roth, 2002; Perrot-Sinal, Ossenkopp, & Kavaliers, 1999; Pohl, Olmstead, Wynne-Edwards, Harkness, & Menard, 2007; Rees, Steiner, & Fleming, 2006; Romeo, Karatsoreos, & McEwen, 2006b). Often, no direct relationship between cort and behaviour is observed (e.g. Morrow et al., 2002; Perrot-Sinal et al., 1999). Many factors contribute to the variability of results, including the type of stressor employed, the species, and the particular cort and behavioural measures

examined. In understanding the variability of results, it is important to recognize that exhibition of behaviour requires bodily movement (or lack of movement), which is initiated by neural activation in particular regulatory brain circuits. The hormonal milieu can change the likelihood that a certain type of behaviour will be initiated, but it doesn't direct behavioural action, *per se*. For this reason, the present set of experiments aims to examine relationships between cort and stress-related behaviour and also leads to an examination of stress-induced changes in dopamine (DA) receptor levels, as DA receptors play a direct role in the regulation of movement.

Allostasis is defined as 'maintaining stability through change' and is used to describe the dynamic process by which the body responds to homeostatic threats. The concept can be extended to include 'allostatic load', the cumulative physical costs of allostasis ('wear and tear' of maintaining homeostasis over time). The frequency and severity of challenges encountered, as well as the efficiency of response systems, will therefore determine how quickly allostatic load accumulates for an individual. An inability to deal with conditions leading to increased allostatic load can culminate in pathological conditions, as a result of excess release of glucocorticoids and other mediatiors of allostasis (B. S. McEwen, 2003, 2004). Glucocorticoids are catabolic, and excessive stress responding promotes development of a diverse array of disease processes, including cardiovascular, metabolic, and mental illnesses (Heuser & Lammers, 2003; B. S. McEwen, 2004). Imbalance of MR and GR can lead to disease states, such as unwarranted levels of anxiety (De Kloet, 2004), by decreasing negative feedback of the HPA axis, resulting in exaggerated stress responses.

As stated previously, limbic and prefrontal brain regions exert important influences over HPA activity. DA is particularly important in regulating prefrontal influences over stress responding, as DA is released in prefrontal regions in response to even mild stressors (Carlson, Fitzgerald, Keller, & Glick, 1993; Ravard, Carnoy, Herve, Tassin, Thiebot, & Soubrie, 1990; Sullivan & Gratton, 2002), such as in the ventromedial prefrontal cortex. Also, there are hemispheric asymmetries in prefrontal cortical DA involvement in the stress circuitry, with release in right prefrontal regions playing a more prominent role in regulating HPA activity (Brake, Sullivan, & Gratton, 2000; Carlson et al., 1993; Sullivan & Gratton, 2002). Gamma-aminobutyric acid (GABA), in association with its type A receptor, appears to play a role in differentiating regional differences in the DAergic response to psychological stress in prefrontal and striatal brain regions (Matsumoto, Togashi, Kaku, Kanno, Tahara, & Yoshioka, 2005). Hippocampal and prefrontal neurons show dendritic retraction when an individual is repeatedly exposed to stress; however, the amygdala, which is specialized for regulating fear responses, shows patterns of growth (B. S. McEwen, 2004).

Considering the catabolic nature of glucocorticoids, it becomes clear that it is critical for long-lived animals to deal with changing conditions *during* a lifespan, and, in many species, major unpredictable perturbations initiate behavioural strategies (for example, proactive/reactive coping styles; flight/fight responses to rapid emergencies) that serve to deal with the perturbation and to avoid chronically high levels of glucocorticoids (i.e. avoid allostatic overload; Wingfield, 2003). The role that developmental programming plays in the organization of such behavioural strategies in

adulthood is unknown. A main objective of this thesis is to evaluate the potential for such programming to occur during the adolescent period.

Few studies have directly compared stress responding in adolescents versus adults. In terms of cort release, adolescents show a different hormonal profile, relative to adults. Although data on adolescent cort responses to cat odour stimuli do not appear to exist at present, Romeo *et al*. (2004, 2006) have shown that adolescent male rats show a more protracted cort response to acute restraint stress and an increased cort response with a faster return to baseline following repeated restraint stress, relative to adult males. Adolescent females also show a more protracted cort response to acute restraint stress, relative to adult females, and ovarian hormones are not necessary for this effect (Romeo, Lee, & McEwen, 2004), although they may play a role in programming the adult cort response. A strong point of the present set of studies is the inclusion of males and females.

1.3. Environmental Programming and Critical Periods

It is proposed that features of the environment relevant for survival, such as resource availability and predation threat, play significant roles in guiding development, and therefore, in modulating expression of adult behaviour. Flexible developmental systems have evolved as a survival strategy allowing species to maximize fitness over a range of suitable habitats. Each potential habitat presents a unique set of advantages and challenges. Thus, behaviours that are adaptive in one habitat, with its unique challenges, may not maximize fitness in another. Evolutionary theory predicts that physiological systems with a lengthy period of developmental flexibility have the capacity to act as

environmental gauges. The stress response repertoire represents such a system because of a lengthy period of development extending into postnatal life, making it particularly amenable to the study of environmental programming.

As outlined above, Romeo *et al*. (2004, 2006) have demonstrated differences between adolescent and adult endocrinological stress responding, which are likely due to underdeveloped negative feedback systems in the adolescents. Indeed, limbic GR continue to increase to adulthood (Meaney, Sapolsky, & McEwen, 1985), and when more juvenile levels of GR are achieved experimentally at adolescence by inducing receptor down-regulation with application of exogenous cort, feedback inhibition of stress responding is disrupted (Meaney et al., 1985). These findings suggest that natural exposure to stressors during the adolescent period may also alter late postnatal development of the stress response system.

To date, most of the research examining developmental programming of stress responding has been conducted using manipulations administered prenatally or during the early postnatal period. For example, exposing rodents to early life stressors, such as repeated maternal separation, impacts the development of stress response systems at neural, endocrine, and behavioural levels (Bock, Gruss, Becker, & Braun, 2005; Levine, 2005; Macri, Mason, & Wurbel, 2004; Meaney, Brake, & Gratton, 2002; Plotsky & Meaney, 1993; Slotten, Kalinichev, Hagan, Marsden, & Fone, 2006). At weaning age, rats exposed to short periods (~one hr) of maternal separation for a few days before or after the stress hyporesponsive period had elevated basal plasma levels of cort and altered dendritic spine densities in pyramidal neurons of anterior cingulate and somatosensory cortices, with the direction of dendritic changes depending on the time frame of exposure

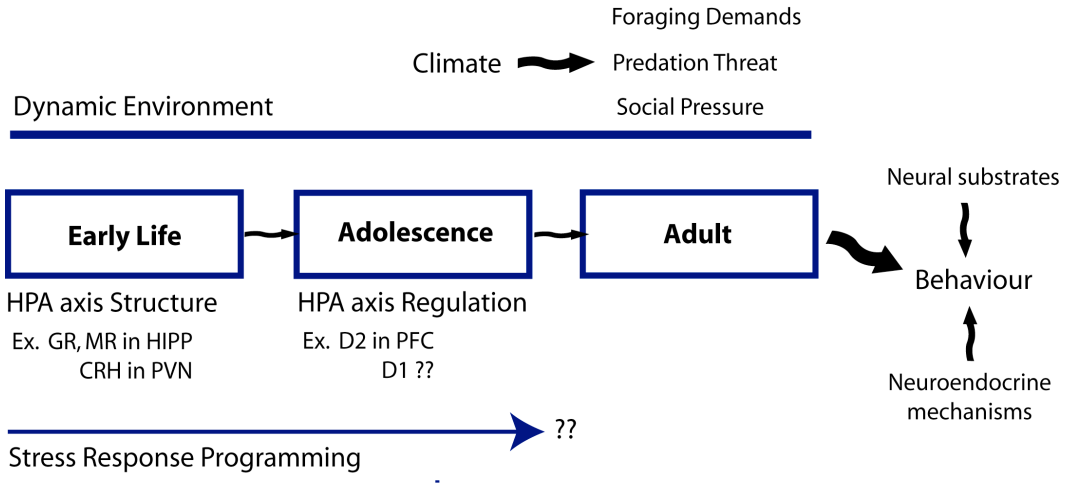
(Bock et al., 2005). In contrast, very brief periods of daily maternal separation (~3-15 min) prior to weaning, usually termed 'early handling', results in lower hypothalamic CRH mRNA levels, higher densities of GR in hippocampus, and blunted cort responses to stress, relative to non-handled animals or animals exposed to longer daily periods of separation (Levine, 2005; Macri et al., 2004; Plotsky & Meaney, 1993). Rats exposed to longer periods of maternal separation during the early life period have also shown a reduction in expression of the dopamine transporter and increased dopamine responses to stress in later life (Meaney et al., 2002).

In contrast to studies examining the early postnatal period, relatively few studies have examined effects of manipulations administered later, during potentially critical periods of adolescent development. This is despite the fact that environmental demands experienced during adolescence (re: habitat with its balance of advantages and challenges) will be a more reliable predictor of the adult environment. Environmental modulation of adolescent development should therefore steer programming toward optimizing fitness for prevailing environmental demands. Such modulation is likely to affect neural systems that are 'under construction' at that time. Figure 1.1 represents a schematic overview of the theoretical framework within which the hypotheses for this thesis are based.

1.4. Adolescent Development

Adolescence as a categorical developmental stage is a loose construct, because it actually consists of overlapping neuroendocrine cascades involving adrenal and gonadal hormones and major restructuring of neuronal architecture in frontal regions (Nagel,

Figure 1.1. A hypothetical model that provides a framework in which to study stress response programming. Across generations, the environment of most animals is dynamic, as major factors such as climate affect critical variables within species-specific ecological niches. For an adult animal (i.e. one that has gained independence) to maximize fitness, behavioural output in response to such changing demands needs to be appropriate. One way in which behavioural responses can be tailored to the present ecological demands is to program the underlying neural and neuroendocrine mechanisms after birth. In other words, physiological systems, such as stress responding, contain a certain level of flexibility that is afforded by epigenetic modulation of components of the system. In the rat, there is ample evidence for a critical period for hypothalamic-pituitary-adrenal (HPA) axis development early in life. For example, certain structural aspects of the HPA axis, such as levels of glucorcorticoid receptors (GR) and mineralocorticoid receptors (MR) in hippocampus (HIPP) and corticotrophic releasing hormone (CRH) levels in paraventricular nucleus of hypothalamus (PVN) are programmed during the first two weeks of life in the rat. Here, we hypothesize that adolescence represents a sensitive period for stress response programming. In particular, we propose that the dopamine (DA) system, which regulates HPA axis function, continues to be programmed throughout adolescence. As we argue in this thesis, adolescence represents the point at which an animal attains independence and has to finally face the environment alone. It is also a time of massive neuroendocrine and neural change, which has consequences for adaptive behaviour. In this thesis, we demonstrate long-lasting changes in adult defensive behaviours and in D2 receptors in prefrontal cortex (PFC) in animals exposed to repeated adolescent predation threat. We expect that, as we and others further investigate programming of the DA system, we will find programming of other aspects of this system during adolescence.



Medina, Yoshii, Schweinsburg, Moadab, & Tapert, 2006; Spear, 2000). While these cascades interact once initiated, each has a relatively independent onset and temporal progression (Spear, 2000).

In mammalian species, the hypothalamic-pituitary-gonadal axis is responsible for initiating a pattern of intermittent secretion of gonadotropin releasing hormone during adolescence, which ultimately results in pubertal production and secretion of androgens and estrogens from the gonads, and the attainment of sexual maturity (Sisk & Zehr, 2005). It is now being recognized that the adolescent brain is susceptible to organizational effects of gonadal hormone levels, and it has been proposed that this susceptibility gradually declines with increasing postnatal age (Schulz, Molenda-Figueira, & Sisk, 2009). It is not fully understood how the specific timing of gonadal hormone secretion interacts with the period of susceptibility to organizational effects to shape adult brain circuits and behaviours within an individual. It is likely of importance what particular brain regions happen to be undergoing intense development when the gonadal hormone levels rise, as the new hormonal milieu may alter the ongoing developmental processes.

As puberty becomes established, there are several systematic alterations in behaviour that occur, coinciding with late maturation of the PFC (Fuster, 2002). These include increases in risk-taking, novelty-seeking, and social behaviours (Spear, 2000), as well as emergence of defensive-type behavioural responses to threat (Hubbard, Blanchard, Yang, Markham, Gervacio, Chun, & Blanchard, 2004), and various other alterations in stress responding (Charmandari, Tsigos, & Chrousos, 2005; Hubbard et al., 2004). In addition, recent findings using impulsive decision-making paradigms indicate

that adolescents evaluate rewards differently than adults (Spear, 2000; Volkow, 2005). Mesocortical DA is critically involved in regulating all of these behaviours; therefore, developmental alterations in PFC affecting components of the DA system might underlie the behavioural changes characteristic of adolescents. Prefrontal D1 and D2 DA receptors increase in density up to 40 days of age in rats, which represents a mid-adolescent period, and are subsequently pruned extensively to levels approximately 60% and 35% lower, respectively, than the 40-day-old peak values by adulthood (100 days of age; (Andersen, Thompson, Rutstein, Hostetter, & Teicher, 2000). Similar patterns of overproduction and elimination of DA receptors have been observed in subcortical brain regions, although, in nucleus accumbens, levels remain high into adulthood (Teicher, Andersen, & Hostetter, 1995). Furthermore, the process of elimination appears to be sex-specific in striatal brain regions, with males overproducing and subsequently pruning DA receptors and females maintaining a more stable level of receptors across development (Andersen & Teicher, 2000). Neither castration nor ovariectomy at 28 days of age, when pubertal hormone levels begin to rise, altered this sex difference (Andersen, Thompson, Krenzel, & Teicher, 2002). It is unknown whether or not there are also sex differences in PFC DA receptor pruning, as data have only been obtained from male rats thus far. In males, however, DA receptor pruning appears to be more extensive and protracted in PFC, relative to striatum (Andersen et al., 2000). Mechanisms regulating developmental changes in DA receptor levels during the adolescent period are not understood, although they do not appear to involve glutamatergic actions at N-methyl-D-aspartate (NMDA) receptors, as it has been shown that antagonism of NMDA receptors from 40 to 60 post natal days of age did not alter DA receptor levels in either males or females (Teicher,

Krenzel, Thompson, & Andersen, 2003). Thus, pronounced, sex-specific developmental changes occur in levels of cortical and subcortical DA receptors from adolescence to adulthood. These changes may or may not be sensitive to environmental conditions but likely contribute to a shift in the balance of mesolimbic and mesocortical dopamine drive that is postulated to occur during adolescence (Spear, 2000).

The temporal progression of cortical development and the behaviours it regulates, many of which emerge during adolescence, invite opportunity for environmental conditions to steer flexible developmental programming toward a context-specific optimum. Two classes of behaviour that increase during adolescence and may be particularly relevant for stress response programming are risk-taking behaviours and peer-directed social interactions. McCormick and colleagues have shown that pubertal social stress can interact with nicotine sensitization to alter the adult cort response to immobilization stress in a sex-dependent manner (McCormick, Robarts, Gleason, & Kelsey, 2004). Furthermore, they have provided evidence that pubertal social stress enhances adult sensitivity to drugs of abuse in both males and females (Mathews, Mills, & McCormick, 2008a; McCormick, Robarts, Kopeikina, & Kelsey, 2005).

1.5. Role of Dopamine in Execution of Cognitive Tasks Mediated by the Prefrontal Cortex

Although responding to a stressor is partly an innate process, it often includes cognitive appraisal/evaluation of the situation. Cognitive processing allows information to be gathered from the environment, so that relevant material can be integrated with internal cues, such as emotional state and level of physiological arousal, in order to adaptively guide behaviour.

PFC-mediated neural processes related to cognitive ability have been investigated using functional magnetic resonance imaging (MRI) studies in humans, as well as electrophysiological and/or behavioural studies in animal models (for reviews, see (Floresco & Magyar, 2006; Postle, 2006). Using these techniques, specific components of cognitive functions can be isolated for study. For example, attention and working memory processes, whereby information is retained "on-line" for short-term use, are concepts intimately related to the idea of a cognitive percept. Brain function is examined during execution of tasks designed to tap these processes, thereby providing neural correlates of cognition. While the primate dorsolateral PFC is of primary importance for execution of cognitive tasks employing attention and working memory processes, the medial PFC (mPFC) plays an analogous role in rodents (see (Floresco & Magyar, 2006). These broader areas are comprised of subregions with distinct cytoarchitecture and connectivity (Ongur & Price, 2000), and these accordingly play differential roles in execution of cognitive tasks.

Work directed toward elucidating neurochemical mechanisms of action underlying goal-directed behaviour points toward an integral role for mesocortical DAergic drive (Chudasama & Robbins, 2004; Floresco & Magyar, 2006; Goldman-Rakic, 1996; Seamans, Floresco, & Phillips, 1998). Various DA receptor subtypes are present within the subregions of PFC, and downstream signal transduction events induced by DA depend on the type and location of the activated receptors.

DA receptors are G-protein coupled receptors embedded in cellular membranes. In general, they are classified as D1-type receptors, which include D1 and D5 subtypes, and D2-type receptors, which include D2, D3, and D4 subtypes. The major difference

between the two types is that activation of D1-type receptors by ligand binding induces changes in membrane potential that bring a neuron closer to its firing threshold (depolarizing effects), whereas activation of D2-type receptors move a neuron further from its firing threshold (hyperpolarizing effects). Therefore, D1-type receptors are considered excitatory and D2-type receptors are inhibitory. D1, D2, and D4 subtypes are all present within PFC (Goldman-Rakic, 1996; Wedzony, Chocyk, Mackowiak, Fijal, & Czyrak, 2000), with D1 receptors being expressed to a greater extent than D2-like on excitatory principle pyramidal neurons (Floresco & Magyar, 2006). Both types are also expressed on GABAergic interneurons, which act to hyperpolarize cells onto which they synapse. Therefore, excitatory and inhibitory receptors exist on excitatory and inhibitory neurons within subregions of PFC. This complexity hints at the varied nature of DA's effects on neural activity within prefrontal circuits.

Postnatal programming of DAergic circuits may alter an individual's capacity for behavioural flexibility, a relatively recent concept that has been shown to rely heavily on PFC DA innervation and is reliant on attentional processes (Floresco & Magyar, 2006). Simple reversal learning, for example, requires alternation between two response choices in order to receive rewards. Learning to alternate in these tasks depends on the integrity of the orbital PFC (McAlonan & Brown, 2003). The mPFC, in contrast, is tapped when rats are trained on more complex tasks requiring different aspects of attention, such as attentional set shifting.

In attentional set shifting tasks, rats are required to first base a decision on information gathered in one 'stimulus dimension' (e.g. visual cue), and then to switch the basis of the next decision to information gathered in a different dimension (e.g. tactile

cue). Successful performance therefore requires both the inhibition of a previous response and the acquisition of a new attentional set in a different sensory modality. Pharmacological studies using local administration of receptor agonists and antagonists have revealed important involvement of both D1 and D2 receptor activation in successful set shifting, as well as a selective effect of D4 receptor activation in antagonizing the effects over behavioural flexibility exerted cooperatively by D1 and D2 (Floresco & Magyar, 2006). More specifically, enhancement of D4 activity leads to persistent focus in one dimension and, thus, impaired set shifting.

In the tasks described above that are used to study the involvement of PFC DA in cognitive ability, subjects are tested under relatively stress-free conditions. Researchers are only now beginning to appreciate the important role of PFC DA in regulating stress responding (see Section 1.6; Herman et al., 2005; Sullivan & Gratton, 2002), which leads to conjecture about how DAergic stress responses impact cognitive abilities.

Rodent behavioural tasks are also used to determine the roles of PFC DA receptors in impulsive decision-making. Impulsivity is an individual factor important in determining levels of risk-taking behaviour, and, thus, may be particularly relevant for the expression of adolescent risk-taking. Tasks that require rats to choose between an immediate small reward and a larger delayed reward are used to provide an index of impulsivity, and systemic administration of DA agonists increases the preference for the larger, delayed reward (van Gaalen, van Koten, Schoffelmeer, & Vanderschuren, 2006). Reward evaluation in this paradigm, however, does not incorporate a punishment component reflective of the 'down-side' that usually exists naturally when reward influences impulsive decision-making. Floresco and Magyar (2006) created a model to

better capture this process using a conditioned punishment paradigm. In rats, a tone that predicts an electric shock creates an aversion to the tone alone. If rats are then trained to press levers for rewards, they will forego the optimal foraging strategy, in order to avoid pressing levers associated with the aversive tone. However, microinfusion of a D1, a D2, or a D4 receptor antagonist into the mPFC abolishes this discriminative ability, demonstrating that D1, D2, and D4 receptors in PFC all contribute to this effect (Floresco & Magyar, 2006). DA signaling in PFC is proposed to mediate a cost-benefit analysis upon presentation of rewards, as altering behaviour to attain rewards disrupts homeostasis.

Thus, the PFC is a brain region that mediates cognitive processes and regulates HPA axis stress responding, and both actions involve DA signaling. Interconnections of prefrontal subregions and connectedness of the PFC with other brain regions continues to develop across adolescence, via alterations in DAergic and other signaling pathways. Thus, although most cognitive tasks may be performed efficiently before adolescent development takes place, the influence of cognition on various behavioural processes, including risk-taking and stress responding, may continue to be refined across the adolescent period. Cognitive processes involve behavioural flexibility, a feature of organisms that may be programmed by environmental conditions during sensitive developmental periods and affected by stress.

1.6. Prefrontal Cortex Dopaminergic Modulation of Stress Responding

DA is a key player mediating stress responding (Sullivan & Dufresne, 2006). DA release in PFC has been confirmed by microdialysis in behaving animals in response to

handling and other psychological stressors (Matsumoto et al., 2005; Pehek, Nocjar, Roth, Byrd, & Mabrouk, 2006). As stimuli become stress provoking via association with punishment, such as in the conditioned punishment paradigm described above, their avoidance is also mediated by DA signaling in PFC (Floresco & Magyar, 2006). These findings demonstrate a role for DA signaling in response to both controllable and uncontrollable stressors. As mentioned above, enhanced DA transmission in PFC increases the tolerance of delay to attain a reward. In accordance with this, DA depletion in PFC differentially alters responses to negative events, depending on whether they are controllable or uncontrollable, with increased punished responding in two paradigms involving controllable stressors (Ravard et al., 1990).

Fear- and anxiety-related behavioural output during stressful conditions has come to be understood in terms of a regulatory balance between limbic drive and cortical drive within the stress response circuitry. The hippocampus and amygdala each connect reciprocally to PFC, and there is evidence that both these limbic structures relay contextual information during stress responding. However, learned contextual associations are stored within the hippocampus when environmental cues come to predict reward (food, mates) or challenge (predators, competitors). The hippocampus is therefore involved in storing training-related information gathered during contextual fear conditioning (Pentkowski, Blanchard, Lever, Litvin, & Blanchard, 2006), while the amygdala appears to play a more general role in memory consolidation of emotionally arousing experiences (Davidson & Irwin, 1999; Malin & McGaugh, 2006). Fear extinction occurs when a conditioned stimulus is repeatedly presented without the original negative association, and this sort of adaptive response is mediated by PFC

output to the amygdala (Sotres-Bayon, Bush, & LeDoux, 2004). The PFC therefore integrates its inputs dynamically during stressful conditions, in order to assess the current emotional and physical state of the organism and direct adaptive coping strategies to minimize allostatic load.

Taken together, these findings imply that DAergic signaling in PFC is very important for cognitive integration of contextual information, and thus, adaptively guiding behaviour associated with stress responding. For example, activation of inhibitory D2 receptors on principle pyramidal neurons increases the change in membrane potential required to induce neuronal firing in these cells (hyperpolarization). In such a hypothetical case, stress responding would not rely as much on input from prefrontal principle pyramidal neurons, because they would require higher levels of stimulation to make a contribution to ongoing responding of the limbic component of the stress circuitry. Therefore, stress responding would be more reflexive and less guided by cognitive appraisal. Alternatively, this type of scenario could occur via activation of excitatory D1 receptors located on inhibitory interneurons that synapse on principle pyramidal neurons in prefrontal cortex.

As discussed above, there are cost-benefit health trade-offs that are inherent in stress responding. A stress response mounted to deal with a particular stressor in one environmental context may not be necessary or sufficient for successful coping within another context. Simply put, stress responding needs to be efficient and specific for the stressor and context encountered. Therefore, organisms with the capability to cognitively fine-tune stress responses in accordance with environmental assessment will be selected for, by avoiding unnecessary energy expenditure. Species occupying broad and varied

niches would be expected to show more fine-tuning capability than species that occupy very specific niches, since environmental features would be more unpredictable.

1.7. Adolescence as a Critical Period for Stress Response Programming

Aspects of the stress response are programmed during early life. Childhood abuse and neglect have long-term effects on adult physical and mental health by altering the HPA axis (Nemeroff, 2004; Nemeroff & Vale, 2005). In laboratory rats, the first two weeks are critical for programming systems important in adult behavioural and neural stress responding (Francis, Champagne, Liu, & Meaney, 1999; Gutman & Nemeroff, 2002; Levine, 1957; Levine, Alpert, & Lewis, 1957; Liu, Diorio, Tannenbaum, Caldji, Francis, Freedman, Sharma, Pearson, Plotsky, & Meaney, 1997). For example, daily short-term (3 - 15 min) separations of pups from the dam during the first 1-2 weeks of life (early handling; EH) results in blunted adrenal stress responses and reduced fear- and anxiety-related behaviour in adulthood, relative to pups left undisturbed until weaning (not handled; NH; Caldji, Diorio, & Meaney, 2000; Levine, 2005). This manipulation also attenuates adult DAergic responses to stress in nucleus accumbens (Brake, Zhang, Diorio, Meaney, & Gratton, 2004).

In humans, adolescent victims of bullying show alterations in patterns of cortisol secretion (Vaillancourt, Duku, Decatanzaro, Macmillan, Muir, & Schmidt, 2007), suggesting potential for changes in late postnatal development of the HPA axis. There is scant literature on HPA axis changes in rats following exposure to stressors during adolescence, although work by McCormick *et al*. (2004) demonstrates that chronic isolation/social stress during the adolescent period can alter adult cort secretion in

response to a heterotypic stressor, depending on sex and further experience of the animal (i.e. whether or not the animal was sensitized to nicotine). However, in a further study, the same paradigm resulted in altered adult cort secretion following chronic isolation/social stress exposure in adulthood but not adolescence (McCormick et al., 2005).

While PFC development during adolescence is being vigorously studied, there is still relatively little conclusive information available on changes in expression levels of DA signaling markers, although adolescent changes in levels of PFC DA receptors have been described (see Section 1.4 above). Nor is there information on whether or not developmental changes are sensitive to environmental factors. In humans, PFC volume increases through to adolescence, with a later peak for white matter increases than for gray matter, reflective of late axonal myelination. As in other cortical and subcortical regions, extensive synaptic pruning occurs in PFC during adolescence, as shown in mammalian animal models (Spear, 2000). This is reflected by decreases in whole PFC (Fuster, 2002) and in white matter volumes (Nagel et al., 2006) towards the latter end of the adolescent period in humans.

Presuming these pruning events are Hebbian in nature, the possibility of neural programming during adolescence is supported. Late myelination may aid in strengthening connections exhibiting increased activity, while those less active die away. Features of the adolescent environment, such as levels of adversity, could impact this process by influencing neuronal activity, through DAergic and/or other neurotransmitter signaling. We know that DA responses are evoked in PFC during adolescence even in response to mild psychological stressors (Elsworth, Morrow, & Roth, 2001), and therefore, levels of

adversity in the adolescent environment could program aspects of the DA system during the adolescent period. In adulthood, the overall effect of stress-induced DA release in PFC is a reduction in neural output from this region, which can effectively augment limbic control over behavioural responses. Because DA receptors are co-localized with various other receptors in PFC, the effects of their activation on neural activity when DA is released are complex and difficult to predict. A stress-induced DA augmentation of limbic drive may be conceptualized to function like a biological gearshift. DA released in response to stress kicks the PFC into low gear, for instance, via interaction with D1 receptors on inhibitory interneurons in stress-responsive subregions. This DA response to stress would establish a requirement for more excitatory neurotransmission (more glutamate release, for example), in order to boost activity in these circuits to pre-stress levels.

While this possibility is only one simplified illustration, it demonstrates a potential mechanism by which levels of environmental adversity could modulate development of circuits that regulate PFC DA-mediated negative feedback on HPA activity. For example, an individual exposed frequently to stressors during adolescence may develop a phenotype optimized for survival in an environment replete with danger and may thus require a lower criterion level of stress for kicking the PFC into low gear and passing control of stress responding over to limbic inputs in adulthood. The infralimbic cortex is thought to play a major role in this effect, is sensitive to early life environmental conditions, and appears to regulate adaptation to repeated stress (Sullivan & Dufresne, 2006). Given the massive and widespread, sex-specific changes that occur in DA system structure during the adolescent period, repeated stress experienced at this time

might be expected to permanently alter synaptic stabilization in specific PFC subregions, by altering the balance of activity between DA-responsive and non-responsive connections. This could provide a means by which the PFC is programmed toward an adult phenotype best suited to cope with stressful conditions.

1.8. Using an Ecologically Relevant Approach to Examine Stress Responding and Developmental Programming During Adolescence

In order to examine the possibility that adverse environmental conditions experienced during adolescence can program aspects of adult stress responding, I designed a model that involves exposing male and female rats to repeated predation threat (cat odour) during the periadolescent period (periadolescent predator odour; PPO). Predation threat is a natural means to mimic adverse environmental conditions in laboratory animals. Predatory cues, such as cat odour, robustly induce well-characterized changes in defensive and non-defensive behaviours in many species of rodent (R. J. Blanchard, Blanchard, Weiss, & Meyer, 1990; R. J. Blanchard, Nikulina, Sakai, McKittrick, McEwen, & Blanchard, 1998; Endler & Mappes, 2004; Kavaliers & Choleris, 2001), as well as in HPA axis function (R. J. Blanchard et al., 1998; Figueiredo, Bruestle, Bodie, Dolgas, & Herman, 2003; File, Zangrossi, Sanders, & Mabbutt, 1993; Perrot-Sinal et al., 1999). As such, predatory cues can be used to assess adaptive stress responding in adult animals. Although other ethologically relevant stressor models have been developed, such as those incorporating chronic social stress (Mathews et al., 2008a; McCormick et al., 2004), this model offers many advantages over other commonly used models of stress, as it involves a purely psychological stressor that induces robust and quantifiable behavioural responses that are measurable *during* the stressor exposure

(Dielenberg, Hunt, & McGregor, 2001; Kavaliers & Choleris, 2001). The more commonly used restraint model, in contrast, does not offer the possibility of measuring behavioural responses during exposure, nor is it a stressor to which rats would be expected to have evolved characteristic defense tactics. In the same vein, another commonly used stressor model, electric footshock, also lacks ecological validity, since most wild rats would never be confronted with this. Thus, we wouldn't expect that such stressors would produce the same effects on developmental programming. A further advantage of the predator odour model is that adult rats continue to exhibit defensive responses in the presence of cat odour stimuli even after three weeks of daily one-hour exposure sessions (Mashoodh et al., 2008).

At this point, few direct comparisons have been made between adolescent and adult responses to predator odour stimuli or to stressors, in general. It is known that sensivity to particular stressors changes across development (Wiedenmayer & Barr, 2001). Juvenile rats as young as 18 days of age have been shown to avoid cat odour stimuli; however, locomotor inhibition and risk-assessment defense tactics do not appear to develop until rats are closer to the age of puberty (Hubbard et al., 2004).

1.9. Overall Objectives

Given the information summarized above, the overall objectives for the present thesis were: 1) to characterize behavioural and endocrine responses of adolescent male and female rats across repeated exposure to cat odour stimuli, 2) to examine long-term changes in defensive behaviours and cort levels in adult rats exposed to stress as

adolescents, and 3) to examine adult levels of DA receptors following repeated adolescent stressor exposure.

CHAPTER 2

COMPARISON OF STRESS RESPONSE PARAMETERS OF RATS EXPOSED DURING ADOLESCENCE TO CAT ODOUR STIMULI

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Abstract

In order to characterize the short- and long-term effects of repeated stressor exposure during adolescence, and to compare the effects of using two sources of cat odour as stressor stimuli, male and female adolescent rats (PND38-46) were exposed on five occasions to either a control stimulus, a J-cloth stimulus containing cat hair/dander, or a section of cat collar previously worn by a cat. Relative to control animals, activity was suppressed by both stressor stimuli, but most consistently in rats exposed to the collar. Adolescent collar-exposed rats also showed increased anxiety-like behaviour and high levels of huddling during exposures. Interestingly, only J-cloth-exposed rats showed elevated levels of corticosterone (cort), and only after repeated stressor exposure. In control rats, social contact during the final adolescent exposure was positively related to cort levels measured after this exposure, and these levels were positively related to adult baseline cort levels. These associations suggest a relationship between cort levels and adolescent social behaviour that is disrupted by repeated adolescent exposure to either stressor stimulus. Rats exposed to the collar stimulus during adolescence continued to show increased behavioural indices of anxiety in adulthood. In this group, time spent in physical contact with a cagemate during the final adolescent exposure was negatively related to stress-induced cort output in adulthood, which suggests that greater use of social support during adolescent stress may support adult behavioural coping without increased cort release. These findings indicate the specificity of cat odour stimuli as stressors and suggest a potential role for social behaviour (huddling) during adolescent stressor exposure in mediating long-term outcomes.

2.1. Introduction

Adolescence denotes the sum of pubertal features, together with behavioural and brain changes, that characterize the transition to adulthood and independence. Notable behavioural changes that occur during this transition include increases in attention span, cognitive capacity, and impulse control, afforded by an extensive developmental remodeling of prefrontal cortex and its connections with other brain regions (Giedd, 2004; Spear, 2000). In addition, adolescence is characterized by increased risk-taking behaviour and peer-directed social activity, and differences are evident in reward processing and stress response systems between adolescents and adults (McCormick & Mathews, 2007; Nagel et al., 2006; Romeo, Bellani, Karatsoreos, Chhua, Vernov, Conrad, & McEwen, 2006a; Spear, 2000). Many of these features are conserved across a broad range of species, including various mammalian models (Spear, 2004), suggesting common neurobiological bases related to adolescent development.

The increased propensity of adolescents to engage in risky behaviour, especially at a time when they are communicating and interacting primarily with one another, has prompted some to suggest this may be a vulnerable period (Adriani & Laviola, 2004; McCormick & Mathews, 2007). Negative life experiences at this time may lead to stress that causes enduring harmful effects, including a decline in lifetime health. Indeed, in rat models of adolescent stress, there is evidence for enhanced cort release in adulthood following stress during the adolescent period (Pohl et al., 2007), but there is also evidence for negligible effects on adult cort levels following adolescent stress (Watt, Burke, Renner, & Forster, 2009). Some have hypothesized that adolescents could in fact

be relatively resilient, when compared with other age groups (Ferris, Messenger, & Sullivan, 2005). These disparate views underscore the need for research designed to examine not only the acute, but also the long-term effects of stress experienced during the adolescent period and how they interact with the social environment. Stressor effects have been demonstrated to be dependent on a number of factors, including the duration/type of stressor, age, sex, and test context (R. J. Blanchard & Blanchard, 2003; McCormick & Mathews, 2009).

Increasingly, predatory odours are being used to investigate the effects of mammalian stress responding (R. J. Blanchard, Yang, Li, Gervacio, & Blanchard, 2001). Such paradigms are advantageous because the stressor is natural and innately evokes ethologically relevant, unconditioned defensive responses that are resistant to habituation (Apfelbach, Blanchard, Blanchard, Hayes, & McGregor, 2005; Hubbard et al., 2004; Mashoodh et al., 2008). Predator odour cues activate the HPA axis (Dielenberg et al., 2001), culminating in the release of cort from the adrenal cortex (Charmandari et al., 2005). Importantly, using predator odour as a stressor stimulus allows for behavioural monitoring *during* stressor exposure, with measures of defensiveness and risk-assessment being quantified while the animal perceives the threat, which isn't always possible when using more traditional stress paradigms, such as restraint or footshock. Because endocrine and behavioural responses to stress can be dissociated (e.g. Mashoodh et al., 2008), examining behaviour in addition to cort output is critical. Additionally, adult males and females tend to show qualitative differences in the way they respond to predator odour cues, making this stressor useful for examining sex differences. In general, females appear more defensive, behaviourally (D. C. Blanchard, Shepherd, De Padua Carobrez, &

Blanchard, 1991; R. J. Blanchard, Yudko, Rodgers, & Blanchard, 1993). Acutely, both males and females inhibit activity; females, however, show a more sustained reduction of activity and increased frequency of risk assessment (head-outs) across exposure sessions and across repeated exposures (Mashoodh et al., 2008).

Holistic predator odour sources, such as cloths coated with cat hair, appear to be more effective at eliciting a broad range of defensive behaviours and the accompanying activation in neural defense circuits, relative to isolated components of predator odours or synthetic derivatives, such as trimethylthiazoline from fox feces, which activate only specific brain regions that are part of the defense pathways and seem to produce weaker behavioural responses that aren't as easily conditioned (McGregor, Schrama, Ambermoon, & Dielenberg, 2002; Siviy, Harrison, & McGregor, 2006; Staples, McGregor, Apfelbach, & Hunt, 2008b). Innate responses of adult rats to cat odours have been characterized for the two sources used most commonly in predator odour exposure studies—cloth rubbed on a cat (e.g. Mashoodh et al., 2008)) and pieces of collar previously worn by a cat (e.g. Dielenberg & McGregor, 1999; Perrot-Sinal, Gregus, Boudreau, & Kalynchuk, 2004).

Despite the similarity of these odour sources, it appears that rats are exquisitely sensitive to features of cat odour stimuli, suggesting that it may be prudent to take note of the specific odour source. For example, when using cat hair-coated cloths as predator stimuli, larger cloths are better able than smaller cloths to instate conditioned defensive behaviours that extinguish slower, demonstrating that behavioural responses are dependent on the quantity of odour molecules delivered (Takahashi, Nakashima, Hong, & Watanabe, 2005). Recent work showing that defensive behaviours that had habituated

with repeated exposure to cloths rubbed on one cat were reinstated when odours from a new cat were presented suggests that rats can even distinguish individual cats, based on unique compositions of odour molecules (Staples, Hunt, van Nieuwenhuijzen, & McGregor, 2008a).

Juvenile rats show immobility in response to urine-soiled cat bedding (Wiedenmayer & Barr, 2001) or cat hair-coated cloth as young as 14 days of age; however, they don't develop conditioned stress responses to the context of predator odour presentation until post-weaning (Hubbard et al., 2004). Siviy *et al*. (2006) have shown that adolescent exposure to cat collar stimuli substantially reduced play behaviours, both during exposures and up to 24 hours later. Adolescent exposure to a social stress paradigm involving repeated periods of isolation and pairing with a new cagemate led to increased cort release in males exposed to swim stress later on in adolescence (Mathews et al., 2008b) but not in males exposed to restraint stress in adulthood (McCormick et al., 2005), indicating that long-term effects of the adolescent stressor manipulation on HPA activity may not be permanent and depend on the stress test used. However, it is unknown whether long-term effects are also specific to the type of stressor experienced during the adolescent period.

The overall goals of the present experiment were: 1) to characterize adolescent responding to two different predator odour stimuli, and 2) to compare the long-term effects of repeated adolescent exposure to these stimuli. For the predator odour cues, we used a cloth stimulus (J-cloth) containing hair and dander from a number of cats and a section of worn cat collar derived from a single cat. Responses to these were compared with responses to a control stimulus, and long-term effects on adult endocrine and

behavioural stress responding were examined. We hypothesized a stronger reaction to the J-cloth stimulus, as well as more robust long-term effects, because it was intended to model a high-risk adolescent environment involving many predators, whereas the collar stimulus involved only one cat whose odours may have become more familiar across repeated exposures.

2.2. Materials and Methods

2.2.1. Subjects

2.2.1.1. General Husbandry Procedures

Subjects were derived from Long Evans hooded rats purchased from Charles River (Quebec, Canada). Rats were housed in 22 x 24 x 48 cm polypropylene cages that were covered with wire lids and contained wood-chip bedding and an ~5-inch section black, polyvinyl-carbonate tubing, provided for enrichment. The colony room was maintained at 20 ± 1 °C, on a 12:12 reverse light:dark cycle (lights off at 0930h). Food (Purina Lab Chow) and tap water were available *ad libitum*. Prior to any experimental manipulation, rats were handled each day for four or five days (at least 2 min per day) by the experimenter. This always occurred during the rats' active phase (subjective night) and entailed being picked up, touched, and held. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals. Effort was made to minimize any excess pain or discomfort that the animals might experience.

2.2.1.2. Breeding

Each male breeder was paired for five days with a female in a standard homecage. Following removal of the male, pregnant females were housed singly until giving birth. Beginning on estimated gestational day 20, females were checked at least once daily for litters, and the first day pups were noted was designated as PND0. Dams were then allowed to nurse their litters undisturbed, except for once-weekly cage changing. Pups were weaned on PND21 and were subsequently housed in same-sex pairs (or groups of three when necessary to avoid isolation, with groups of three distributed evenly amongst experimental treatments).

2.2.1.3. Experimental Group Designation

Each cage of weaned rats was randomly assigned to one of three adolescent treatment groups: 1) Exposure to strips of J-cloth freshly coated with cat hair/dander (ADOL-J-cloth; N = 6 males and 8 females), 2) Exposure to sections of collar previously worn by a cat (ADOL-collar; N = 7 males and 10 females), or 3) Exposure to strips of clean cloth (ADOL-control; N = 11 males and 11 females). Details of the stimuli are provided in the ***Adolescent Stressor Manipulation*** section below. Individuals were identified using non-toxic ink markings applied to the tail at weaning and reapplied as necessary (~weekly).

Fifty-three additional animals were used in a second experiment examining acute adolescent cort secretion (see below; N = 8 males and 8 females designated to the ADOL-J-cloth group, N = 12 males and 8 females designated to the ADOL-collar group, and N = 8 males and 9 females designated to the ADOL-control group).

2.2.2. Experimental Design

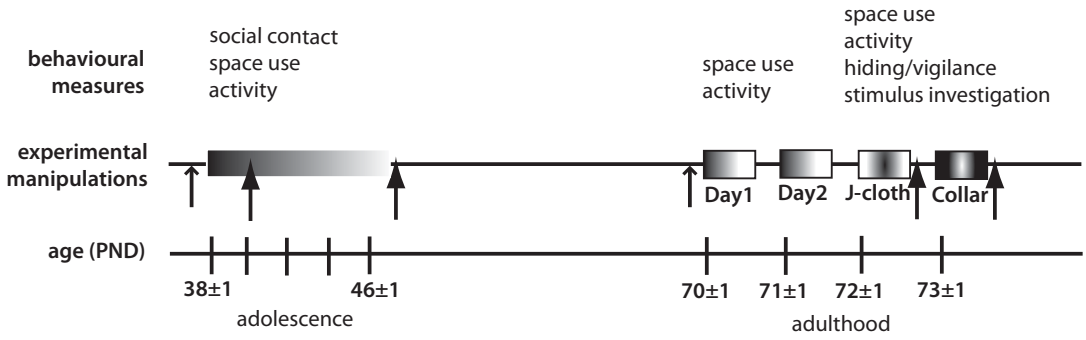
The experimental groups were created to achieve the objective of comparing behavioural and cort responses to the two different stressor stimuli, in relation to the control stimulus. Behaviours (described in the ***Adolescent Behavioural Assessment*** section below) were quantified within each exposure context during the first and final (fifth) exposure sessions, and cort responses were measured in response to the final exposure. Animals repeatedly exposed with cagemates to stressor or control stimuli during the adolescent period were then raised to adulthood, and behaviours and cort levels were measured for each rat individually in a series of open field sessions, including exposure of individual rats to each of the two cat odour stressor stimuli. Figure 2.1A illustrates the experimental design.

2.2.2.1. Adolescent Stressor Manipulation

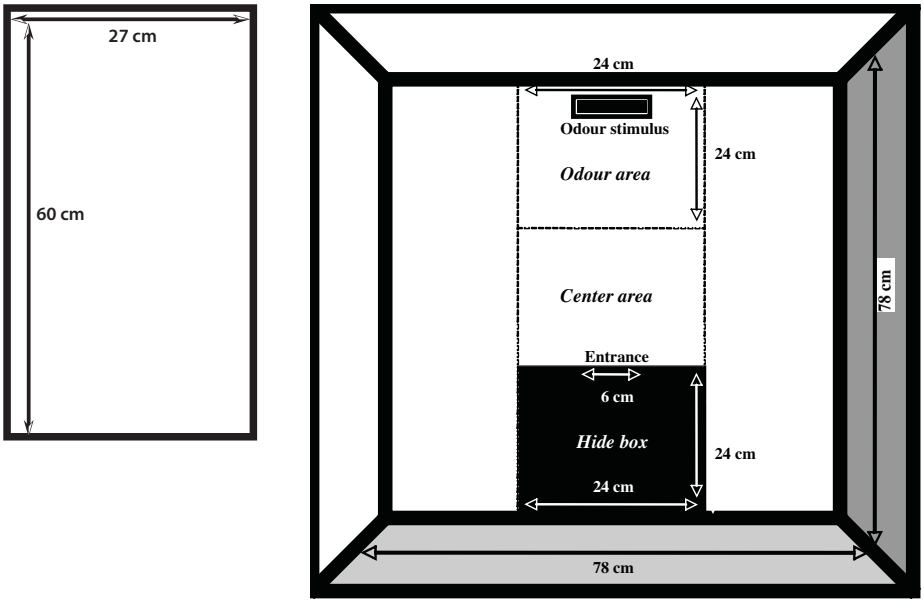
Each animal was exposed, together with the cagemate(s), to its designated stressor stimulus (J-cloth, collar, or control) for 30 min on five occasions across the mid- to late adolescent period (Hodes & Shors, 2005; Spear, 2004), beginning on PND38 ± 1 and re-occurring every 2nd day until PND46 ± 1. A 30-min duration of exposure to predator odour has been shown to enhance HPA axis output in adults (Masini et al., 2005). Rats were exposed in a social context, rather than individually, since the manipulation is intended to model natural conditions, and juvenile rats are highly social and show signs of stress when isolated (Frisone, Frye, & Zimmerberg, 2002). Exposures took place

Figure 2.1. A) Schematic timeline of the experimental procedures used in this experiment and the behaviours measured. During adolescence (postnatal day (PND) ~38-46), male and female rats were exposed on five occasions to a stressor (cat collar worn by a cat, J-cloth containing cat dander, or control; see text for details) and levels of various behaviours measured. Blood samples were taken at various points to assess levels of corticosterone. In adulthood (~PND70), behavioural testing was performed on four consecutive days: Day1 - novel open field; Day2 – familiar open field; J-cloth – exposure to J-cloth covered with cat hair/dander; Collar – exposure to a collar previously worn by a cat. A small arrowhead indicates times at which blood was taken in the evening during the rats' circadian nadir of corticosterone secretion (baseline sample). A large arrowhead indicates times when blood was taken immediately following stressor exposure sessions (experimental sample). Note that blood samples taken after the first and last adolescent stressor session were taken from different cohorts of animals. **B)** Schematic illustrations of the arena employed for adolescent exposures (left) and the behavioural test apparatus employed for each open field test session in adulthood (right). The open field apparatus contained a hide box across from the position at which an odour stimulus could be attached. For the purpose of analyzing space use, the box was divided into the hide box area, the center area, and the odour area. Locomotor activity was measured by analyzing movement from one area to another, including the areas outside of these 3 main areas.

A.


behavioural measures
social contact
space use
activity
space use
activity
space use
activity
hiding/vigilance
stimulus investigation
experimental manipulations
Day1
Day2
J-cloth
Collar
age (PND)
38±1
46±1
70±1
71±1
72±1
73±1
adolescence
adulthood

B.


27 cm
60 cm
24 cm
Odour stimulus
Odour area
24 cm
Center area
78 cm
Entrance
6 cm
Hide box
24 cm
24 cm
78 cm

during the dark (active) phase of the light:dark cycle, in rectangular, clear Plexiglas arenas that measured 35.5 cm high, 27 cm wide, and 60 cm long (Fig. 2.1B). Arena were situated side-by-side on opaque white Plexiglas floors and were covered by clear Plexiglas lids with ventilation holes. They did not contain hide boxes or any other form of environmental enrichment besides the stressor or control stimuli, which were attached to one end wall, using an alligator clip centered 6.5 cm from the top of the arena. Visual barriers were taped to the long sides of each arena, in order to prevent rats from viewing others in neighbouring arenas. Control exposures took place in a room separate from J-cloth and collar exposures, in order to minimize the possibility of odour contamination, but all other aspects of cat odour exposures were maintained for the control group.

J-cloth stimuli were prepared each day an exposure occurred by coating a blue and white striped cloth with fresh hair and dander from 2-4 gonadally intact, female, domestic cats housed communally in the Psychology Department, and cutting the cloth into 2.5 x 15 cm strips. Cats were selected for J-cloth stimulus preparation from a pool of six cats, such that odours from different individual cats were used from one exposure to the next. This was done to create a relatively unpredictable stimulus, compared with the collar, since rats are able to distinguish individual cats based on odour (Staples et al., 2008a). Control stimuli were smaller strips (2.5 x 5 cm) of yellow and white striped cloth. Cat collar stimuli were obtained from Avenue® nylon cat collars that had been worn by one particular intact female cat for two weeks, and these were cut into ~1-inch sections and stored at -20°C until use. In all cases, great care was taken to not contaminate control stimuli.

2.2.2.2. Adolescent Behavioural Quantification

The first (PND38 ± 1) and last (PND46 ± 1) adolescent exposure sessions were videotaped using a Sony 8 mm digital camera (CCD-TRV65 or CCD-TRV108) suspended directly above the arenas, and behaviours expressed during the first 10 min of each session were later quantified for each rat using The Observer 5.0.3.1 software (Noldus, Netherlands). Frequency and duration of the following behaviours were scored:

Rearing (exploratory, non-defensive)—Front legs leave contact with the floor of the arena. They may rest on a wall during rearing.

Grooming Bout (self only; non-defensive)—A series of uninterrupted motions using the mouth and/or paws to clean any part of the body (minimum 1 s duration).

Odour Stimulus Contact (exploratory)—Bodily contact (excluding tail) with the odour source.

Social Contact—Contact with the body of a cagemate (excluding tail). Animals could be in passive contact (ex. huddled together), and this would result in contact being scored for each animal. Animals could also be actively contacting the cagemate with their face or limbs, in which case the animal *receiving* the contact would not be simultaneously scored as contacting the partner.

Space Use—Usage of three virtual regions within the arena: the odour area (exploratory; the third of the arena containing the stimulus), the middle, and the safe area (defensive; the third of the arena furthest from the odour source).

Linecrosses—A crude measure of general activity was taken as the number of virtual linecrosses (entrances of greater than 50% of the body, excluding the tail, into a new region).

2.2.2.3. Adolescent Endocrine Assessment

Plasma cort levels were assayed for three adolescent points in time: i) a physiological baseline measure was taken on the evening prior to the first stimulus exposure (PND37 ± 1), at the circadian nadir of basal cort secretion (2130 h; light period onset), and ii) an experimental sample was taken following the final stimulus exposure (PND46 ± 1; Last Exposure). Additional samples were taken from a second cohort of animals: i) on the evening prior to an acute stimulus exposure (PND37 ± 1) to assess physiological baseline levels, and (ii) immediately following an acute stimulus exposure (PND38 ± 1; First Exposure). All blood samples were taken following transport of animals in the homecage to a room separate from the colony and stimulus exposure rooms, but in close proximity (~ 15 s transport time). To collect a blood sample, a hind leg was shaven, pressure was applied to the saphenous vein, which was pricked with a 21-gauge needle (Becton Dickinson, United States) and subsequently bled into a 600 μl Microtainer plasma separator tube containing lithium heparin (Becton Dickinson, United States). Tubes were kept on ice until all samples for a particular time point were collected, and then all samples were centrifuged at 6000 x g for two minutes at 4 ˚C. Blood plasma was aliquoted into three 60 μl samples, and these were frozen at -80 ˚C until assay. Samples were diluted 1:50 or 1:40 with assay buffer (tris-buffered saline with sodium azide preservative) and assayed using the Correlate-EIA Corticosterone Enzyme Immunoassay Kit (Assay Designs, Michigan, USA). Each 96-well plate was assayed as per the manufacturer's instructions, except that displacement reagent was not added. The addition of displacement reagent was eliminated in order to minimize the

contribution of bound (inactive) cort to the results. Samples were maintained on ice throughout the assay. Intra- and inter-assay coefficients of variation for this kit range between 6.6 - 8.0% and 7.8 - 13.1%, respectively, and the lower limit of detection is 32 pg/mL.

2.2.2.4. Open Field Behavioural Testing in Adulthood

Following adolescent manipulation, rats were raised to adulthood and tested individually in a series of four 12 min open field (OF) sessions administered on consecutive days, beginning on PND70 ± 1. The OF apparatus was constructed from 1/4-inch black Plexiglas, measured 35.5 cm high, 79 cm wide, and 79 cm long, and contained a black Plexiglas hide box (HB; 22 cm high, 24 cm wide, and 21.5 cm long, with a 6 cm x 6 cm door at the front) in the center of one wall during testing sessions (see Fig. 2.1B). An alligator clip was attached ~6.5 cm from the top, in the centre of the wall opposite the hide box, and this was used to secure predator odour stimuli. A clear Plexiglas lid containing ventilation holes covered each apparatus during testing, and the sessions were videotaped from above, using a Sony 8 mm digital camera (CCD-TRV65 or CCD-TRV108), so that behaviours could later be scored using The Observer 5.0.3.1 software.

Animals were tested between 0930h and 1500h, during the dark phase of the light:dark cycle, in the same order on each day. The apparatus was cleaned thoroughly between animals, by washing with unscented laboratory soap, rinsing, and drying with paper towel. The purpose of the first session was to test behavioural responses to the novelty of the OF (OF1), while the second session was used to investigate habituation to the familiar arena 24 hours later (OF2). The final two sessions (OF3-J-cloth and OF4-

collar) were used to test adult endocrine and behavioural responses to cat odour stimuli. The stimuli were obtained exactly as for adolescent manipulations.

Frequencies and durations of the following behaviours were scored for the first 10 min of each OF session:

Rearing (exploratory, non-defensive)—Front legs leave contact with the floor of the arena. They may rest on a wall during rearing.

Grooming Bout (non-defensive)—A series of uninterrupted motions using the mouth and/or paws to clean any part of the body (minimum 1 s duration).

Head-out (defensive)—The head of the rat protrudes from the hide box. A head-out ends when half the body is beyond the opening or when the head retreats into the hide box. The area midway between the forelimbs and hindlimbs is considered to represent the halfway point along the rostro-caudal body axis.

Odour Stimulus Contact (exploratory)—Bodily contact (excluding tail) with the odour source.

Space Use—Usage of ten virtual regions within the open field.

Linecrosses—A measure of general activity was taken as the number of virtual linecrosses (entrances of greater than 50% of the body, excluding the tail, into a new region).

2.2.2.5. Adult Corticosterone Responses to Cat Odour Stimuli

Plasma samples were collected as described previously and cort levels assayed for three timepoints in adulthood: i) a physiological baseline measure was taken the evening following OF2 (2130h), in a room directly adjacent to the colony room (~15 s transport

time), ii) two experimental samples were taken immediately following OF3-J-cloth and OF4-collar sessions, in a room in very close proximity to the testing rooms (~15 s transport time). All adult samples were diluted 1:50 for assay.

2.2.3. Data Manipulation and Statistical Analyses

Adolescent behavioural data were statistically analyzed using repeated measures analysis of variance (ANOVA) for each of the behaviours listed above, with SEX (female, male) and GROUP (ADOL-J-cloth, ADOL-collar, and ADOL-control) as between-subject factors and first and last exposure sessions as the repeated measure. Adult behavioural data were analyzed in the same manner, with the open field sessions (OF1, OF2, OF3-J-cloth, and OF4-collar) as the repeated measure. Because OF1 and OF2 sessions did not involve exposure to cat odour stimuli, whereas OF3-J-cloth and OF4-collar sessions did, separate repeated measures ANOVAs were also conducted for cat odour vs. non-cat odour conditions, to verify whether or not a GROUP effect existed in one situation but not the other. The results for the OF1 and OF2 analysis and the OF-3-J-cloth and OF4-collar analysis are presented in Appendix I.

Adolescent cort data were excluded if the intra-sample coefficient of variation was higher than 20% variance. Baseline data was also excluded if it exceeded 4x the mean value for the appropriate sex. This occurred for three females in the ADOL-J-cloth group (remaining N = 12), one female in the ADOL-control group (remaining N = 15), and two males in the ADOL-J-cloth group (remaining N = 11). Experimental adolescent cort data were also examined for values that exceeded 4x the group mean; however, none did.

Three separate adolescent cort measures were compared among groups using univariate ANOVA, with SEX (female, male) and GROUP (ADOL-J-cloth, ADOL-collar, and ADOL-control) as between-subject factors. The three measures were: 1) adolescent baseline cort, 2) circulating cort following the initial adolescent stimulus exposure (First Exposure), and circulating cort following the final adolescent stimulus exposure (Last Exposure).

Similarly, adult baseline and experimental cort values were excluded if the coefficient of variation indicated higher than 20% variance. Data were also examined for values that exceeded 4x the appropriate group mean. One baseline value was excluded from each of the following groups: female ADOL-control group (remaining N = 6), male ADOL-J-cloth group (remaining N = 5), and male ADOL-control group (remaining N = 8). Adult baseline data were compared among groups using univariate ANOVA, and adult cort responses to OF3-J-cloth and OF4-collar exposures were compared among groups using repeated measures ANOVA, with EXPOSURE (OF3-J-cloth and OF4-collar) as the within-subject factor and SEX (female, male) and GROUP (J-cloth, collar, and control) as between-subject factors.

Heterogeneity of variance for all data was assessed using Levene's test. Post-hoc analyses for main effects of GROUP were conducted using Tukey's test if group variances were not significantly different, or the Games-Howell test if they were.

Additionally, correlation analyses were conducted for each treatment group to examine relationships among adolescent line cross rates and adolescent social contact during the first and last exposure sessions, and adult line crosses, centre area usage, contact with the stimuli, hide box usage, and head-out posturing during the J-cloth and

collar tests, together with adolescent and adult baseline and experimental cort measures. Separate analyses were conducted for rate measures and for duration measures, and Pearson correlation coefficients are reported.

2.3. Results

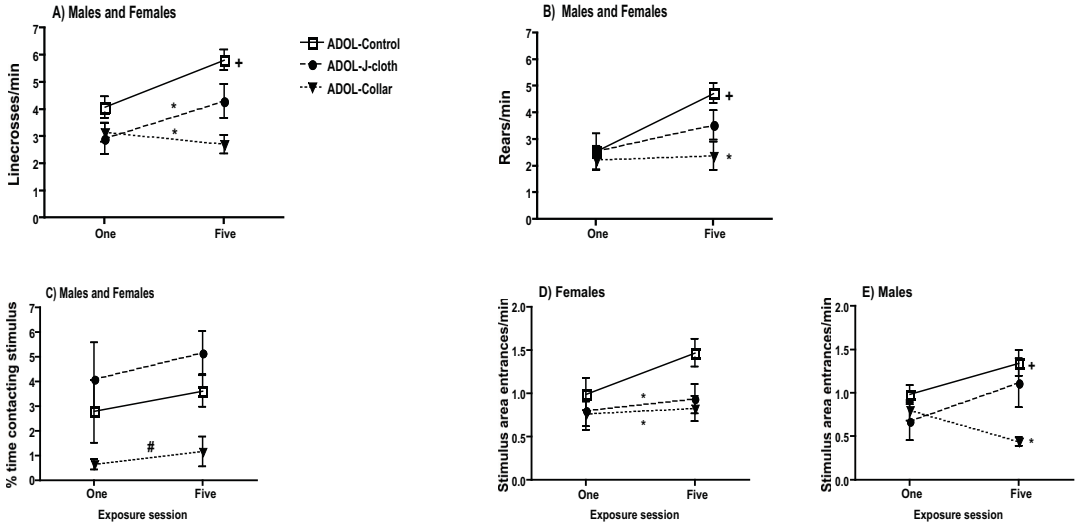
2.3.1. Adolescent Behavioural Responses across Repeated Stimulus Exposures

2.3.1.a. General and Exploratory Activity: The rate of line crossing into the three virtual regions of the exposure arena was used as a general measure of locomotion. There was a main effect of GROUP, $F(2, 31) = 13.56$, $P < 0.001$, and the results of post-hoc testing indicated that both the ADOL-J-cloth group ($P = 0.019$) and the ADOL-collar group ($P < 0.001$) exhibited less movement overall, relative to ADOL-controls (Fig. 2.2A). Only the ADOL-control group significantly increased line cross rates across the exposure period, $F(1, 14) = 7.14$, $P = 0.018$.

An EXPOSURE DAY x GROUP interaction was observed for rates of rearing, $F(2, 31) = 3.70$, $P = 0.036$. Simple effects analyses and post-hoc testing revealed that a GROUP effect was not present during the first exposure ($P = 0.760$) but had emerged by the final exposure ($P = 0.005$), with the ADOL-collar group rearing less frequently than controls ($P = 0.003$), and only the ADOL-control group increased rearing rates from the first to the last exposure ($P = 0.001$; Fig. 2.2B).

A main effect of EXPOSURE DAY for grooming rates revealed that this behaviour increased across the adolescent exposure period, $F(1, 31) = 8.66$, $P = 0.006$ (see Appendix I). There was a GROUP x SEX interaction for duration of time spent

Figure 2.2. Measures of activity and stimulus investigation in male and female adolescent rats during the first (One) and last (Five) exposure session to a control stimulus (ADOL-control), a j-cloth containing cat dander (ADOL-J-cloth), or a cat collar previously worn by a cat (ADOL-collar). In general, responses to the collar stimulus were more pronounced, particularly in males. ***** = different from control group; **+** = different from same group during the first exposure; # = different from control and J-cloth groups. All data are represented as mean ± standard error of the mean.



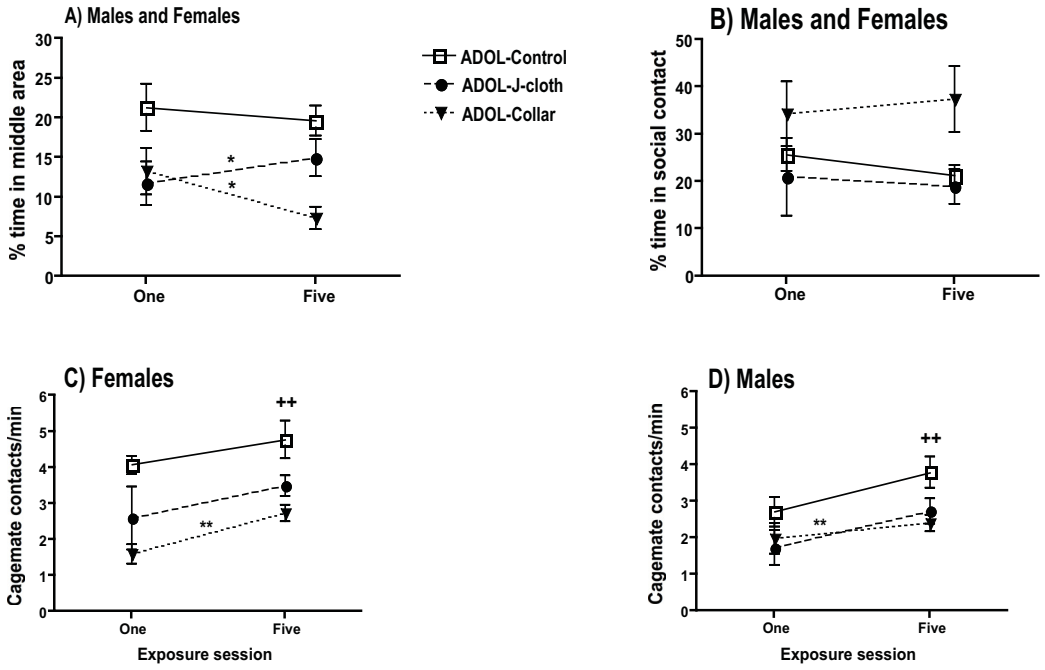
grooming, $F(2, 31) = 3.43$, $P = 0.045$, which was driven by a GROUP effect for males, $F(2, 15) = 4.40$, $P = 0.031$. This effect could not be further resolved by post-hoc testing.

The rate of stimulus investigation increased across the adolescent exposure period, $F(1, 31) = 12.23$, $P = 0.001$. ADOL-collar animals spent a shorter overall duration investigating the stimuli, relative to both the ADOL-J-cloth group ($P = 0.030$) and the ADOL-control group ($P = 0.027$), across adolescent exposure sessions, as revealed by post-hoc testing following a main effect of GROUP, $F(2, 31) = 4.58$, $P = 0.018$ (Fig. 2.2C).

An EXPOSURE DAY x GROUP x SEX interaction was observed for stimulus area entrance rates, $F(2, 31) = 4.70$, $P = 0.016$. Separate analyses for each sex revealed a GROUP effect in the females, $F(2, 16) = 6.27$, $P = 0.010$. Post-hoc tests indicated that both ADOL-J-cloth females ($P = 0.044$) and ADOL-collar females ($P = 0.012$) entered the stimulus area less frequently than ADOL-control females across exposure sessions (Fig. 2.2D). In the males, there was an EXPOSURE DAY x GROUP interaction, $F(2, 15) = 10.14$, $P = 0.002$, with the ADOL-collar males entering less frequently than ADOL-control males during the final exposure day only ($P = 0.001$; Fig. 2.2E). Only ADOL-control males increased stimulus area entrance rates across exposure sessions ($P = 0.003$; Fig. 2.2E).

2.3.1.b. Space Use: There was a main effect of GROUP for durations of time spent in the middle area, $F(2, 37) = 12.25$, $P < 0.001$, with both ADOL-J-cloth ($P = 0.002$) and ADOL-collar ($P < 0.001$) animals spending less time in the middle, relative to ADOL-controls (Fig. 2.3A). An EXPOSURE DAY x GROUP interaction was found for middle

Figure 2.3. Measures of **A-C)** space use and **D-F)** social contact in male and female adolescent rats during the first (One) and last (Five) exposures to a control stimulus (ADOL-control), a j-cloth containing cat dander (ADOL-J-cloth), or a cat collar previously worn by a cat (ADOL-collar). In general, cat odour stimuli reduced time spent in the open area of the test arena and reduced social contact rate. * = different from control group; ** = different from control group, collapsed across both sexes, which are displayed separately; ++ = different from same group during the first exposure, collapsed across both sexes, which are displayed separately. All data are represented as mean ± standard error of the mean.



area entrance rates, $F(2, 31) = 3.45$, $P = 0.044$, with post-hocs showing reduced entrance rates in the ADOL-collar group during the final exposure ($P < 0.001$), relative to ADOL-controls.

There was a main effect of SEX for rates of entrance into the safe area, $F(1, 31) = 6.28$, $P = 0.018$, with females entering more frequently than males, as well as an EXPOSURE DAY x GROUP interaction, $F(2, 31) = 4.42$, $P = 0.020$. Further exploration, using simple effects analyses followed by post-hoc testing, revealed a GROUP effect specific to the final exposure: the ADOL-collar group entered the safe area less frequently than both the ADOL-J-cloth group ($P = 0.047$) and the ADOL-control group ($P < 0.001$). Only the ADOL-control group increased safe area entrance rates across exposure sessions ($P = 0.007$).

2.3.1.c. Social Behaviour: A trend toward a GROUP effect did not reach statistical significance for durations spent contacting the cagemate(s), $F(2, 31) = 2.72$, $P = 0.082$ (Fig. 2.3B); however, there was a GROUP effect for rates of cagemate contact, $F(2, 31) = 11.09$, $P < 0.001$, whereby the ADOL-collar group contacted cagemates less frequently than the ADOL-control group ($P < 0.001$; Fig. 2.3C-D). The overall rate of cagemate contact increased across the adolescent exposure period, $F(1, 31) = 17.64$, $P < 0.001$, and females contacted cagemates more frequently than males, $F(1, 31) = 6.04$, $P = 0.020$.

2.3.2. Adolescent Corticosterone Responses Following an Acute Stimulus Exposure

Baseline levels of circulating cort were generally very low in the adolescents. No group or sex differences were observed in baseline cort levels at this time point. In

samples taken following an acute adolescent stimulus exposure, however, there was a main effect of SEX, $F(1, 60) = 11.91$, $P = 0.001$, with adolescent males showing higher circulating cort levels, relative to adolescent females (Fig. 2.4A-B).

2.3.3. Adolescent Corticosterone Responses Following Repeated Stimulus Exposures

In samples taken following the final adolescent stimulus exposure, females showed higher levels of cort relative to males ($F(1, 40) = 10.94$, $P = 0.002$). There was also a GROUP effect ($F(2, 40) = 4.20$, $P = 0.023$), whereby ADOL-J-cloth animals had higher levels of circulating cort, relative to ADOL-control animals ($P = 0.010$).

2.3.4. Adult Behaviours during Open Field Testing

2.3.4.a. General and Exploratory Activity: There was a main effect of DAY for rates of line crossing, $F(3, 26) = 14.22$, $P < 0.001$ (Fig. 2.5A and Appendix I).

There was a SEX x DAY interaction for durations spent grooming, $F(3, 26) = 4.19$, $P = 0.015$. Separate analyses for each sex revealed that females increased durations spent grooming on Day 2, relative to Day 1 ($P < 0.043$), but then decreased time spent grooming during the J-cloth and collar open field sessions, relative to Day 2 (P's $\leq$ 0.004); this pattern was not evident in males. There were also main effects of DAY for grooming durations, $F(3, 26) = 3.16$, $P = 0.041$, grooming rates, $F(3, 26) = 8.75$, $P < 0.001$, and rearing rates, $F(3, 26) = 7.16$, $P = 0.001$ (see Appendix I).

2.3.4.b. Space Use: Adolescent stressor exposure impacted adult rates of centre area entrance across all test days, $F(2, 28) = 5.62$, $P = 0.009$, with ADOL-collar animals

Figure 2.4. Levels of corticosterone measured in plasma of male and female adolescent rats exposed to either a control stimulus, a J-cloth containing cat dander, or a cat collar previously worn by a cat (depicted in the legend). Measurements were taken during the circadian nadir of the light/dark cycle (Baseline), following the first exposure (Acute), and following five exposures (Repeated). ** = different from control group, collapsed across both sexes, which are displayed separately. All data are expressed as mean ± standard error of the mean.

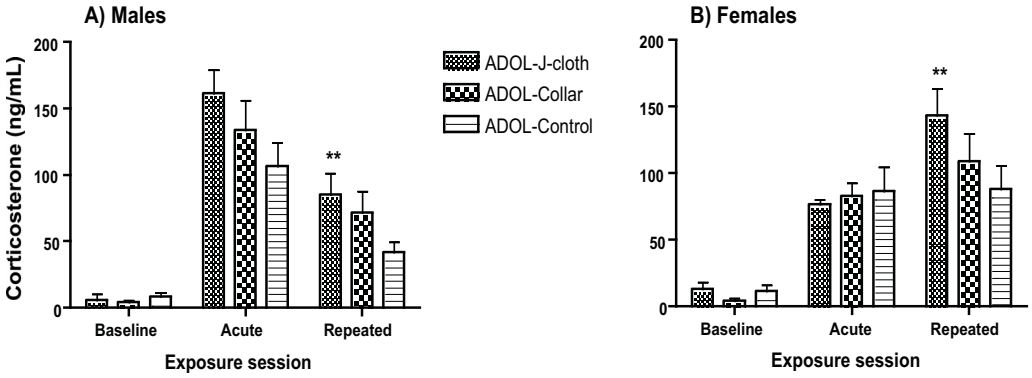
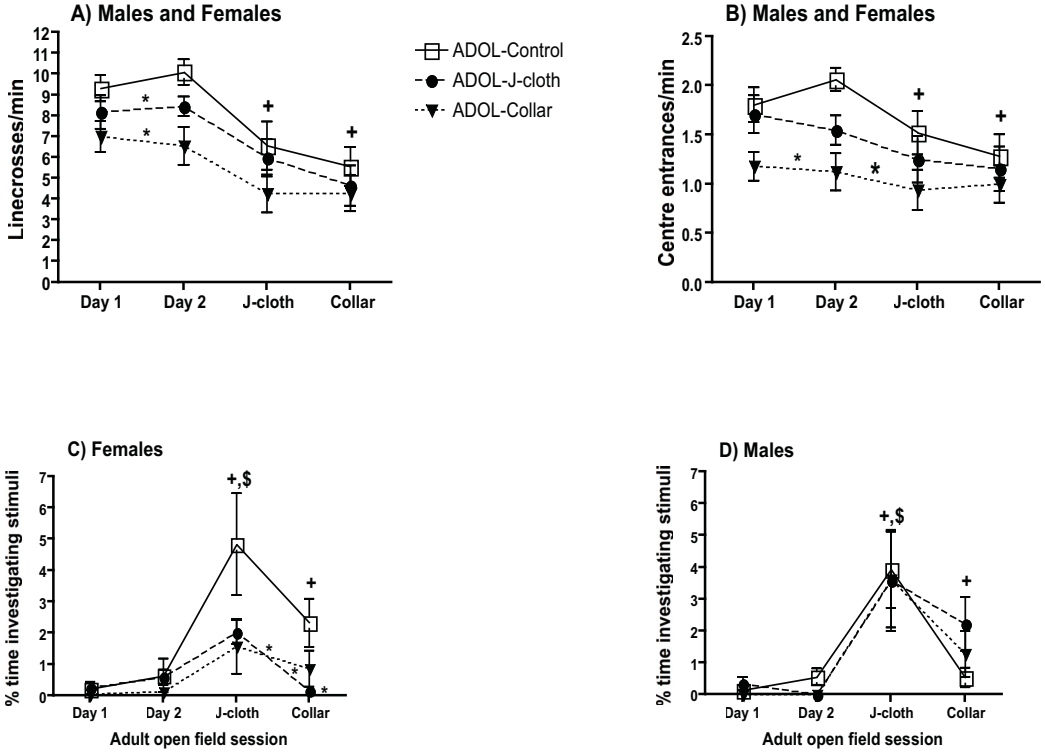


Figure 2.5. Measures of **A)** activity, **B)** anxiety-like behaviour, and **C-D)** stimulus investigation in adult male and female rats that had been exposed to either a control stimulus (ADOL-control), a j-cloth containing cat dander (ADOL-J-cloth), or a cat collar previously worn by a cat (ADOL-collar), as adolescents. For group effects, a large asterisk on the centre of a line indicates that the effect is collapsed across all sessions. A smaller asterisk on a line between Day 1 and Day 2, or between the J-cloth test session and the collar test session, indicates that the effect was derived from an analysis of variance specific to either the open field test days or the cat odour test days, respectively. Symbols directly above session points apply to all groups, while the symbol to the side is group-specific for open field day four (collar exposure). ***** = different from control group; **+** = different from open field days one and two; **$** = different from open field day four (collar exposure). All data are represented as mean ± standard error of the mean.



entering the centre less frequently, relative to ADOL-control animals ($P = 0.038$; Fig. 2.5B). There was a main effect of DAY, $F(3, 26) = 4.91$, $P = 0.008$, and simple effects analyses revealed that the centre was entered more on Day 1 and Day 2, relative to both the J-cloth and collar open field sessions (P's ≤ 0.015). For durations of time spent in the centre, there was a SEX x DAY interaction, $F(3, 26) = 3.29$, $P = 0.037$, and simple effects analyses revealed that this was driven by an effect of DAY in females, $F(3, 13) = 5.43$, $P = 0.012$, which could not be further resolved, although durations females spent in the centre during the collar open field session approached criterion for being significantly less than all other open field days (P's ≤ 0.078). There was also a main effect of DAY, $F(3, 26) = 6.16$, $P = 0.003$, with durations spent in the centre on Day 1 greater than on all other days (P's ≤ 0.033).

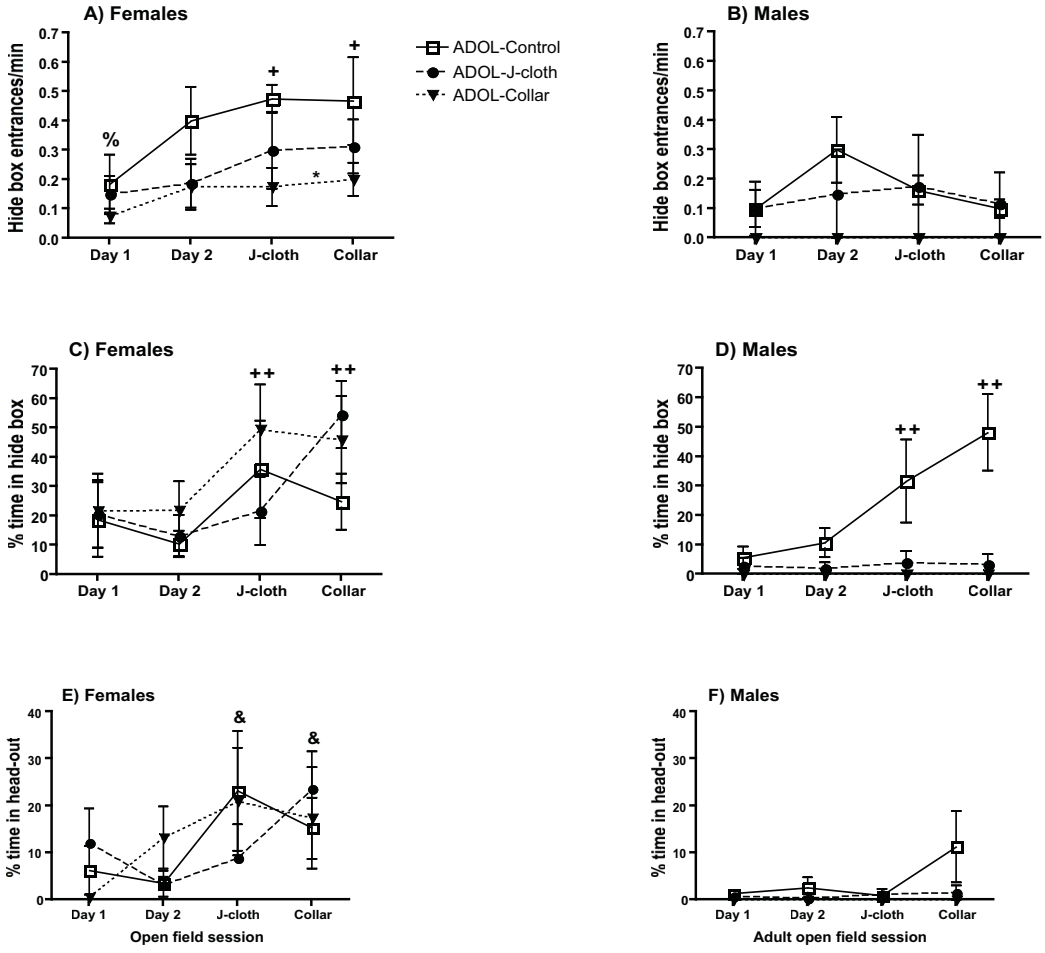
There were main effects of DAY for rates of entrance into the stimulus area, $F(3, 26) = 9.91$, $P < 0.001$, and durations of time spent there, $F(3, 26) = 4.44$, $P = 0.012$ (see Appendix I). The stimulus area was entered less frequently on predator odour exposure days, relative to Days 1 and 2 and less during the collar exposure, relative to the J-cloth exposure (P's ≤ 0.019). Interestingly, animals spent overall longer durations investigating the J-cloth stimulus, relative to the empty alligator clip on Days 1 and 2 (P's ≤ 0.013).

Time spent investigating the stimulus (and/or the alligator clip to which the stimuli were attached) was influenced by sex and adolescent treatment, as indicated by a DAY x SEX x GROUP interaction, $F(6, 50) = 2.87$, $P = 0.017$ (Fig. 2.5C-D). Simple effects analyses revealed that the interaction was driven by the behaviour of the ADOL-J-cloth females, who investigated the collar stimulus for less time than ADOL-control

females ($P = 0.022$) during the collar open field session. The alligator clip was investigated for less time during Day 1 and Day 2, relative to the cat odour stimuli during each of the J-cloth and collar open field sessions, and the J-cloth stimuli were consistently investigated for longer during the J-cloth open field session than the collar stimuli during the collar session, in both females (P's ≤ 0.043) and males (P's ≤ 0.009). In addition, there were main effects of DAY for rates of stimulus investigation, $F(3, 26) = 12.13$, $P < 0.001$, as well as for time spent investigating the stimuli, $F(3, 26) = 12.39$, $P < 0.001$ (see Appendix I).

2.3.4.c. Defensive Behaviours: There was a GROUP effect for rates of entering the hide box, $F(2, 28) = 4.72$, $P = 0.017$; however, the source could not be identified definitively by post-hoc testing, due to the complex patterns of heterogeneity of variance among groups and sexes. There was also a DAY x SEX interaction, $F(3, 26) = 3.19$, $P = 0.040$, and a main effect of DAY for entrance rates, $F(3, 26) = 3.02$, $P = 0.048$, as well as a main effect of DAY for time spent in the hide box, $F(3, 26) = 6.13$, $P = 0.003$. Separate analyses for each sex revealed that females increased their hide box entrance rates in the presence of the cat odour stimuli during the J-cloth and collar open field sessions, relative to Day 1 (P's ≤ 0.027; Fig. 2.6A); males did not show this pattern (Fig. 2.6B). Females entered the hide box more than males overall, $F(1, 28) = 6.80$, $P = 0.014$ (Fig. 2.6A-B), and spent more time overall within the hide box, relative to males, $F(1, 28) = 5.07$, $P = 0.032$ (Fig. 2.6C-D). For overall durations spent within the hide box, more time was spent in the safety of the hide box during each of the J-cloth and collar open field sessions, relative to both Day 1 and Day 2 (P's ≤ 0.013; Fig. 2.6C-D).

Figure 2.6. Measures of **A-F)** defensive behaviour in adult male and female rats that had been exposed to either a control stimulus (ADOL-control), a j-cloth containing cat dander (ADOL-J-cloth), or a cat collar previously worn by a cat (ADOL-collar), as adolescents, in response to a novel open field (Day 1), a familiar open field (Day 2), and two stressor stimuli (J-cloth – cloth coated with cat fur and dander; Collar – cat collar previously worn by a cat). The asterisk on the line between the J-cloth test session and the collar test session indicates a group effect that was derived from an analysis of variance specific to those test sessions. Symbols directly above session points apply to all groups. ***** = different from control group; **%** = different from open field days three (J-cloth exposure) and four (collar exposure); **+** = different from open field days one and two; **++** = different from open field days one and two, collapsed across both sexes, which are displayed separately; **&** = different from open field day two. All data are represented as mean ± standard error of the mean.



There was a DAY x SEX interaction for time spent in a head-out position, $F(3, 26) = 3.57$, $P = 0.028$, as well as a main effect of SEX, $F(1, 28) = 8.06$, $P = 0.008$, with females spending longer in the position, relative to males. Separate analyses for each sex revealed that females increased time spent in the head-out posture during the J-cloth and collar open field sessions, relative to Day 2 (P's ≤ 0.019); this pattern was not evident in males (Fig. 2.6E-F). There was a main effect of DAY, $F(3, 26) = 4.23$, $P = 0.015$, which reflected the pattern observed in females (see Appendix I). Females also showed increased rates of head-out display, compared with males, $F(1, 28) = 8.17$, $P = 0.008$. A GROUP effect for head-out rates, $F(2, 28) = 3.56$, $P = 0.042$, could not be further resolved in post-hoc testing.

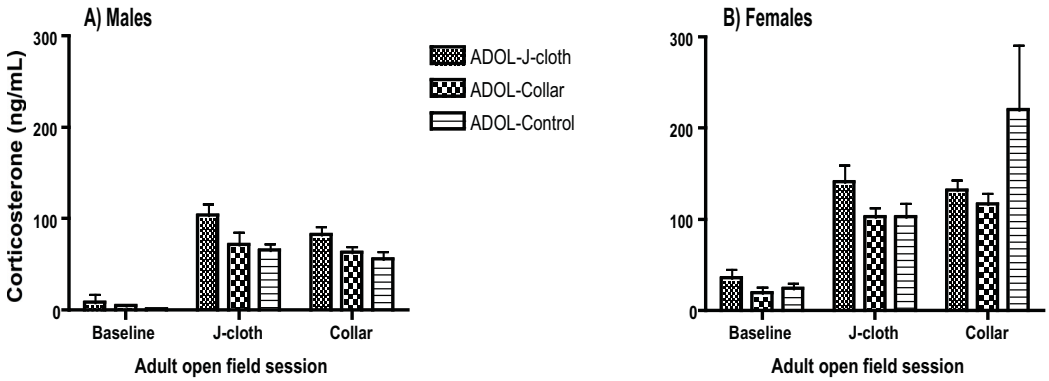
2.3.5. Corticosterone Responses to Cat Odour Stimuli in Adulthood

As depicted in Figure 2.7A-B, adult baseline levels of cort were low, with significantly higher levels in females relative to males, $F(1, 31) = 11.26$, $P = 0.002$. There was also a main effect of sex for the experimental samples, $F(1, 30) = 17.46$, $P < 0.001$, whereby females secreted higher levels of cort, relative to males.

2.3.6. Correlation Analyses

A number of factors were significantly correlated with one another. Many of these were expected (e.g. a behaviour exhibited during the J-cloth test would be positively correlated with the same behaviour exhibited during the collar test); however, the complete set of correlations can be found in Appendix II. A subset of correlations

Figure 2.7. Levels of corticosterone measured in plasma of male and female adult rats exposed repeatedly during adolescence to either a control stimulus, a J-cloth containing cat dander, or a cat collar previously worn by a cat (depicted in the legend). Measurements were taken during the circadian nadir of the light/dark cycle (Baseline), following exposure to a J-cloth stimulus in the open field (J-cloth), and following exposure to a collar stimulus in the open field (Collar). All data are expressed as mean ± standard error of the mean.



deemed to be relevant to other results obtained in the present study is presented in Table 1.

2.4. Discussion

Very few studies have used cat odour as a naturalistic stressor model in adolescent rats; thus, the present study was conducted to investigate the use of this model for examining acute and long-term effects of adolescent stress. We chose to do this by employing two similar but distinct cat odour stimuli, both of which have been used successfully to induce neural, endocrine, and/or behavioural stress responses in adult rats (Dielenberg et al., 2001; Dielenberg & McGregor, 2001; Mashoodh et al., 2008; McGregor et al., 2002; Staples et al., 2008a). We expected that the J-cloth stimulus would be perceived as more dangerous, but our results are not entirely consistent with that.

2.4.1. The Cat Collar Stimulus Induced a More Pronounced Inhibitory Response in Adolescent Rats across Repeated Exposure Sessions, but Only the J-cloth Stimulus Induced a Corticosterone Response Following the Final Exposure

Control animals demonstrated inhibited activity during the initial exposure session, a typical response when rodents are introduced to a novel environmental context. After repeated exposure sessions, however, control rats habituated to the arena, as indicated by increased movement (line crosses, rearing and cagemate contact rates). Initial inhibitory responses exhibited during adolescent J-cloth or collar exposures are

Table 2.1. Significant correlations among a variety of measures from adolescence (ADOL) and adulthood (ADUL) in male and female rats exposed repeatedly (5 exposures) during adolescence to a control stimulus (ADOL-cont), a J-cloth stimulus (ADOL-J-cloth; cloth covered with hair/dander from a cat), or a collar stimulus (ADOL-collar; collar previously worn by a cat) and tested in adulthood in a novel open field (Day 1), a familiar open field (Day 2), in response to the J-cloth stimulus (J-cloth), and in response to the collar stimulus (Collar). See Figure 2.1 for a timeline. Abbreviations: ADOLBAS – baseline corticosterone (cort) levels measured on the night prior to the first adolescent exposure (~PND37); SOCCON – social contact exhibited during the fifth adolescent exposure (~PND46); ADOLEXPT – cort levels measured immediately following the fifth adolescent exposure; ADULBAS – baseline cort levels measured on the night prior to J-cloth exposure in adulthood; ADULJCloth - cort levels measured after exposure to the J-cloth stimulus in adulthood; ADULCollar - cort levels measured after exposure to the collar stimulus in adulthood; D – duration; R – rate.

	Factor 1	**Factor 2**	**Correlation coefficient (*p*-value)**
ADOL-control			
	ADOLBAS	SOCCON (R)	0.706 (0.002)
	ADOLBAS	SOCCON (D)	0.730 (0.001)
	ADOLEXPT	SOCCON (R)	0.538 (0.032)
	ADOLEXPT	ADULBAS	0.729 (0.001)
ADOL-collar			
	SOCCON (D)	ADULCollar	-0.579 (0.038)
	ADULJCloth	ADULCollar	0.740 (0.004)

therefore assumed to be due to both the novelty of the arena and to the presence of the stressor.

With repeated sessions, animals exposed to either stressor stimulus responded by showing inhibited activity and reduced centre time, relative to controls. By the final exposure session, collar-exposed animals also reared less frequently and males entered the stimulus area less often, indicating a more pronounced wariness and perception of threat, following habituation to the exposure arena itself. These findings suggest that rats perceive a threat in the presence of either stressor stimulus, but the collar is the most effective for inducing inhibition of activity in the adolescents. Collar-exposed animals also investigated the collar stimuli for less time overall during exposures, compared with both the J-cloth and control stimuli, indicating a stronger and more persistent avoidance of this stressor stimulus.

As mentioned above, animals exposed to either stressor stimulus avoided spending time in the middle of the arena. Thigmotaxic behaviour (preference for close proximity to a wall) is associated with anxiety in rodents (Mashoodh, Sinal, & Perrot-Sinal, 2009; McGrath, Campbell, Veldman, & Burton, 1999; Simon, Dupuis, & Costentin, 1994; Treit & Fundytus, 1988; Wagner, Postal, Darrah, Chen, & Khan, 2007), while time spent in an exposed area, away from walls or shelter, is indicative of bravery. The reluctance of both adolescent stressed groups to occupy open space during the exposures suggests that each stimulus generates an anxiogenic response.

Adolescent behavioural responses to J-cloth exposures were typically intermediary between collar and control exposure responses, suggesting that the J-cloth stimulus is less potent or that it is interpreted to signify a less imminent form of predation

threat. For example, the fur and dander present on the cloth stimulus might serve as an indication to a rodent that a predator has passed through an area (and could potentially return). Interestingly, adolescents appeared to be just as interested in exploring the J-cloth stimulus as the control stimulus. In adulthood, too, both males and females spent more time investigating the stimulus during the J-cloth open field test, relative to the collar and the empty alligator clip on open field days 1 and 2. It is unknown why rats are willing to investigate a stimulus containing fresh cat hair and dander but will not approach a piece of collar previously worn by a cat; one might speculate that the collar, after being worn close to sebaceous glands around the neck, becomes infused with a more potent odour than the hair-coated cloth, much in the same way a person's clothing will become redolent of their body odours after a few days' wear, but pieces of hair and skin collected freshly may not have as strong a scent. It may also be the case that the J-cloth necessitates investigation because it is comprised of odour molecules from more than one cat. A future experiment should attempt to sort out effects of odour source vs. cat identity by using one cat for both the J-cloth and collar stimuli.

Interestingly, only the J-cloth stimulus enhanced cort secretion during the last (but not the first) adolescent exposure, relative to the control group. This was similar to the findings in a recent study that used a social defeat model across five days beginning on PND35: there were no differences in circulating cort levels between control and defeated rats on the first day; however, by the fifth day, cort levels were higher in the defeated group (Watt et al., 2009).

Overall, then, the collar is deemed to be the most effective of the two odour stimuli at eliciting inhibitory and avoidance responses in adolescent rats of either sex, but only the J-cloth stimulus induced a cort response in this study.

2.4.2. Adolescent Social Behaviour in Cat Collar-exposed Animals may Buffer the Corticosterone Response to a Homotypic Stressor Challenge Encountered in Adulthood

Social behaviour increases during adolescence (Pellis & Pellis, 1990) and is regulated in part by the prefrontal cortex (Pellis, Pellis, & Whishaw, 1992), a region that undergoes intense developmental modification at this time (Spear, 2000). Gonadal steroid hormones modulate some of these developmental processes (Primus & Kellogg, 1989), but others appear to be independent of hormones (Andersen et al., 2002) and can be altered by adolescent manipulation (Wright, Hebert, & Perrot-Sinal, 2008). It is frequently suggested that the rise in social contact observed during adolescence assists the individual in transitioning from the natal territory toward independence and reproductive maturity (Spear, 2004). A number of negative effects have been described following adolescent social isolation, with consequences related to the development of an effective and efficient adult stress response system (Hall, 1998; Yee, Cavigelli, Delgado, & McClintock, 2008). However, it is unclear whether or not individual differences in social behaviour during adolescence relate directly to ongoing developmental processes, and in particular, development of the stress response. As a first step to understanding the potential importance of social behaviour during adolescence for development of stress responding, we quantified the amount of social contact that occurred during adolescent

exposures and examined how it related to exposure responses, as well as to long-term outcomes.

We first examined significant correlations in control animals as a benchmark to compare those that were disrupted in adolescent stressor-exposed animals. In the control animals, baseline cort was strongly positively associated with both rates and durations of social contact on the last day of exposure, and social contact rates during this exposure were also positively correlated with cort levels circulating after the exposure. Cort levels following the final adolescent exposure were further positively related to adult baseline cort levels. In humans, higher basal cortisol levels have been associated with better memory formation for emotional cues (Preuss, Schoofs, & Wolf, 2009), suggesting some benefit for higher basal levels under stressful conditions. Although correlational, our results suggest an important connection between adolescent social behaviour and circulating levels of stress hormones in adolescence and into adulthood, with higher levels of circulating cort found in those who engage in more contact. Future studies will be designed to examine causal relationships – but our present results represent an important contribution, as they demonstrate a connection between cort levels and social behaviour exhibited during adolescence and suggest an involvement of this connection in ongoing development of the stress response system.

In contrast to the relationships observed in control animals, adolescent social behaviour exhibited by collar-exposed animals, who showed potent and persistent behavioural responses during adolescence, was associated with a *reduction* in cort output following exposure to a homotypic stressor in adulthood, as indicated by a significant negative correlation. This is consistent with work in male hamsters in which social

subjugation during adolescence is associated with blunted cortisol levels in response to an agonistic encounter in adulthood (Ferris et al., 2005). Collar-exposed animals spent over a third of their exposure time in close social contact and showed a cautious behavioural profile during the adult open field tests. Specifically, collar-exposed animals showed increased inhibition in the open field, as well as a sustained avoidance of the centre of the arena, and for females, of the stressor stimuli during the J-cloth and collar tests. Therefore, collar-exposed animals showed increased behavioural indices of anxiety in adulthood. However, in the collar-exposed group exclusively, time spent in physical contact with a cagemate during the final adolescent exposure was negatively related to cort output in response to the collar test in adulthood. Altogether, these findings suggest that adolescent collar exposure increased the propensity toward anxiety in adulthood, although greater use of social support during the exposures may prepare the individual to cope behaviourally with a homotypic threat cue encountered in adulthood without requiring secretion of high levels of cort. Future studies should explore differences in stressor-induced neural activation of stress responsive brain regions that are also involved in regulating social behaviours, such as prefrontal cortex, between animals that do and do not use social support during adolescent exposure to a cat odour stressor.

2.4.3. Repeated Stressor Exposure During Adolescence Increased Adult Inhibition in the Novel Open Field and Wariness of Predator Odour Cues

Adult rats showed anxiogenic responses during the J-cloth test and the collar test. Regardless of adolescent treatment condition, rats entered the centre less and spent more time hiding on these test days, relative to days 1 and 2 in the open field. This demonstrates that the stressors were effective as administered in the open field context.

Interestingly, however, a number of correlations among behaviour and cort in adulthood were disrupted if animals had been exposed to either stressor during adolescence.

For example, in the collar-exposed group only, animals that showed bravery during the J-cloth test (increased time in the centre or investigating the stimulus) were more vigilant during the collar test, as indicated by time spent in a head-out position. Additionally, head-out rates and cort output were positively correlated during the collar test in adolescent control-exposed animals only. Additionally, groups exposed to either of the adolescent stressor treatments showed differences in behaviour during the open field tests, relative to the control group. They crossed fewer lines on the first two days of open field testing and spent less time investigating the stressor stimuli during the J-cloth and collar tests, demonstrating an increased inhibitory response to the novelty of the open field, as well as increased wariness of cues of predation threat, regardless of whether they were homotypic or heterotypic with what had been encountered across the stressor exposure period during adolescence. Taken together, there is evidence for long-term changes in stress responding and anxiety in animals stressed during adolescence, and evidence that for some adult measures, the specific nature of the adolescent stressor manipulation may not matter.

These behavioural differences between adolescent stressed and control animals were not accompanied by long-term alterations in adult cort output in response to the open field stress tests. In a recent study using a social defeat paradigm across five days beginning on PND35, previously defeated male rats showed conditioned risk-assessment and inhibitory responses to the defeat context as young adults, as well as decreased

dopamine in medial prefrontal cortex, but not increased circulating cort (Watt et al., 2009).

2.4.4. Sex Differences

One of the goals of the present study was to systematically investigate sex differences in all measures. Other work, using different models of adolescent stress, has resulted in differential findings for males and females. For example, when adolescent rats are isolation-housed or repeatedly exposed to a combined isolation/social stress paradigm, only females showed increased depressive behaviours in response to a heterotypic swim stress test (Leussis, Lawson, Stone, & Andersen, 2008; Mathews et al., 2008b). In the present study, females showed some evidence of increased anxiety, relative to males, although there were no profound sex differences. Adolescent control-exposed females spent more time in the safe area and also entered it more frequently than males during the first exposure session, and they contacted cagemates more frequently than males. In adulthood, females entered the hide box more frequently and spent more time within it, relative to males. Females also showed increased head-out rates and spent longer in the position, relative to males.

In terms of physiological sex differences, adolescent males showed higher cort responses to an acute predator stimulus exposure administered on PND38, which is very interesting, given that higher cort levels are consistently observed in females during other age periods (Atkinson & Waddell, 1997; Beiko, Lander, Hampson, Boon, & Cain, 2004; Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963; Mashoodh et al., 2008). McCormick *et al*. (2006) have similarly found higher cort levels in males, relative to

females, on PND30 after an acute social isolation stressor and pairing with a new cage mate (McCormick, Merrick, Secen, & Helmreich, 2007). It appears that the adult sex difference emerges during this critical time, as higher levels of circulating cort were observed in females following the final repeated adolescent stimulus exposure administered about a week later, and this persisted in adult baseline and experimental samples.

2.5. Summary and Research Direction

Although exposure to either stressor stimulus induced long-term alterations in adult behaviours, the collar stimulus induced both a more potent anxiogenic response in the adolescents and a wider profile of long-term changes. The adolescent collar-exposed animals exhibited a slightly greater generalized increase in anxiety across all adult test situations, as they entered the centre less across all test days. However, the J-cloth stimulus was also effective in eliciting anxiogenic behaviours in adolescent rats and in inducing long-term increases in cautious behaviour in females. Also, a cort response was mounted against only the J-cloth stimulus, relative to the control stimulus, following repeated administration in adolescence. Future studies should further examine relationships among adolescent social behaviour, responses to cat odour stressors, and long-term alterations in adult defensive behaviours and medial prefrontal cortical function.

It was initially hypothesized that the J-cloth stimulus would be more potent than the collar stimulus, in terms of inducing stress responses in rats. Our results did not support this hypothesis but do demonstrate important differences in the effects produced

by each stressor stimulus. While the J-cloth stimulus induced more subtle effects on defensive behaviours, its effects were more widespread in that it induced both behavioural and physiological responses. The collar stimulus, on the other hand, induced more robust behavioural responses across the exposure period and increased adult defensive behaviours more profoundly than the J-cloth stimulus. The reason(s) why rats showed different responses to the two sources of cat odour used in this study are still elusive; however, our characterization of these differences allows a conceptualization of when one cat odour source may be more useful than others when investigating stress responding in a rodent model.

In Chapter 3, responses to repeated administration of cat odour stimuli are compared between adolescent rats and young adult rats that were housed for ~ three weeks following exposures and then tested for long-term effects. The collar stimulus was used for this study, which is deemed most appropriate, since a major study objective was to examine the specificity of the adolescent period for stressor induction of long-term effects on defensive behaviours in adulthood. In Chapter 4, the examination of long-term effects of repeated adolescent stressor exposure is extended to include effects on levels of dopamine receptors in prefrontal cortical brain regions of adult rats. In this case, the J-cloth stimulus was used and was found to be effective in eliciting long-term changes in adult neural protein levels, as well as defensive behaviours.

CHAPTER 3

ENHANCED STRESS RESPONSES IN ADOLESCENT VERSUS ADULT RATS EXPOSED TO CUES OF PREDATION THREAT AND PEER INTERACTION AS A PREDICTOR OF ADULT DEFENSIVENESS

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Abstract

Development of the hypothalamic-pituitary-adrenal (HPA) axis is influenced by external factors during early life in mammals in a fashion that optimizes adult function for predicted conditions. We have hypothesized that adolescence represents a sensitive period for the development of some aspects of adult stress response regulation. This was based on prior work showing that repeated exposure of rats to a stressor across part of the adolescent period increases fearfulness in a novel environment in adulthood. Here, we further our investigation of both acute and long-term effects of repeated adolescent stressor exposure on physiological (i.e. corticosterone) and behavioural (i.e. defensive behaviour) measures of stress responding in male and female rats. Furthermore, we compared outcomes with those following identical manipulations administered in early adulthood and found that animals exposed to cues of predation threat during adolescence showed the most robust defensive responses to a homotypic stressor encountered in adulthood. Peer interaction during control manipulation in adolescence was identified as an important individual characteristic mediating development of adult defensive strategies.

3.1. Introduction

The ability to respond effectively to environmental stressors is an important aspect of fitness, and the HPA axis is the major regulator of mammalian stress responding. Its activation, beginning at stress-responsive neurons in the PVN, eventually leads to the release of glucocorticoids from the adrenal glands into the circulation. There is a high degree of individual variability in the adult stress response (B. McEwen & Lasley, 2003; B. S. McEwen & Stellar, 1993; Sapolsky, 2000), which is partially attributable to developmental programming during critical periods. It has become accepted that HPA responsiveness to stressors is shaped by events that occur during early development, making the early environment one source of individual variability in adult stress responses in humans (De Bellis & Thomas, 2003; Nemeroff & Vale, 2005), non-human primates (Coplan, Andrews, Rosenblum, Owens, Friedman, Gorman, & Nemeroff, 1996), and rodents (Francis et al., 1999; Meaney & Szyf, 2005).

Mammalian brain development continues throughout adolescence, a phase about which we know relatively little, in terms of stress response programming, compared with early childhood. Presently, most animal models are focused on the role of the neonatal environment in stress response programming, neglecting later developmental periods that are important for vulnerability to disease (Adriani & Laviola, 2004) and for the continued development of the PFC (Andersen & Teicher, 2000; Andersen et al., 2000; Giedd, 2004), a brain region involved in adult stress response regulation, decision-making, social interaction, and emotional processing (Cerqueira, Almeida, & Sousa, 2008; Floresco & Magyar, 2006; Herman et al., 2005; Pellis et al., 1992; Spencer, Buller, & Day, 2005; Sullivan, 2004; Sullivan & Gratton, 2002). We, and others, have put forth the hypothesis

that adolescence represents a 'sensitive period' for stress response programming and have developed animal models to test that hypothesis (McCormick & Mathews, 2007; McCormick et al., 2005). While the developmental phase representing adolescence is by nature loosely defined, it is generally accepted to fall within the period between 28 and 48 days of age in the rat (Spear, 2000). One of the objectives of the present study was to directly assess the specificity of the adolescent period for mediating long-term effects on adult stress responding. We hypothesize that some long-term effects of repeated stressor exposure on adult stress responding are specific to having been exposed during the adolescent period.

The model we have developed in rats is based upon adolescence being recognized as a stressful, transitional period, during which individuals are establishing their independence (Spear, 2000). Gaining independence is thought to be facilitated by increased novelty seeking and peer-directed social behaviours (Douglas, Varlinskaya, & Spear, 2003, 2004; Spear, 2000, 2004; Varlinskaya & Spear, 2008). Indeed, social behaviour is one of the major features most often used to describe the adolescent period (Spear, 2000). In rats, play behaviour is much more notable amongst adolescents (Pellis et al., 1992; Siviy et al., 2006), relative to their adult counterparts, and social deprivation has been shown to alter prefrontal brain development and adult social interaction (Ferdman, Murmu, Bock, Braun, & Leshem, 2007; Hall, 1998; Leussis et al., 2008; Varlinskaya & Spear, 2008). However, increased social contact is also cited as a major source of stress for adolescents, and administering a social stressor during adolescence in rats increases social anxiety in adulthood (Vidal, Bie, Granneman, Wallinga, Koolhaas, & Buwalda, 2007). These findings imply an important connection between social contact

and adolescent development of the stress response system. Thus, a second objective of the present study was to examine relationships among social contact levels, stress-induced cort elevations, and defensive behaviour in individual subjects. While we don't make specific predictions as to how these factors are related, we do hypothesize that significant associations will be observed, and these associations may provide clues as to the relevance of social contact for optimum adolescent development.

HPA axis responses to stressors are known to be different during adolescence, relative to adulthood. During early adolescence, cort elevations in response to an acute stressor are prolonged, and after repeated exposure they show higher peaks but a more rapid decline to baseline, before taking on the adult profile in late adolescence/early adulthood (McCormick & Mathews, 2007; Romeo et al., 2006a; Romeo et al., 2006b; Romeo, Lee, Chhua, McPherson, & McEwen, 2004). Defensive behaviours in response to predation threat also change as juvenile rats age, with responses of younger animals characterized mainly by avoidance of the stressor stimulus and active defense strategies emerging later (Hubbard et al., 2004). Changes in cort release between adolescents and adults have been connected to maturation of the hippocampus, notably GR levels, which peak at puberty, concurrent with the emergence of negative feedback inhibition of the HPA axis (McCormick & Mathews, 2007; Meaney et al., 1985; Sapolsky et al., 1985).

Thus, the HPA axis is in flux for much of early to mid-adolescence, possibly due, in part, to the lack of full development of brain regions that modulate stress responding, including the hippocampus and the PFC (Sullivan, 2004; Sullivan & Gratton, 2002). The extent to which these changes also underlie the development of the adult behavioural defense repertoire is unknown. As a step toward better characterizing adolescent versus

adult stress responses to repeated cat odour exposure, a third objective of the present study was to directly compare these responses, in terms of behaviours displayed during stressor exposure and cort levels following exposure. Because adolescents often display risky behaviour and because the full adult defense repertoire is not yet established during the adolescent phase of development, we hypothesize that adolescent animals will show less defensive behaviour than adults in response to cues of predation threat. However, because adolescent rats are known to show higher spikes in hormone levels than adults directly following a final half-hour session of repeated restraint stress (Romeo et al., 2006a), we predict higher cort levels in adolescents, relative to adults, following repeated half-hour exposures to cat odour.

The three main objectives were addressed as follows: 1) Adolescent responses to our main experimental manipulation (repeated cat odour exposure) were examined, in comparison to adult responses, 2) Relationships among social contact, cort levels, and defensive behaviours were examined within individual subjects, and 3) Long-term effects of stressor exposure were examined and compared between animals receiving exposure as adolescents and those receiving exposure as adults.

3.2. Materials and Methods

3.2.1. Subjects

3.2.1.1. General Husbandry Procedures

Rats were housed in same-sex pairs in a standard colony room in the Psychology Department. Cages were 22 x 24 x 48 cm polypropylene models covered with wire lids, and each contained wood-chip bedding and an ~5-inch piece of polyvinyl-carbonate

black tubing for enrichment. The colony room was maintained at 20 ± 1 °C with a 12:12 reverse light:dark cycle (lights off at 0930h). Food (Purina Lab Chow) and tap water were available *ad libitum*. Prior to any experimental manipulation, rats were handled each day for five days (at least 2 min per day) by the experimenter. This always occurred during the rats' active phase (subjective night), and entailed being picked up, touched, and held. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals. Effort was made to minimize any suffering the animals may have experienced.

3.2.1.2. Breeding

Male and female Long-Evans rats purchased from Charles River, Quebec, Canada, were used as breeders. Male breeders were paired with a female in a standard homecage for 5 days, at which time the male was removed.

Females were housed singly during pregnancy and were checked daily for litters, beginning 21 days after the first day of pairing. Deliveries proceeded naturally, and the day of birth was designated as PND0. On PND0, litters were culled to twelve pups, maintaining, when possible, an equal number of males and females. Dams/pups were not manipulated in any way, except for once-weekly cage changing, until offspring were weaned on PND21. Weaned pups were housed in same-sex, littermate pairs, and individuals were identified using non-toxic ink markings applied to the tail during handling and retouched approximately once per week.

3.2.1.3. Experimental Group Designation

Animals were randomly assigned to two groups based on age during stressor/control exposures (adolescence vs. adulthood). These two groups were further divided based on whether they received exposure to stress or exposure to a control condition, resulting in the formation of four groups (stressor-exposed adolescents; control-exposed adolescents; stressor-exposed adults; control-exposed adults). All of these groups contained both males and females (N = 6-8 of each sex) from at least three different litters.

3.2.2. Apparatus and Procedure

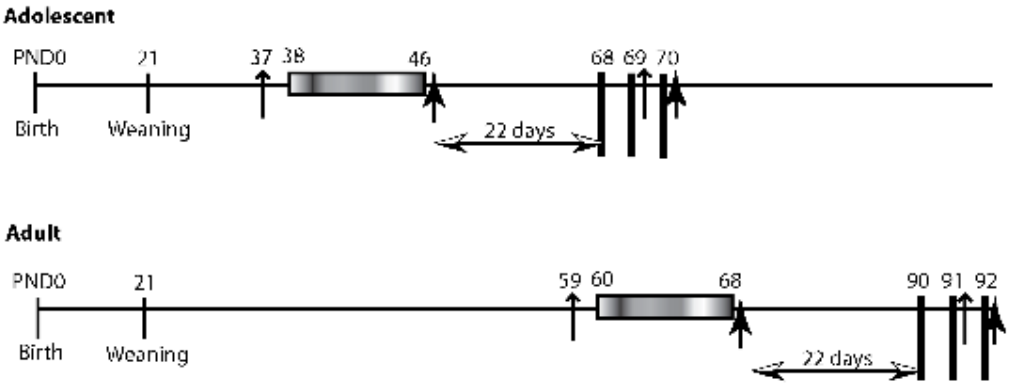
3.2.2.1. Stressor and Control Exposures

For animals exposed during adolescence, exposures began on PND38, and for animals exposed during adulthood, exposures began on PND60. For adolescent exposures, PND38 was chosen because it corresponds with the latter phase of adolescence (Hodes & Shors, 2005; Spear, 2004) and is consistent with prior work from our lab. The adult age of PND60 was chosen to ensure that animals in both age groups would undergo behavioural testing in young adulthood, following an ~ three-week (22 days) interval after the final exposure session, so as to minimize any confounding effects of aging (Fig. 3.1A).

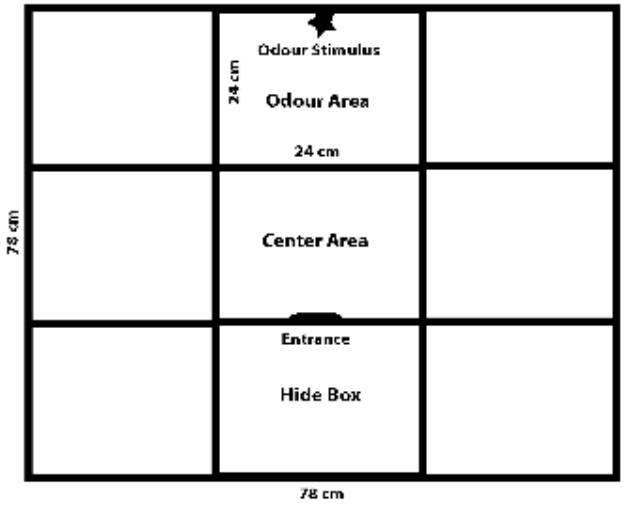
Stressor/control exposures took place on every second day for both age groups (i.e., PND38, 40, 42, 44, and 46 for adolescents and PND60, 62, 64, 66, and 68 for adults). During each exposure, animals were placed in a small, bare environment (clear

Figure 3.1. A) Experimental timeline for animals exposed as adolescents or as adults. Rats at both ages were exposed (five 30-minute exposures) to a stress (cat odour) or control condition (gray-scale horizontal bars) followed 22 days later by three sessions of behavioural testing (thick black vertical bars), during which responses to two stressors (novel open field and predator testing) were assessed. The small-headed arrow indicates times at which blood was taken to determine physiological baseline levels of corticosterone (cort), while the large-headed arrow indicates times during which blood was taken to determine stress-related levels of cort (experimental). **B)** Schematic representation of the adult behavioural testing apparatus. Note that the odour stimulus was only in place during predator testing on day 3.

A.


Adolescent
PND0
21
37
38
46
68
69
70
Birth
Weaning
22 days
Adult
PND0
21
59
60
68
90
91
92
Birth
Weaning
22 days

B.


Odour Stimulus
24 cm
Odour Area
24 cm
78 cm
Center Area
Entrance
Hide Box
78 cm

60 x 27 x 35.5 cm Plexiglas box with lid containing ventilation holes) together with the cagemate for 30 min. Animals were exposed with the cagemate rather than alone, because isolation during the adolescent period is itself considered stressful (Frisone et al., 2002), and because we were interested in how social behaviour exhibited during the exposures moderated long-term effects. Within this environment, stressor-exposed adolescent animals and stressor-exposed adult animals were exposed to a piece of cat collar, approximately one cm long, attached via an alligator clip to one end wall of the box, approximately five cm from the top. The pieces of collar for stressed animals (predator odour stressor) came from a collar that had been worn for at least two weeks by a reproductively intact domestic female cat housed communally in the Psychology Department. This duration of predator odour exposure induces activation of the HPA axis in adults (Masini et al., 2005). Control-exposed adolescents and control-exposed adults were exposed in an identical manner to a piece of cat collar never worn by a cat. Stress and control exposures were carried out in different rooms to avoid cross-contamination of odour, and all exposures occurred during the dark phase of the animals' light/dark circadian cycle, when rats are physically active.

During the first and last exposure sessions, adolescents were videotaped using Sony 8 mm digital cameras (CCD-TRV65 or CCD-TRV108) suspended directly above the exposure arenas. Behaviours were later quantified for the first 10 min of exposure, with the aid of The Observer 5.0.31 software (Noldus, Netherlands). For the purposes of analysis, the exposure arena was divided into three compartments of equal area, termed: the odour area (area that contained the cat collar), the middle area, and the safe area. Movement from one area into another was scored as a linecross, and rate of linecrosses

was taken as a measure of general activity. Rates of entry into each area were also scored, along with durations of time spent in each area. Finally, rates and durations of contact with the cagemate (bodily contact excluding the tail; termed social contact) were scored for each animal. Animals could be in passive contact (e.g. huddled together), and this would result in contact being scored for both members of the pair. However, animals could be actively contacting the cagemate with their face or limbs, in which case the animal *receiving* the contact would not be simultaneously scored as contacting the partner.

3.2.2.2. Behavioural Testing

Behavioural testing began on PND68 for animals exposed during adolescence and PND90 for animals exposed in adulthood (see Fig. 3.1A). The testing apparatus consisted of a 79 x 79 x 35.5 cm black Plexiglas box. A 21.5 x 24 x 22 cm black Plexiglas hide box with a 6 x 6 cm opening in the center of the front wall was placed in the center of one wall (Fig. 3.1B). Tests consisted of two open field exposures on consecutive days (open field day 1, OF1; open field day 2, OF2) followed by a predator stress test (PT) on the third day. During OF1, animals were exposed to the testing apparatus for the first time. Such a novel environment has been shown to induce HPA axis activity (Morrow et al., 2002; Rees et al., 2006). During OF2, the apparatus was somewhat familiar, which tends to induce more exploratory behaviour. During the PT on the third day, a piece of collar measuring approximately one cm, from a cat collar worn for at least two weeks (processed as described above for stressor exposures), was affixed with an alligator clip to the wall opposite the hide box. Animals were placed in the open field for 12 min each

day during the dark (active) phase of the animals' light/dark cycle. Behaviours elicited during OF1, OF2, and PT sessions were recorded using a Sony 8 mm digital camera suspended above each test arena. Behaviours during the initial 10 min of each session were later quantified with the aid of The Observer 5.0.3.1 software (Noldus, Netherlands).

For the purpose of quantifying behaviour, the apparatus was divided into ten unequal areas (see Fig. 3.1B). Rates and durations were quantified for the following non-defensive and defensive behaviours: *Rearing* (exploratory, non-defensive) – Front legs leave contact with the floor of the apparatus and may rest on a wall; *Grooming bout* (self-maintenance, non-defensive) – A series of uninterrupted motions using the mouth and/or paws to clean any part of the body (minimum 1 s duration); *Head-out* (risk assessment, defensive) – The head of the rat protrudes from the hide box, such that the rat is in a protected surveillance position. A head-out ends when half the body is beyond the hide box opening or when the head retreats into the hide box. The area midway between the forelimbs and hindlimbs is considered to represent the halfway point along the rostro-caudal body axis; *Odour Stimulus Contact* (exploratory, defensive) – Bodily contact (excluding tail) with the odour source; *Space Use* - Amount of time spent in, and rate of entry into the hide box, the centre area, and the odour area (see Fig. 3.1B); *Linecross* (exploratory, non-defensive) – Entry from one area into another was scored as one linecross. The animal was defined as leaving one area and entering another when more than half of its body was in the new area (see Fig. 3.1B).

3.2.2.3. Steroid Hormone Assays

For blood sampling, a hind leg was shaved, and pressure was applied to the saphenous vein, which was pricked with a 21-gauge needle (Becton Dickinson, United States). The vein was bled into a 600 μl Microtainer plasma separator tube containing lithium heparin (Becton Dickinson, United States), and the samples were centrifuged at 6000 x g for two minutes at 4 ˚C. Blood plasma was collected and aliquoted into three 60 μl samples, which were frozen at -80 ˚C until assay.

3.2.2.3.a. Basal Corticosterone Levels: Changes in physiological baseline levels (nadir of the circadian cycle; (Atkinson & Waddell, 1997) of cort were assessed at two time points during the experiment. Samples were taken on evenings prior to the first stressor/control exposure and prior to the last behavioural testing session between 1800 h and 2200 h, at the beginning of the light phase of the circadian cycle. For animals exposed during adolescence, the two samples were taken on PND37 and PND69, respectively. For animals exposed as adults, the two samples were taken on PND59 and PND91, respectively.

3.2.2.3.b. Experimental Corticosterone Levels: Blood samples were also collected immediately following the last stressor/control exposure and immediately following the PT, which was the last behavioural testing session, during the dark phase of the animals' light/dark cycle. For animals exposed during adolescence, these two samples were taken on PND46 and PND70, respectively, while for animals exposed in adulthood, these samples were taken on PND68 and PND92, respectively.

3.2.2.3.c. Corticosterone Assay: Duplicate samples were diluted 1:40 with assay buffer (tris-buffered saline with sodium azide preservative), and unbound cort levels were assayed using the Correlate-EIA Corticosterone Enzyme Immunoassay Kit (Assay Designs, Michigan, USA). The primary antibody showed 100% cross-reactivity with corticosterone, 21.3% cross-reactivity with deoxycorticosterone and 21% cross-reactivity with desoxycorticosterone. Each 96-well plate was assayed as per the manufacturer's instructions, with the exception that the Steroid Displacement Solution was not used (thus, measurement of unbound steroid). Samples were maintained on ice throughout. Assay sensitivity was determined to be 37.032 pg/ml. Some baseline samples were below the level of detection, and, in these cases, the assay sensitivity value was taken as a conservative estimate. Intra- and inter-assay coefficients of variation for this kit range between 6.6 - 8.0% and 7.8 - 13.1%, respectively.

3.2.2.3.d. Testosterone Assay: Bound and unbound testosterone levels were measured in male baseline and experimental plasma samples that corresponded to PND37/59 and PND46/68 for animals exposed during adolescence and adulthood, respectively. Testosterone was assayed using a Correlate-EIA™ Testosterone kit (Assay Designs, Ann Arbor, MI). The primary antibody was a mouse monoclonal antibody to testosterone. The secondary antibody was a goat anti-mouse IgG antibody. The primary antibody showed 100% cross-reactivity with testosterone, 14.6% cross-reactivity with 19-hydroxytestosterone, 7.20% cross-reactivity with androstendione and 0.72% cross-reactivity for dihydrotestosterone. All other cross-reactivities were less than 0.5%.

Samples were diluted 1:20. The manufacturer's instructions were followed. A set of standards was run on each plate and all samples and standards were run in duplicate. Assay sensitivity was determined to be 5.67 pg/ml. Intra- and inter-assay coefficients of variation for this kit range between 7.8 – 10.8% and 9.3 – 14.6%, respectively.

3.2.3. Data Manipulation and Statistical Analyses

Statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS; Version 11.0.4).

3.2.3.a. Behavioural Data: Analyses were conducted for three separate datasets: i) behaviours exhibited during exposures, ii) behaviours measured in the open field (OF1 and OF2), and iii) behaviours measured during the PT. For the first dataset, repeated measures ANOVAs were conducted for each dependent variable, with two levels of exposure session (SESSION; first and last) as the repeated measure and sex (SEX; female or male) and exposure condition (TREATMENT; stress or control) as between-subject variables. For the second dataset, for each dependent measure, ANOVA was performed with two levels of open field day (DAY; OF1 and OF2) as the repeated measure, and SEX, age (AGE; adolescent, adult), and TREATMENT as between-subject variables. ANOVAs were conducted similarly for each dependent variable in the third dataset, except that there was no repeated measure for this analysis.

3.2.3.b. Steroid Hormone Levels: Due to high individual variability in circulating hormone levels, cort levels in rodents are often normalized to their respective

physiological baseline levels and expressed as a percentage of baseline level. However, natural increases in baseline cort and testosterone during the adolescent period muddy this type of comparison among our experimental groups. Instead, we chose to present raw baseline and raw experimental (post-stressor/control exposure) data, in order to sort out more directly how stress exposure may alter adolescent development of adrenocortical parameters. Separate repeated measures ANOVAs were conducted for baseline samples and for experimental samples, with SAMPLE (exposure phase, adult test phase) as the repeated measure and SEX, AGE, and TREATMENT as between-subject factors. Using the same dataset, separate repeated measures ANOVAs were also conducted for the exposure phase and for the adult test phase, with SAMPLE (baseline, experimental) as the repeated measure and SEX, AGE, and TREATMENT as between-subject factors. This second analysis was used to clarify differences in hormone levels between adolescents and adults, which could be compared directly during the exposure phase.

3.2.3.c. Correlations: Relationships among cort and testosterone measures, levels of social interaction exhibited during exposure sessions, and measures of adult defensiveness or bravery were examined, and Pearson correlation coefficients were tabulated for each group and are displayed in Appendix III.

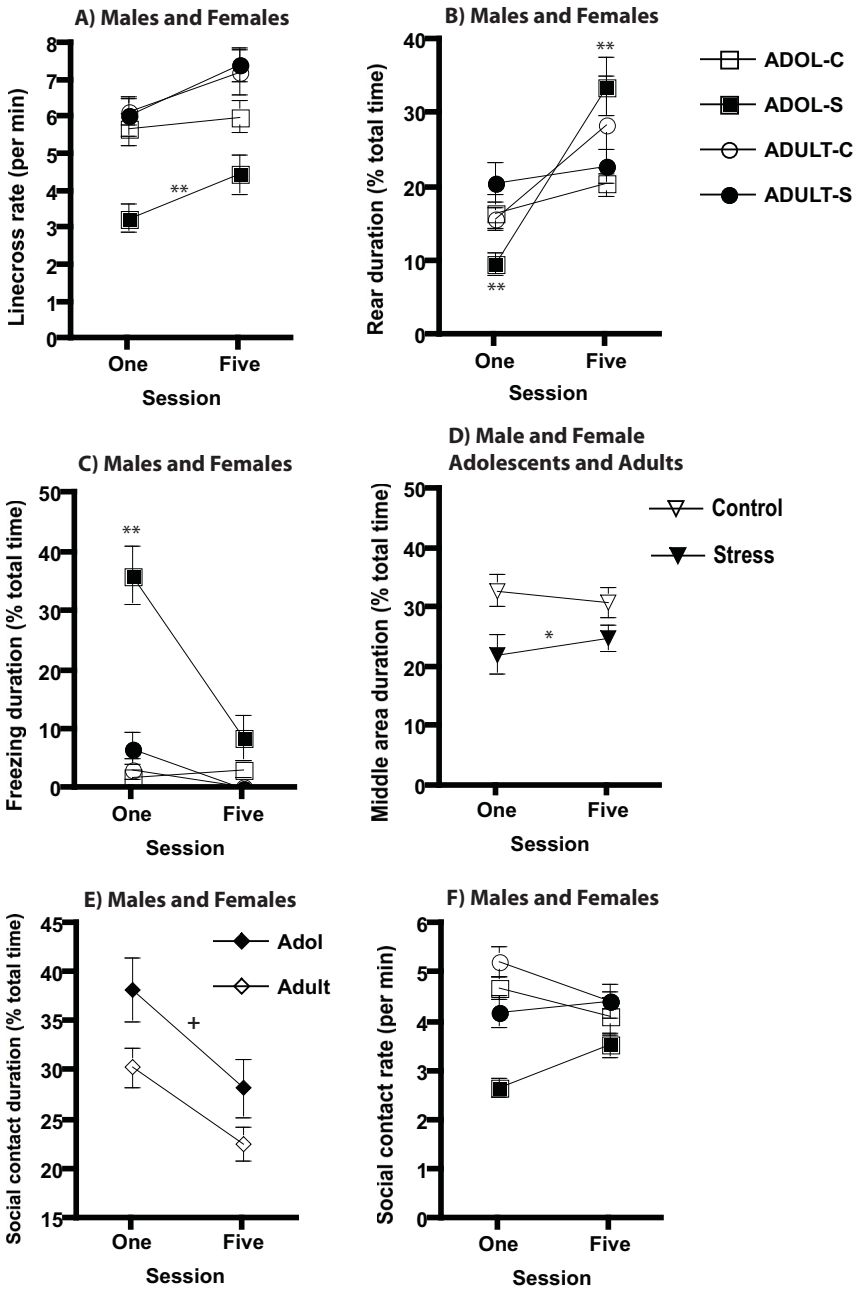
3.3. Results

3.3.1. Behaviours Observed During Exposures

3.3.1.a. Animals Exposed to a Stressor during Adolescence Display Stronger Inhibition of Activity than those Exposed as Adults: In general, exposure to a cat odour stressor during adolescence resulted in greater inhibition of activity, relative to that observed in response to the same stressor administered during adulthood. Simple effects analyses of an AGE x TREATMENT interaction, $F(1, 34) = 7.48$, $P = 0.010$, revealed that linecrossing was inhibited in the stressor-exposed animals, relative to control-exposed, for the adolescents ($P = 0.001$) but not the adults ($P = 0.966$; Fig. 3.2A). In general, rates of linecrosses increased across exposure sessions, $F(1, 34) = 13.79$, $P = 0.001$.

This inhibition of activity in stressor-exposed adolescents was further reflected in the time spent rearing during the first exposure session. Simple effects analyses of a SESSION x AGE x TREATMENT interaction, $F(1, 32) = 8.68$, $P = 0.006$, revealed that stressor-exposed adolescents spent less time rearing during the first exposure session ($P = 0.022$) but more time rearing during the final exposure session ($P = 0.008$), relative to control-exposed adolescents (Fig. 3.2B). There was also a SEX x AGE x TREATMENT interaction, $F(1, 32) = 7.40$, $P = 0.010$, which arose because control-exposed adult males spent more time rearing than control-exposed adolescent males ($P = 0.019$). In addition to durations, frequencies of rearing were also affected. There were SEX x TREATMENT, $F(1, 34) = 7.96$, $P = 0.008$, and AGE x TREATMENT, $F(1, 34) = 6.79$, $P = 0.014$,

Figure 3.2. Exposure to stress during adolescence (ADOL-S) resulted in inhibition of activity relative to exposure to control during the same age period (ADOL-C) and relative to animals exposed as adults to stress (ADULT-S) or control (ADULT-C). **A)** The rate of line crossing was significantly reduced during the first and last exposure session in males and females exposed to stress during adolescence (filled squares) relative to those exposed to control; **B)** Similarly, males and females exposed to the stressor during adolescence (filled squares) spent less time rearing during the first exposure session. During the final exposure session, however, these same animals spent more time rearing; **C)** Males and females exposed as adolescents to a stressor (filled squares) also spent more time freezing during the first exposure session; **D)** Regardless of age and sex, animals exposed to the stressor spent less time in the center of the exposure arena, relative to those exposed to control; **E)** Adolescents (squares) spent more time in contact with the cage mate during exposures relative to adults (circles); **F)** In contrast, adolescents (squares) contacted the cage mate less frequently relative to adults (circles) during exposures. **significantly different from control-exposed adolescents, $p<0.05$; *significantly different from control, $p<0.05$. **+**significantly different from adults, $p<0.05$. Symbols placed over a line indicate effects collapsed across both exposure sessions.


A) Males and Females
Linecross rate (per min)
Session
One
Five
B) Males and Females
Rear duration (% total time)
ADOL-C
ADOL-S
ADULT-C
ADULT-S
C) Males and Females
Freezing duration (% total time)
D) Male and Female
Adolescents and Adults
Middle area duration (% total time)
Control
Stress
E) Males and Females
Social contact duration (% total time)
Adol
Adult
F) Males and Females
Social contact rate (per min)

interaction effects for rates of rearing, whereby stressor-exposed females showed lower rearing rates than control-exposed females ($P < 0.001$), but males showed no difference ($P = 0.917$), and stressor-exposed adolescents reared less frequently than control-exposed adolescents ($P = 0.006$), but adults showed no difference ($P = 0.935$; Table 1). Rates of rearing increased from the first to the last exposure session, $F(1, 34) = 27.56$, $P < 0.001$.

Adolescent animals exposed to the stressor also displayed more freezing. For time spent freezing, there was a SESSION x AGE x TREATMENT interaction, $F(1, 34) = 7.18$, $P = 0.012$, and simple effects analyses revealed that stressor-exposed adolescents spent longer durations freezing, relative to control-exposed adolescents, during the first exposure session (Fig. 3.2C; $P < 0.001$). For rates of freezing, there was a SEX x AGE x TREATMENT interaction effect, $F(1, 34) = 5.86$, $P = 0.021$, whereby stressor-exposed adolescent females froze more frequently than control-exposed adolescent females ($P = 0.001$), while stressor-exposed adult males froze more frequently than control-exposed adult males ($P = 0.038$; Table 1). Although there was also a SESSION x TREATMENT interaction, $F(1, 34) = 5.45$, $P = 0.026$, stressor-exposed animals froze more frequently than control-exposed animals during both the first ($P < 0.001$) and the last ($P = 0.032$) exposure sessions (Table 1). The interaction arose due to a reduction in freezing rates across exposure sessions in stressor-exposed animals ($P = 0.011$), which was not significant in control animals ($P = 0.080$).

3.3.1.b. Space Use Was Affected by Stressor Exposure and Exposure Age: Regardless of exposure age or sex, animals exposed to the stressor spent less time overall within the middle area, compared with control-exposed animals, $F(1, 32) = 7.88$, $P = 0.008$ (Fig.

Table 3.1. Means (number per minute for rates and percent total time for durations) and standard errors of the mean for various behaviours quantified in adolescents (Adol) and adults, in response to stress (S) or control (C), administered during two exposure sessions. Within the first section are variables for which there were significant interactions among combinations of all four factors (Sex, Age, Treatment, Session). Within the second section, variables are presented for which Treatment is collapsed, while in the third section, variables are presented for which Sex is collapsed. Please see Results for details of statistics.

Dependent Variable	Sex	Age/Treatment	Session 1	Session 5
Rear rate	F	Adol-C	5.71±0.65	7.81±0.54
	M	Adol-C	3.69±0.51	4.14±0.44
	F	Adol-S	2.36±0.57	3.71±0.83
	M	Adol-S	2.69±0.40	4.64±0.92
	F	Adult-C	5.05±0.48	5.93±0.89
	M	Adult-C	4.40±0.21	5.53±0.52
	F	Adult-S	4.39±0.58	7.07±0.34
	M	Adult-S	4.32±1.18	6.40±0.43
Freeze rate	F	Adol-C	0.14±0.06	0.19±0.14
	M	Adol-C	0.57±0.22	0.29±0.13
	F	Adol-S	1.21±0.22	1.02±0.37
	M	Adol-S	1.0±0.1	0.5±0.18
	F	Adult-C	0.21±0.13	0.07±0.04
	M	Adult-C	0.48±0.15	0.11±0.11
	F	Adult-S	0.39±0.26	0.11±0.11
	M	Adult-S	1.25±0.26	0.12±0.04
Safe area duration	F	Adol-C	42.2±4.3	42.2±5.2
	M	Adol-C	50.4±6.0	49.5±8.3
	F	Adol-S	58.7±3.9	68.2±5.3
	M	Adol-S	66.2+11.9	52.6±5.4
	F	Adult-C	39.6±4.1	39.4±3.1
	M	Adult-C	35.0±3.6	41.1±4.6
	F	Adult-S	57.8±6.3	42.5±7.4
	M	Adult-S	35.8±1.8	32.4±1.6

Dependent Variable	Age/Sex	Session 1	Session 5
Odor area duration	Adol-F	29.4±2.9	20.7±2.5
	Adol-M	11.8±2.4	20.6±2.9
	Adult-F	26.3±2.7	31.1±3.7
	Adult-M	28.0±4.4	32.2±1.6

Dependent Variable	Age/Treatment	Session 1	Session 5
Middle area rate	Adol-C	2.87±0.21	3.05±0.21
	Adol-S	1.65±0.19	2.23±0.26
	Adult-C	3.11±0.17	3.66±0.30
	Adult-S	3.05±0.26	3.77±0.24
Odour area rate	Adol-C	1.36±0.12	1.38±0.11
	Adol-S	0.70±0.13	1.06±0.14
	Adult-C	1.49±0.10	1.75±0.15
	Adult-S	1.52±0.18	1.89±0.16

3.2D), providing support for thigmotaxis in response to a stressful context. Stressor-exposed adolescent animals entered the middle area of the exposure arena less frequently than control-exposed adolescents ($P = 0.001$), as indicated by simple effects analyses following an AGE x TREATMENT interaction, $F(1, 34) = 7.88$, $P = 0.008$ (Table 1). The frequency of entering the middle area increased from the first to the last exposure session, $F(1, 34) = 14.52$, $P = 0.001$.

Simple effects analyses of a SESSION x SEX x AGE interaction, $F(1, 32) = 10.95$, $P = 0.002$, revealed an effect of exposure age for time spent within the odour area, with adolescent males spending less time in the odour area, relative to adult males, during both the first ($P = 0.003$) and last ($P = 0.003$) exposure sessions (Table 1). Similarly, adolescent females spent less time in the odour area, relative to adult females, during the last exposure session only ($P = 0.025$). A sex difference was also revealed for adolescent animals only, with males spending less time in the odour area, relative to females, during the first exposure session ($P < 0.001$). Analyses of an AGE x TREATMENT interaction, $F(1, 32) = 4.69$, $P = 0.038$, revealed a trend toward reduced time spent in the odour area in the stressor-exposed adolescents, relative to control-exposed adolescents ($P = 0.067$). Stressor treatment reduced frequencies of entry into the odour area in the adolescents ($P = 0.002$) but not the adults ($P = 0.633$), as indicated by simple effects analyses conducted in follow-up of an AGE x TREATMENT interaction effect, $F(1, 34) = 7.72$, $P = 0.009$ (Table 1). There was also a SESSION x TREATMENT interaction for rates of entry into the odour area, $F(1, 34) = 7.40$, $P = 0.010$, whereby the odour area was entered less frequently by stressor-exposed animals, relative to control-exposed animals, during the first exposure session ($P < 0.010$) but not the last ($P = 0.381$; Table 1).

Subsequent analyses of a SESSION x SEX x AGE interaction for time spent in the safe area, $F(1, 32) = 6.81$, $P = 0.014$, revealed a similar pattern of results as observed for time spent in the odour area, with adolescent males spending more time in the safe area, relative to adult males, during both the first ($P = 0.008$) and last ($P = 0.014$) exposure sessions (Table 1). Stressor-exposed animals spent longer in the safe area across exposure sessions, relative to control-exposed animals, $F(1, 32) = 5.59$, $P = 0.024$ (Table 1). There was a significant AGE x TREATMENT interaction effect, $F(1, 34) = 4.68$, $P = 0.038$, for rates of entrance into the safe area, with stressor-exposed adolescents showing less frequent entry into the safe area, relative to control-exposed adolescents ($P = 0.014$), but stressor- and control-exposed adults showing no difference.

Grooming wasn't prominent during exposure sessions (e.g. 0.52 ± 0.10 events per min for control-exposed adolescents and 0.50 ± 0.10 events per min for control-exposed adults during the last exposure session); nonetheless, it increased from the first to the last session, in terms of both rate, $F(1, 34) = 11.31$, $P = 0.002$, and duration, $F(1, 32) = 10.51$, $P = 0.003$ (data not shown). There was also a SEX x AGE interaction effect for both grooming rates, $F(1, 34) = 9.13$, $P = 0.005$, and durations, $F(1, 32) = 5.93$, $P = 0.021$, whereby adult females groomed more frequently and for longer than adolescent females (P's ≤ 0.016). This resulted in a sex difference in adult grooming rates and durations, with females grooming more than males (P's ≤ 0.048), an effect that was not yet present during the adolescent period.

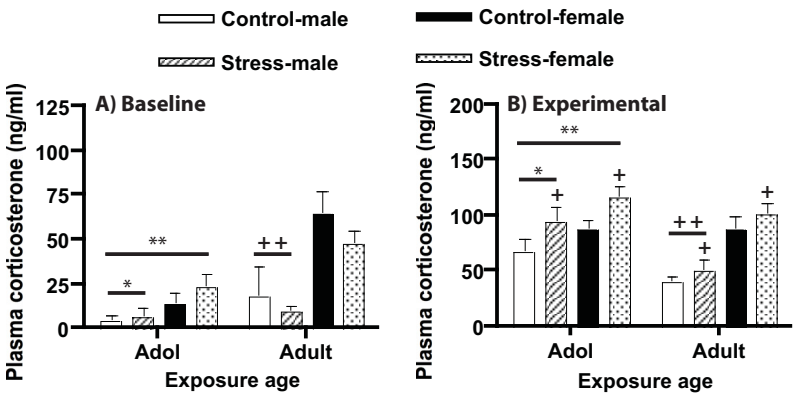
3.3.1.c. Adolescents Spend More Time in Social Contact than Adults: Adolescents spent longer durations in contact with the cagemate than adults did, $F(1, 32) = 9.311$, $P = 0.005$

(Fig. 3.2E), but adolescents contacted cagemates less frequently than adults did, $F(1, 34) = 13.86$, $P = 0.001$ (Fig. 3.2F). Overall, animals decreased their time spent in contact with a cagemate during the final exposure session, relative to the first, $F(1, 34) = 8.72$, $P = 0.006$. Stressor exposure reduced cagemate contact rates, relative to control exposure, during the first ($P < 0.001$) but not the last ($P = 0.427$) exposure session, as was revealed in simple effects follow-up of a SESSION x TREATMENT interaction, $F(1, 34) = 11.27$, $P = 0.002$.

3.3.2. Plasma Corticosterone Levels Analyzed During the Exposure Phase

3.3.2.a. Adolescents Circulate Lower Baseline and Higher Experimental Cort Levels than Adults after Repeated Exposure Sessions: Including sample (baseline and experimental) as the repeated measure, there were SAMPLE x TREATMENT, $F(1, 35) = 6.85$, $P = 0.013$, and SAMPLE x AGE, $F(1, 35) = 26.89$, $P < 0.001$, interaction effects for levels of cort circulating during the exposure phase. Simple effects analyses revealed that an AGE effect was present both in baseline cort levels prior to exposure, $F(1, 35) = 13.39$, $P = 0.001$, and in cort circulating after the final exposure, $F(1, 38) = 8.08$, $P = 0.007$, with adults circulating higher baseline cort levels than adolescent animals (Fig. 3.3A) but adolescents showing higher levels following exposure (Fig. 3.3B). There was also a SEX x AGE interaction, $F(1, 35) = 9.46$, $P = 0.004$, with adolescent males circulating higher levels of cort than adult males ($P = 0.026$) but no significant effect in females ($P = 0.055$; Fig. 3.3A-B).

Figure 3.3. Levels of plasma corticosterone (cort) measured **A)** at the circadian nadir of cort secretion on the evening prior to the final exposure session (Baseline) and **B)** immediately following the final exposure session (Experimental) in male and female animals exposed as adolescents (Adol) or exposed as adults (Adult) to repeated stress or control. Regardless of exposure, cort levels were higher in adolescent males, relative to adult males, across baseline and experimental samples. In adults, cort levels were higher in females than in males. In experimental samples only, animals exposed to stress had higher levels of cort relative to those exposed to control. *significantly different from adults of same sex, collapsed across samples, $p<0.05$; **significantly different from adults, $p<0.05$; +significantly different from control, $p<0.05$; ++significantly different from females of same age, collapsed across samples, $p<0.05$.


Control-male
Stress-male
Control-female
Stress-female
A) Baseline
B) Experimental
Plasma corticosterone (ng/ml)
125
100
75
50
25
0
200
150
100
50
0
**
*
+
++
Adol
Adult
Exposure age

3.3.2.b. Stressor Exposure Increased Circulating Cort Levels in both Adolescents and Adults: As would be expected, the effect of TREATMENT was not observed in the baseline samples ($P > 0.05$). In the experimental samples, however, higher cort levels were observed in those derived from stressor-exposed animals, relative to control-exposed animals, $F(1,38) = 7.47$, $P = 0.009$. A SEX effect did not quite reach significance in the adolescent samples ($P = 0.050$), but females were circulating overall higher cort levels than males in the adult samples, $F(1, 19) = 45.78$, $P < 0.001$ (Fig. 3.3A-B).

3.3.3. Correlational Analyses for Exposure Phase Measures

All significant correlations for exposure phase measures are tabulated in Appendix III. Here, we highlight some of the relationships we found most interesting. In adolescent control animals, time spent freezing was positively correlated with time in contact with the cagemate during both the first and last exposure sessions (P's ≤ 0.015), indicating that adolescent animals huddled together while resting immobile. For stressor-exposed adolescents, during the first exposure session, when durations spent freezing were very high (see Fig. 3.2C), there was no correlation between freezing durations and cagemate contact durations, indicating that adolescents responded to the initial stressor exposure by freezing whether in contact with a cagemate or not. After repeated exposure to the stressor, however, adolescents huddled together while freezing, as indicated by a positive correlation between freezing durations and cagemate contact durations for this group during the final exposure session ($P = 0.045$).

3.3.4. Adult Behaviours Observed During Days 1 & 2 of Testing in the Open Field

In general, adult open field behaviours were minimally affected by prior stressor exposure. There was a SEX x AGE x TREATMENT interaction for linecross rates from OF1 to OF2, $F(1, 38) = 4.33$, $P = 0.044$ (Table 2). Simple effects analyses revealed that a TREATMENT effect was present in females exposed to the stressor during adolescence. They showed inhibited locomotor activity across open field sessions, relative to females exposed to the control stimulus during adolescence ($P = 0.022$). This effect was not seen in animals exposed during adulthood or in males exposed during adolescence (P's > 0.05). Animals exposed during adolescence generally showed inhibition across OF1 and OF2, relative to those exposed during adulthood, within each sex (across stress treatments) and within each stress treatment (across sexes; P's ≤ 0.038). Females generally exhibited more activity than males (P's ≤ 0.043), an effect that was abolished by stressor exposure during adolescence ($P > 0.05$).

Levels of hide box usage were low and very variable during both days of testing in the open field. Although there was a significant four-way interaction for durations spent within the hide box, $F(1, 38) = 4.50$, $P = 0.041$, this was driven mostly by consistently long durations of hiding observed in females exposed to the control stimulus during adolescence, relative to other females, during OF2. During OF2, females exposed to the control stimulus during adolescence spent more time in the hide box (mean % total test duration ± s.e. = 15.7 ± 4.2), relative to females that were stressor-exposed during adolescence (mean % total test duration ± s.e. = 3.5 ± 2.9; $P = 0.037$) and females exposed to the control stimulus as adults (mean % total test duration ± s.e. = 0.53 ± 0.53; $P = 0.005$). Females that were stressor-exposed as adults spent comparatively

Table 3.2. Means (number per minute) and standard errors of the mean for rates of line crossing collapsed across Days 1 and 2 of adult open field testing in animals previously exposed to stress or control as adolescents (Adol) or adults. Please see Results for details of statistics.

Dependent Variable	Age/Sex	Control	Stress
Linecross rate	Adol-F	10.9±0.42	8.5±0.63
	Adol-M	7.7±0.31	8.5±0.88
	Adult-F	11.3±0.70	11.6±0.51
	Adult-M	10.1±0.45	9.3±0.53

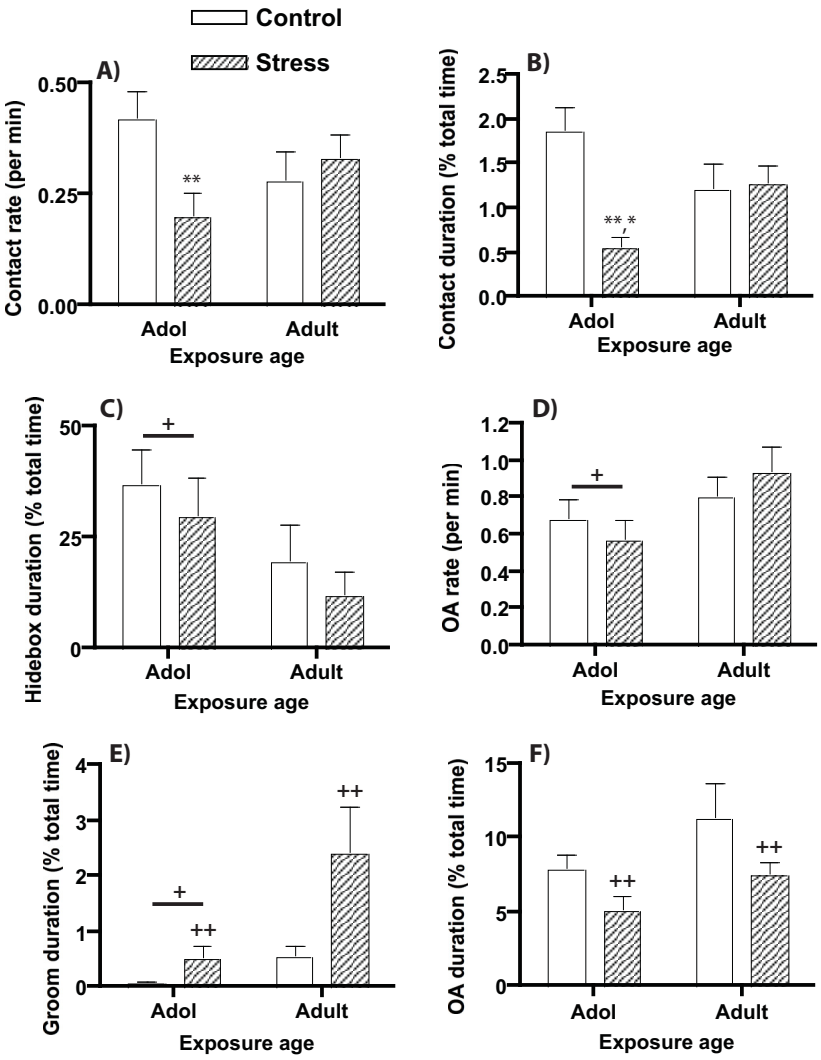
intermediate durations hiding (mean % total test duration ± s.e. = 8.6 ± 5.8). Male groups spent on average (± s.e.) between 0.33 ± 0.27 and 19.2 ± 16.0 % of the total test duration hiding in OF2. During OF1, each male and female group spent on average (± s.e.) between 0 ± 0 and 7.4 ± 4.3 % of the total test duration hiding.

A simple effects follow-up of a DAY x AGE interaction for the time spent grooming, $F(1, 38) = 12.42$, $P = 0.001$, revealed that animals exposed during adulthood increased their grooming durations in OF2, relative to OF1 ($P = 0.016$), while those exposed during adolescence correspondingly *decreased* time spent grooming ($P = 0.020$), resulting in more time spent grooming by animals exposed in adulthood (mean % total test duration ± s.e. = 2.3 ± 0.6) versus adolescence (mean % total test duration ± s.e. = 0.6 ± 0.1; $P = 0.004$) in OF2. In OF1, animals exposed during adolescence spent on average (± s.e.) 1.2 ± 0.2 % of the total test duration grooming, while those exposed in adulthood spent 0.9 ± 0.2 % of the total test duration grooming.

3.3.5. Adult Behaviours Observed During the Predator Test

3.3.5.a. Animals Exposed to Cat Odour during Adolescence were Highly Wary of the Stimulus during the Adult Predator Test: In animals exposed to the stressor during adolescence, specifically, decreased rates of contacting the collar stimulus, $F(1, 23) = 6.79$, $P = 0.017$ (Fig. 3.4A), as well as durations of time spent investigating it, $F(1, 23) = 19.22$, $P < 0.001$ (Fig. 3.4B), were observed during the PT, relative to control-exposed adolescent animals. Animals that were stressor-exposed during adolescence also spent less time contacting the collar stimulus in the PT, relative to those stressor-exposed as adults, $F(1, 21) = 8.22$, $P = 0.010$ (Fig. 3.4B).

Figure 3.4. Behaviours measured during the adult predator test (PT) were affected by adolescent (Adol) stressor exposure, adolescent exposure in general, and stressor exposure regardless of exposure age. The **A)** rate, and **B)** duration of contacting the stimulus during the PT were significantly reduced, specifically in adolescent males and females exposed to stress, relative to those exposed to control. This pattern was not observed in animals exposed to stress as adults; **C)** The duration of time spent in the hide box was increased, while **D)** the rate of entering the odour area (OA) was decreased in animals exposed to either stress or control as adolescents, relative to those exposed as adults; **E)** Animals exposed as adults spent longer grooming than those exposed as adolescents, and, across both age groups, prior exposure to stress resulted in an increase in the time spent grooming and **G)** a decrease in the time spent in the odour area (OA), during the PT. **significantly different from control-exposed adolescents, $p<0.05$; *significantly different from stress-exposed adults, $p<0.05$; **+**significantly different from control and stress-exposed adults, $p<0.05$; **++**significantly different from control-exposed animals, $p<0.05$.


Control
Stress
A)
Contact rate (per min)
0.50
0.25
0.00
**
Adol
Adult
Exposure age
B)
Contact duration (% total time)
2.5
2.0
1.5
1.0
0.5
0.0
** *
Adol
Adult
Exposure age
C)
Hidebox duration (% total time)
50
25
0
+
Adol
Adult
Exposure age
D)
OA rate (per min)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+
Adol
Adult
Exposure age
E)
Groom duration (% total time)
4
3
2
1
0
+
++
++
Adol
Adult
Exposure age
F)
OA duration (% total time)
15
10
5
0
++
++
Adol
Adult
Exposure age

3.3.5.b. Adolescent Manipulation (Stressor or Control Exposure) Increased General Defensiveness in the Adult Predator Test: Some measures quantified during the adult PT were affected generally by prior exposure (stressor or control) in adolescence but not in adulthood. For example, animals exposed during adolescence spent more time within the hide box, $F(1, 45) = 4.61$, $P = 0.038$ (Fig. 3.4C), and entered the odour area less frequently, $F(1, 45) = 5.18$, $P = 0.029$ (Fig. 3.4D), relative to those exposed during adulthood.

A sex difference in the effect of adolescent exposure on head-out posturing was also evident during the PT. Males but not females exposed during adolescence spent longer in the head-out position, relative to males exposed as adults, ($P = 0.008$; data not shown), as revealed in follow-up of a SEX x AGE interaction for head-out durations, $F(1, 45) = 5.35$, $P = 0.026$.

3.3.5.c. Prior Cat Odour Exposure Resulted in Avoidance of the Odour Area during the Adult Predator Test: In the adult PT, previously stressed animals spent more time grooming, relative to control-exposed animals, $F(1, 45) = 8.27$, $P = 0.007$ (Fig. 3.4E). Also, animals exposed during adulthood spent more time grooming in the PT, relative to those exposed during adolescence, $F(1, 45) = 8.94$, $P = 0.005$ (Fig. 3.4E). Regardless of exposure age, prior stressor exposure resulted in less time spent near the cat odour stimulus during the PT, relative to those previously exposed to control stimuli, $F(1, 45) = 4.58$, $P = 0.039$ (Fig. 3.4F).

3.3.6. Plasma Corticosterone Levels Analyzed During the Test Phase

In comparison to the results of the exposure phase cort analysis, the SAMPLE x TREATMENT and SEX x AGE interaction effects had dissipated (*P*'s > 0.05) during the adult behavioural test phase analysis. The SAMPLE x AGE interaction effect, however, reemerged, $F(1, 34) = 4.89$, $P = 0.034$, but the effect was difficult to resolve, as the effect of AGE did not reach significance in either baseline or experimental samples taken during the adult behavioural test phase (*P*'s > 0.05), and there were higher levels of cort in experimental samples, relative to baseline samples, in groups exposed at either age (*P*'s < 0.001). However, in animals exposed during adolescence, cort levels in experimental samples were over three times the levels in baseline samples (mean ± s.e. = 108.83 ng/ml ± 11.27 vs. 29.31 ng/ml ± 7.97), whereas in animals exposed during adulthood, the difference between levels in the experimental and baseline samples was not as great (mean ± s.e. = 86.24 ± 9.86 ng/ml for experimental samples and 40.18 ± 7.83 ng/ml for baseline samples). There was a main effect of SEX, $F(1, 34) = 31.28$, $P < 0.001$, with females circulating higher levels of cort than males.

3.3.7. Plasma Corticosterone Levels Analyzed Separately for Baseline Samples and Experimental Samples

There was a SESSION x TREATMENT interaction in the baseline samples, $F(1, 33) = 5.18$, $P = 0.030$. Simple effects analyses revealed that no *a priori* group differences existed between stressed and control animals in basal circulating levels of cort; however, stressor exposure increased baseline cort levels, as revealed by a TREATMENT effect in samples collected the evening prior to the adult PT, $F(1, 41) = 4.31$, $P = 0.045$, and an effect of SESSION in the stressor-exposed animals, regardless of exposure age, $F(1, 15)$

= 9.55, $P = 0.001$ (Fig. 3.5A). Thus, repeated cat odour exposure increased baseline cort levels without affecting levels circulating after the final stressor exposure or the adult PT. This suggests a reduction in stressor-induced cort output following repeated exposure to a homotypic stressor stimulus.

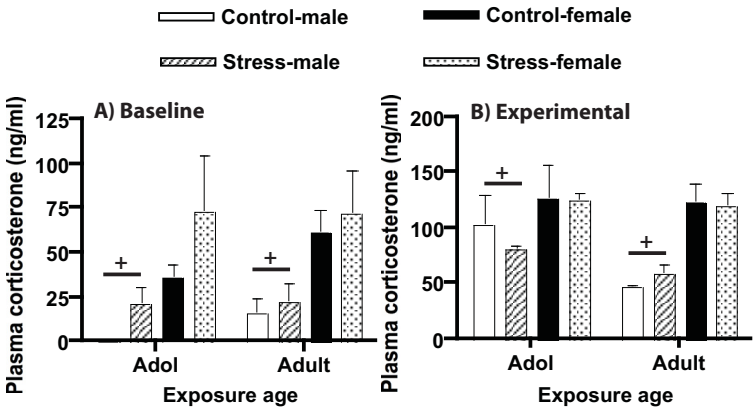
3.3.8. Plasma Testosterone Levels

Baseline levels of testosterone were higher in adult males (mean ± s.e. = 8.67 ± 1.28 ng/ml) than in adolescent males (mean ± s.e. = 3.49 ± 0.189 ng/ml) prior to exposure, $F(1, 23) = 15.13$, $P = 0.017$. Once all males had reached adulthood, however, no group differences were observed.

3.3.9. Correlations Among Exposure Phase Measures and Adult Test Phase Measures

3.3.9.a. Adolescent Social Contact in a General Context Predicts Adult Levels of Defensive Behaviour: Significant correlations among all measures examined are tabulated in Appendix III, and those of interest are highlighted here. In adolescent control animals, time spent in contact with the cagemate was correlated with various measures of defensiveness in the adult PT. Specifically, time spent in contact with the cagemate during each of the exposure sessions was negatively correlated with odour stimulus contact durations and odour area entrance rates in the adult PT (P's ≤ 0.041) and positively correlated with durations spent in the hide box (P's ≤ 0.021). These relationships were not observed in animals that were stressor-exposed during adolescence or in either group exposed during adulthood, suggesting that adolescent social contact in

Figure 3.5. Levels of plasma corticosterone (cort) measured **A)** at the circadian nadir of cort secretion on the evening prior to the predator test (PT; Baseline) and **B)** immediately following the PT (Experimental) in male and female animals exposed as adolescents (Adol) or exposed as adults (Adult) to repeated stress or control. In both baseline and experimental samples from animals of both exposure ages, males had lower levels of cort, relative to females. Note also that baseline cort levels in panel **A)** are significantly increased in groups stressed at either age, relative to the levels displayed in Figure 3.3. **+**significantly different from females, $p<0.05$.


Control-male
Control-female
Stress-male
Stress-female
A) Baseline
B) Experimental
Plasma corticosterone (ng/ml)
125
100
75
50
25
0
200
150
100
50
0
+
Adol
Adult
Exposure age

a context devoid of potent stressors may be predictive of defensive behaviour in adulthood.

Cort levels following the PT were positively correlated with those following the final exposure session in all groups (P's ≤ 0.041), except that which was stressor-exposed during adolescence (P = 0.172).

3.3.9.b. Adolescent and Adult Social Contact are Differentially Related to Adult Steroid Hormone Levels: The durations of time that adolescent control animals spent in contact during the first and final exposure sessions were negatively correlated with baseline cort levels in adulthood (P's ≤ 0.007). Animals that were stressor-exposed during adolescence, however, only showed this correlation for the first exposure session (P = 0.006). Interestingly, animals that were stressor-exposed during adulthood showed the opposite pattern; that is, a positive relationship between duration of time spent in contact during the initial exposure session and baseline cort prior to the PT (P = 0.034).

Males that were stressor-exposed during adolescence also showed a negative correlation between duration of time spent in contact during the first exposure session and adult testosterone levels (P < 0.001). Furthermore, adult baseline cort levels were positively correlated with adult testosterone levels in these males (P = 0.045), while males that were stressor-exposed in adulthood correspondingly showed a *negative* correlation between adult baseline cort and adult testosterone (P = 0.030).

3.4. Discussion

In the present study, a naturalistic model of predator threat was used to invoke a period of heightened stress in groups of male and female rats. This was done by repeatedly exposing cagemate pairs, either during adolescence or during early adulthood, to either cat odour cues or to control stimuli within an enclosed environment. Three main questions were addressed: 1) How do the behavioural and hormonal stress responses of adolescent-aged rats compare with those of adult rats across the exposure period? 2) Does repeated stressor exposure alter subsequent stress responding in adulthood, and, if so, are the effects specific to having been exposed during adolescence, as opposed to exposure in adulthood? 3) As a measure of social behaviour, is adolescent physical contact related to the acute and long-term effects of stressor exposure?

3.4.1. Hormonal and Behavioural Responses of Adolescent Rats to Predator Odour

3.4.1.1. General Response of Rats to Cat Odour Stimuli

In general, the cat odour stimulus used in this study was effective in eliciting behavioural and hormonal responses during the exposure sessions. Both adolescents and adults showed elevated cort levels following the final stressor exposure, relative to control exposure, demonstrating a hormonal response to the stressor in both age groups that does not habituate after five sessions of cat odour exposure. Behaviourally, stressor-exposed animals, relative to control-exposed animals, spent more time in the safe area and less time in the middle of the exposure arena during exposures, consistent with defensive responses observed in other studies employing predation threat cues as a stressor in an environment without a hide area (Apfelbach et al., 2005; McGregor et al.,

2002). In general, and especially when confronted with predation threat, rodents prefer to be in contact with walls, rather than to occupy open space (Simon et al., 1994; Treit & Fundytus, 1988). We have previously found that adult rats continue to show defensive responses to cat odour cues, even after three weeks of daily exposure (Mashoodh et al., 2008).

3.4.1.2. Response of Adolescent Rats to Cat Odour Stimuli

Others have posited that adolescents respond differently to stressful stimuli, relative to adults (Ferris et al., 2005; Marquardt, Ortiz-Lemos, Lucion, & Barros, 2004; Romeo). Adolescence is currently thought of as a developmental phase characterized by increased novelty seeking and risky behaviours, as individuals transition away from the natal territory toward independence (Spear, 2000). Since risky behaviour is prominent amongst adolescents, we might expect that adolescents would show more bravery and less defensive responding in the presence of predation threat. In terms of cort secretion, however, there is evidence that prepubertal animals maintain elevated cort levels longer than adults do after exposure to an acute stressor and show higher peak cort levels following repeated exposure (McCormick & Mathews, 2007; Romeo et al., 2006a; Romeo et al., 2006b). This is thought to reflect less efficiency in gluccocorticoid receptor-mediated negative feedback loops at the cortical level, which are still in an immature state, as the cortex continues to develop throughout adolescence (Andersen et al., 2002; Andersen et al., 2000; Leussis et al., 2008; Spear, 2000).

As mentioned above, in the present study, cort was elevated relative to control animals in both adolescents and adults following the final stressor exposure. We were

unable to determine whether or not cort remained elevated for longer in the adolescents than in adults; however, our data revealed higher cort levels in adolescent males, relative to adult males, after the final exposure session (see Sex Differences section below). Also, adolescents circulated much lower baseline levels of cort than adults did. As far as we can tell, this difference has not been documented with published data, as stress-induced cort levels are typically expressed as a percentage of baseline levels, and baseline levels *per se* are not shown. The low baseline cort levels in adolescents relative to adults likely contributes to the differences in stress-induced cort dynamics between the two age groups.

Behaviourally, stressor-exposed adolescents in this study clearly mounted a more vigorous defensive response than adults to cat odour exposure. They showed greater inhibition of activity, spent less time rearing (an exploratory posture), and spent much more time completely unmoving (freezing) during the initial exposure session. During the final exposure session, much of their time freezing was spent huddling, a response that has been observed following restraint stress in prepubertal males (Romeo et al., 2006b). In contrast, adults did not engage in much freezing. Adult rats have shown freezing responses to predator stimuli (McGregor et al., 2002) or other psychological stressors (Matsumoto et al., 2005) in prior studies; however, in those studies, animals were exposed individually and not together with a cagemate. The presence of the cagemate during exposures in the present study may have mitigated the freezing response in adults. By the final exposure session, stressor-exposed adolescents were no longer freezing more than adults or control animals; however, their mobility was still reduced, as indicated by their continued lower linecross rates and rearing rates. At this point, though, they were

spending more time than adolescent control animals in the rearing posture. Together, these behavioural and cort findings support the idea that adolescents are more sensitive than adults to the acute effects of stressor exposure, even after repeated exposure sessions. By the fifth exposure session, they had adopted a stance of cautious surveillance in responding to the threat.

Altogether, our findings indicate that adolescents are more sensitive than adults to the acute physiological and behavioural effects of stressor exposure. This is in line with the findings of others (Marquardt et al., 2004). Some have posited that adolescence is a period of vulnerability to stressors (Charmandari et al., 2005), which suggests that adolescents who experience repeated stressors may be at increased risk for developing psychopathological conditions (Romeo, 2010) and/or may suffer negative long-term consequences in adulthood. Others have found that adolescence may in fact be a period of relative resilience (Ferris et al., 2005). For instance, adolescent animals are more resilient than adults to the effects of locomotor-activating drugs (Bowen, Charlesworth, Tokarz, Wright, & Wiley, 2007). Such resilience may allow adolescents to sample the environment more freely, so that adolescent development may be guided by environmental cues, in order to optimize the adult phenotype for present conditions. Mechanisms by which adolescent stressor exposure may guide development are still not well understood. However, prefrontal cortical control over stress responding develops across adolescence, as neural relays among prefrontal and limbic areas mature and project to the HPA axis. There is likely some degree of plasticity in these connections that contributes to adolescent fine-tuning of stress sensitivity.

3.4.2. Long-term Effects of Stressor Manipulation

Long-term effects of stressor manipulation were most prominent when animals were exposed again to the stressor during the PT. Subtle effects were also observed in the context of the open field during Days 1 and 2 of adult testing. There were effects that were specific to having received stressor exposure during the adolescent period, as well as more general effects attributable to repeated stressor exposure or adolescent manipulation (stress or control).

3.4.2.1. Effects Specific to Adolescent Stressor Exposure

Prior repeated exposure to cat odour during either adolescence or adulthood reduced the duration of time spent in the odour area of the open field during the adult PT. However, animals stressed during adolescence were very reluctant to contact a homotypic stressor stimulus in adulthood, whereas those stressed in early adulthood were not as wary of contacting the stimulus later on in adulthood. These findings suggest that there are aspects of responding to stressors that signify predation threat for which adolescence is a sensitive developmental period, since adults exposed repeatedly to cat odour stimuli as adolescents show behavioural defense strategies in the presence of a homotypic stressor that enhance protection against further threat of predation. This enhanced behavioural defensiveness is displayed despite any increase in cort levels beyond that observed in animals that were control-exposed during adolescence. Enhanced defensiveness may come at the cost of increased risk for allostatic overload and thus, stress-induced disease (B. S. McEwen, 2004; Romeo, 2010), especially if the adult animal must cope with an environment replete with threat cues. However, if the animal

is able to fine-tune a behavioural defense repertoire to particular stressors that are encountered in adolescence that does not require excessive or prolonged elevations in cort, it could maximize adult fitness, in terms of stress sensitivity, for a chosen territory. This would allow the animal to respond appropriately to dangers that characterize the chosen territory, while minimizing responses to cues that don't pose as great a threat.

Interestingly, cort output following the adult PT was positively correlated with cort output following the final exposure in all groups, except the group that was repeatedly stressed during adolescence. The disruption of this association in the adolescent stressed group indicates that those adolescents showing the highest cort levels after the final stressor exposure were not necessarily those that showed the highest levels following the PT in adulthood. This provides evidence for a unique hormonal profile and stress responsiveness during the adolescent developmental phase. This profile includes differences in cort levels that may involve differences in production, usage, and/or clearance of cort and also suggests that hormonal responses to stressors may not be correlated between adolescence and adulthood, particularly if the individual has been exposed to the stressor repeatedly during adolescence.

3.4.2.2. General Effects of Stressor Manipulation

Having previously experienced repeated exposure to cat odour resulted in increased baseline levels of circulating cort and enhanced defensive responding, as indicated by reduced durations of time spent in the odour area of the open field during the PT, regardless of exposure age.

Baseline cort appears to be related to social behaviour (see Adolescence and Social Behaviour section below). These findings imply that repeated stressor exposure could affect future social interaction, which could be particularly relevant for adolescents. Other studies have demonstrated increased adult social anxiety in rats exposed to a repeated social stress paradigm in adolescence (Vidal et al., 2007; Watt et al., 2009).

3.4.2.3. General Effects of Adolescent Manipulation

The exposure context itself can be considered mildly stressful for rats, as the control manipulation involved brief handling and the arenas were bare of bedding material or hide space. Also, animals manipulated during the adolescent period had blood drawn for cort assay at this time. This procedure is inherently stressful, as it involves pricking open the saphenous vein for a brief bleed. Although animals recover rapidly, cort levels can reach ceiling levels that mask any prior difference between stressed and control animals, if blood is not collected within rigorous time limits after disturbing the animal (~30 s). If adolescents are more sensitive than adults to any enduring consequences of stressors, then we might expect the mild stress of the control manipulation and blood collection procedure to impact the adolescents more than the adults. Other studies have shown lasting effects of mild experimental manipulation, such as handling or repeated subcutaneous saline injection, during adolescence (Maldonado & Kirstein, 2005a, 2005b; Raap, Morin, Medici, & Smith, 2000).

Indeed, in the present study, animals exposed during adolescence showed more fear-related behaviour and higher levels of cort following the adult PT than those exposed during early adulthood, regardless of whether or not the stressor stimulus was involved.

Specifically, they entered the odour area less and spent longer durations hiding. Also, animals exposed during adolescence showed inhibited activity in the general context of the open field across days 1 and 2 of adult testing. Furthermore, they spent less time grooming in the open field, suggesting that adolescent manipulation resulted in animals allocating less time to self-maintenance behaviours in adulthood.

3.4.3. Adolescence and Social Behaviour

Adolescence is often referred to as a developmental phase characterized by increased peer-directed social contact (Spear, 2000). However, there is a dearth of data to support this claim in laboratory studies of rodents, as these animals are not normally at liberty to interact with others of their choice. In the present study, adolescents did spend more time in contact than adults did. Since social behaviour is especially prominent in adolescents, it may play a role in adolescent development of stress response systems and in levels of defensive behaviours displayed in adulthood. Indeed, social deprivation during adolescence has been shown to exert long-lasting effects on behaviour (Einon & Morgan, 1977). Similar relationships have been postulated previously, in both human (Caldwell, Rudolph, Troop-Gordon, & Kim, 2004) and animal studies (Ferris et al., 2005; Vidal et al., 2007; Watt et al., 2009), and neurotransmitter and/or hormone levels are oft studied in relation to the behavioural phenotype. While many aspects of social behaviour could modulate stress responding, we chose to focus on physical contact as a direct and simple measure of social interaction. Specific forms of physical contact have been shown in other contexts to modulate stress responding and also play a role in the development of the HPA axis (Weaver, Cervoni, Champagne, D'Alessio, Sharma, Seckl, Dymov, Szyf, &

Meaney, 2004). For example, maternal licking and grooming of rat pups influences stress-induced cort output and levels of hippocampal gluccocorticoid receptors in adulthood (Liu et al., 1997). Also, acute stressor exposure suppresses social play behaviour in juvenile rats (Romeo et al., 2006b).

In the present study, adolescent control animals were likely to be huddled in contact with the cagemate while motionless in the freezing position, a relationship not observed in the adults. The longer these animals spent in contact during exposures, the more robust their defensive responses to the PT in adulthood. (i.e. time in contact during exposures was negatively correlated with time contacting the stimulus and frequencies of entrance into the odour area in the PT, but it was positively correlated with time spent hiding in the PT). These associations were disrupted in the adolescent stress-exposed group and not observed in the adult groups.

Upon initial exposure, adolescents exposed to the stressor froze whether in contact with the cagemate or not, and thereby contacted each other less frequently. These findings suggest that stressor exposure may alter typical patterns of adolescent social interaction, which may influence adolescent development and have long-term consequences for adult stress responding and social behaviour. Males who spent long durations in contact during the initial adolescent stressor exposure had lower testosterone levels in adulthood. This may have implications for adult sexual behaviour in these males, as adult testosterone levels are an indicator of sexual motivation in rodents (Ferris et al., 2005).

Interestingly, in the animals exposed during adulthood, there were negative associations between baseline cort at the beginning of the exposure phase and time in

contact during exposures, such that those circulating low baseline cort levels spent longer durations in contact. Adolescents circulate very low baseline cort levels and spend longer durations in contact than adults do. These findings suggest that baseline cort levels may mediate social contact levels. Since adolescent stressor exposure increases baseline cort levels, it may also decrease adult levels of social contact.

3.4.4. Sex Differences

Females generally circulate higher cort levels than males (Bland, Schmid, Der-Avakian, Watkins, Spencer, & Maier, 2005; Bowman, Beck, & Luine, 2003; Critchlow et al., 1963), and these sex differences in HPA function emerge across the adolescent period (McCormick & Mathews, 2007). Accordingly, in the present study there was no sex difference in adolescent baseline cort levels at the beginning of the exposure phase, but higher cort levels were measured in females, relative to males, after the final adolescent exposure and at both sampling points in adulthood. As mentioned above, others have shown higher stress-induced cort level peaks in adolescents, relative to adults, after repeated exposure to a stressor (Romeo et al., 2006a). This is likely more prominent in males than in females, as females begin to circulate higher cort levels than males and continue to do so into adulthood. Indeed, the Romeo *et al*. (2006) study was conducted using males only, and, in the present study, adolescent males but not females circulated higher cort levels, relative to their adult counterparts, after the final exposure session.

There were no profound sex differences in defensive behaviours, although there were subtle differences in the way in which males and females responded during the adult PT. Males showed more exposure age differences in behaviours than females, a finding

that is in line with other studies (Douglas et al., 2003). For example, males exposed in adolescence showed inhibited activity in the PT, and males stressed as adolescents spent longer in the head-out position than males stressed as adults. Females did not show this pattern of effects in the PT, suggesting that males may be more sensitive to the enduring effects of adolescent stressor exposure. There are also studies that show increased sensitivity in females; for example, females exposed repeatedly during adolescence to a mixed isolation/social stress paradigm showed increased locomotor sensitization to nicotine in adulthood (McCormick et al., 2005). Indeed, in the present study, females stressed during adolescence showed inhibited activity in the general context of the open field across Days 1 and 2 of adult testing, but males did not. This suggests that repeated adolescent stressor exposure may enhance adult defensive responding within a similar stressful context in males, but in females the effects may generalize to other contexts.

Testosterone was only measured in males and, as expected, was higher in adults than in adolescents.

3.4.5. Concluding Remarks

In summary, it may be concluded that adolescents respond more robustly than adults to repeated cat odour exposure and also show more enduring fear-related behaviours three weeks later, once they've reached adulthood. The stressor manipulation increased baseline cort levels in both age groups, but manipulation (whether stress or control) during adolescence was the factor in our study that appeared to enhance future defensive responding most profoundly.

3.5. Summary and Research Direction

In Chapter 3, we have demonstrated that adolescence is a sensitive period for development of adult defensive behaviours. Although adolescents are considered to engage in more risky behaviour than adults, they are in fact more sensitive than adults to olfactory cues of predation threat, and exposure to such cues across the adolescent period leads to a more cautious behavioural profile in adulthood. Indeed, even the mild stress of repeated exposure to the stressor context during adolescence increases defensive behaviour in adulthood, relative to animals exposed to the stressor context during a time frame beyond the age of adolescence. While repeated stressor exposure during either age period increased baseline levels of circulating cort three weeks later, this endocrine alteration may have more profound consequences for adult cort dynamics when induced during the adolescent period, given that developmental change in cort levels also occurs at this time.

Chapters 2 and 3 have both indicated important connections among measures of adolescent social behaviour and stress-induced cort output and defensive behaviours in adulthood. In Chapter 4, we begin to explore potential neural bases for some of these connections. Specifically, stress responses are examined in adolescent rats exposed repeatedly to cat odour stimuli, and then adult stress responding is later examined in conjunction with a quantification of dopamine receptor protein levels in subregions of prefrontal cortex. The selected brain area undergoes major developmental modifications during adolescence and into adulthood, including changes in levels of dopamine

receptors, and is known to play important roles in adult stress responding and social behaviour. Thus, it was hypothesized to be an area involved in mediating adolescent developmental programming of the adult stress response, potentially via stress-induced changes in adult levels of dopamine receptor subtypes.

CHAPTER 4

PERIADOLESCENT STRESS EXPOSURE EXERTS LONG-TERM EFFECTS ON ADULT STRESS RESPONDING AND EXPRESSION OF PREFRONTAL DOPAMINE RECEPTORS IN MALE AND FEMALE RATS

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Abstract

Recent research has demonstrated that experiential/environmental factors in early life can program the adult stress response in rats, and this is manifest as altered hypothalamic-pituitary-adrenocortical activity and behaviour in response to a stressor. Very little work has been devoted to investigating whether the environment during adolescence plays a similar role in modulating ongoing developmental processes and how this might affect adult stress responding. Periadolescent predator odour (PPO) exposure was used here as a naturalistic model of repeated psychological stress. Behavioural and endocrine responses to PPO changed across the exposure period, and behavioural alterations persisted into adulthood. While adolescent rats showed pronounced avoidance responses upon initial PPO exposure, hyperactivity increased across the exposure period, especially in females. Corticosterone (cort) responses to stressor exposure also changed in females, with higher physiological baseline levels observed at the end of the exposure period. In adulthood, relative to rats that had received a control manipulation during adolescence, PPO-exposed rats were more fearful in a novel open field and displayed altered responses to a predator odour stress test. Moreover, lower levels of the D2 dopamine (DA) receptor were measured in prefrontal (infralimbic and dorsopeduncular) cortices of PPO-exposed rats. These findings suggest that the adolescent period may represent a sensitive period during which developmental programming of the stress response occurs.

4.1. Introduction

Stress responding is regulated by a set of integrated sub-systems that prepare the body and orchestrate action when a threat is encountered. One major element is the HPA axis, which interacts with other components of the stress circuitry and ultimately regulates release of glucocorticoids (corticosterone in rats; cortisol in humans) into the bloodstream (Herman et al., 2005). The circulating steroid hormone can then mediate various effects via interaction with receptors localized throughout the brain and the periphery (Charmandari et al., 2005). Although extensive exposure to glucocorticoids increases the allostatic load on the individual, which can lead to medical illness over time, or if combined with predisposing factors (B. S. McEwen, 2004), stress response repertoires generally enhance an individual's fitness. A more useful concept of stress might, therefore, lie in the promotion of a healthy stress response system that combats threat effectively and auto-regulates efficiently. Although the present study does not directly test this, it will aid in understanding how adult stress response repertoires may become fine-tuned to conditions that were present during adolescent development, and this information may be incorporated into strategies for maintaining a healthy adult system.

Optimal stress responding will be context-specific, since different types of stressors will necessitate different response strategies. Flexibility accordingly exists at various levels within the system, and environmental/experiential factors in early life can 'program' the mature system (Caldji et al., 2000; Liu et al., 1997; Meaney et al., 2002; Plotsky & Meaney, 1993). In rodents, for example, the level of maternal care experienced

during neonatal life alters expression of HPA activity markers and behavioural responses to stress in adulthood (Caldji et al., 2000). Maternal behaviour is flexible and can be altered by environmental factors, suggesting that it could convey ecological information to the developing individual by gauging environmental adversity (McLeod, Sinal, & Perrot-Sinal, 2007).

Interestingly, some stress response sub-systems continue developing well beyond the neonatal period, and carefully controlled studies indicate that adolescence represents a period of heightened stress sensitivity (Maldonado & Kirstein, 2005a, 2005b; Raap et al., 2000). The mesocorticolimbic DA system is integral to stress responding in the adult (Brake et al., 2000; Matsumoto et al., 2005; Sullivan & Gratton, 2002) and undergoes major developmental modifications during puberty. These include sex-dependent receptor pruning processes in striatum and PFC (Andersen & Teicher, 2000; Andersen et al., 2002; Andersen et al., 2000; Teicher et al., 1995; Teicher et al., 2003). Also during adolescence, defensive behaviours are added to the rats' battery of stress response tactics, which is dominated by avoidance behaviours at earlier ages (Hubbard et al., 2004). In adulthood, dopaminergic neurons in PFC are differentially responsive to stress, depending on whether a stressor is controllable or not (Carlson et al., 1993; Ravard et al., 1990). This suggests that their activation plays a role in cognitive processing, which matures during adolescence, and might regulate defensive behaviours that emerge concurrently.

The adolescent period is also marked by increased social behaviour, risk-taking, and novelty-seeking, behaviours thought to prepare the individual for independence (Douglas et al., 2003; Spear, 2000, 2004); these behaviours are all tied to mesocortical

DA function. For all of these reasons, we propose an involvement of DA receptor pruning processes in the behavioural changes characteristically emergent at adolescence. Our theoretical framework suggests that such processes might be sensitive to features of the adolescent environment, specifically those signifying threat, which would guide development in order to produce a system optimized for prevailing conditions. Capitalizing on the fact that mammalian neuroendocrine systems are 'hard-wired' to respond to species-specific psychological and/or social stressors, including predatory odours (Morrow et al., 2002; Perrot-Sinal et al., 1999), we have designed a stressor paradigm consisting of five episodic exposures to cat odour stimuli during the latter phase of adolescence (PND40-48; periadolescent period). This adolescent manipulation is an advantageous model of psychological stress, because it does not involve a physical component as do other commonly used stressor paradigms, such as restraint and social defeat. Furthermore, it allows evaluation of behaviour *during* stressor exposure, and defensive components of behavioural responses to predator stimuli do not habituate across repeated exposures in adulthood. Using cat odour stimuli as threat cues is becoming increasingly common in laboratory studies on stress responding, and results of these studies corroborate the ethological validity of the stressors (R. J. Blanchard et al., 2001; Hubbard et al., 2004; Perrot-Sinal et al., 1999). Stimuli used in the present study were derived from a number of different cats. While it is acknowledged that a trade-off exists between using varied and complex odorant stimuli and using a more pure stimulus with a known chemical composition, such as the commonly used fox odorant 2,5-dihydro-2,4,5-trimethylthiazoline, the former were chosen for the present study, in order to better simulate unpredictability in a natural environment with a high density of

predators. Furthermore, individual differences exist in the degree of response to specific predator odourants (Venton, Robinson, & Kennedy, 2006), and therefore, using stimuli composed of many odorant molecules may increase the likelihood of invoking a fear response in all exposed animals.

In the present study, we evaluated behavioural responses to this stressor across the adolescent period, and then investigated adult behaviour, cort levels, and expression of DA D1 and D2 receptors in PFC (infralimbic and dorsopeduncular cortices) and striatum. We also examined adolescent cort levels at the beginning and end of the stressor paradigm, in a separate group of animals, as there are currently no known published data on changes in cort levels following exposure of adolescents to olfactory cues of predation threat.

4.2. Materials and Methods

4.2.1. Subjects

4.2.1.1. General Husbandry Procedures

Rats were housed in same-sex pairs (unless otherwise indicated) in a standard colony room in the Psychology Department (Life Sciences Center). Cages were 22 x 24 x 48 cm polypropylene models covered with wire lids, and each contained wood-chip bedding and an ~5-inch-piece polyvinyl-carbonate black tubing for enrichment. The colony room was maintained at 20 ± 1 °C with a 12:12 reverse light:dark cycle (lights off at 0930h). Food (Purina Lab Chow) and tap water were available *ad libitum*. Prior to any experimental manipulation, rats were handled each day for five days (at least 2 min per day) by the experimenter. This always occurred during the rats' active phase

(subjective night), and entailed being picked up, touched, and held. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

4.2.1.2. Breeding

Male and female Long-Evans rats purchased from Charles River, Canada, were used as breeders. Male breeders were paired with a female in a standard cage for 5 days, at which time the male was removed.

Females were housed singly during pregnancy and were checked daily for litters, beginning on estimated gestational day 20. Deliveries proceeded naturally, and the day of birth was designated as PND0. Dams/pups were not manipulated in any way, except for once-weekly cage changing, until offspring were weaned on PND22. Experimental litters were not culled; all pups were destined for participation in either Experiment 1 (n = 48) or Experiment 2 (n = 45). Weaned pups were housed in same-sex, littermate pairs or groups of three when necessary to avoid isolation housing, and individuals were identified using non-toxic ink markings applied to the tail during handling and retouched approximately once per week. Littermate pairs were randomly allocated to adolescent treatment condition.

4.2.1.3. Experimental Group Designation

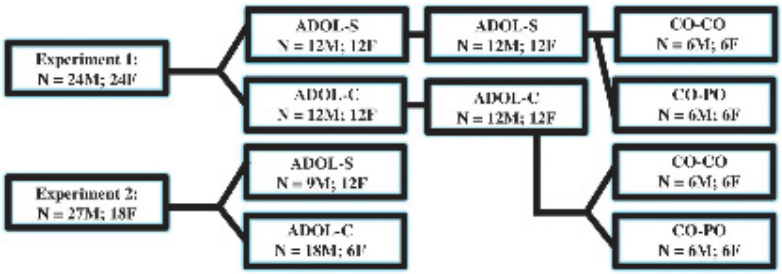
From six litters, forty-eight rats (n = 24 males and 24 females) were randomly selected at PND22 for participation in Experiment 1, and the remaining 45 rats

constituted the sample for Experiment 2. Each experimental group contained rats from 3 different litters. Figure 4.1 illustrates the experimental design. The objective of Experiment 1 was to examine acute and long-term behavioural responses to stressor exposure during adolescence (PND40-48; see below for details of the stressor paradigm). Twelve males and 12 females constituted each of the adolescent stressed (ADOL-S) group and adolescent control (ADOL-C) group. These animals were monitored for behaviour during adolescence and then followed into adulthood to assess: 1) behaviour in an open field test; 2) behaviour in response to exposure to a cat odour stressor (n = 6 males and 6 females) or a control stimulus (n = 6 males and 6 females), 3) circulating levels of plasma cort after the final behavioural test, and 4) levels of expression of D1 and D2 DA receptors in dorsal striatum and mPFC in adult control-exposed animals only. The objective of Experiment 2 was to examine plasma cort levels at the beginning and end of the adolescent stressor exposure period, as described below (n = 9 adolescent males and 12 adolescent females exposed to the stressor regimen, n = 18 control males and 6 control females). For Experiment 2, groups were created from the offspring remaining from six litters, following designation of males and females for Experiment 1. This, along with requirements that each experimental group contain subjects from at least three different litters and cagemate pairs were to receive the same adolescent treatment, resulted in unequal sample sizes for Experiment 2.

4.2.2. Adolescent Stress Paradigm

Males and females in the ADOL-S group were exposed in a bare environment with no hide space (see below for description) to a cat odour stimulus (predator odour;

Figure 4.1. Schematic illustration of the experimental design and timeline used for Experiment 1 and Experiment 2. For both experiments, rats were exposed repeatedly to cat odour (ADOL-S; see Methods) or a control condition (ADOL-C) during the late adolescent period (post natal day (PND) 40-48; ADOL-stress). Experiment 1 rats were tested for behavioural responding in adulthood using 2 open field (OF) exposures (Day 1 and Day 2) and a predator odour test (PT). As indicated, they all received control odour (CO) during the first part of the test and then received either predator odour (CO-PO) or control odour (CO-CO) during the second phase of PT. At sacrifice, blood was taken for measurement of corticosterone levels and brain samples were dissected for assessment of dopamine receptor levels. Rats in Experiment 2 were used to assess physiological baseline changes in plasma corticosterone levels and changes in levels following stressor exposure across the ADOL-stress period.



PND0	PND22	PND40-48	PND60-61	PND62
Birth	•Weaning •Group designation	•ADOL-Stress exposure •Blood sampling	•OF Day 1-2	•PT 0 - 5 min - Habituation 6 - 15 min - CO (all animals) 16 - 25 min - CO (half animals) *or* PO (half animals) •Sacrifice - blood sampling and tissue microdissection

PO), together with 1 or 2 littermate(s) for 30 min. This duration of predator odour exposure has been shown previously to induce activation of the HPA axis in adults (Masini et al., 2005). Exposure occurred on five occasions between PND40 and PND48 (PND40, 41, 44, 47 and 48), during the active (dark) phase of the light:dark cycle. Adolescents were exposed with the littermate(s) rather than alone, since isolation during this developmental stage is itself considered stressful (Frisone et al., 2002). The cat odour stimulus used in this experiment was acquired from 2-4 gonadally intact, domestic cats housed communally in the Psychology Department at Dalhousie University. Cat odour stimulus was comprised of hair and dander, removed from the cats each day an exposure session occurred. The hair and dander were rubbed onto 2.5 x 15 cm strips of yellow non-antibacterial disposable cloth. Rats in the ADOL-C group were exposed in an identical manner except that clean strips of cloth were used as a control odour stimulus (control odour; CO). See Figure 4.1 for an experimental timeline.

Exposures took place in a clear, rectangular, Plexiglas arena measuring 35.5 cm high, 27 cm wide, and 60 cm long. A 2.5 cm alligator clip was centered and positioned 6.5 cm from the bottom of one end wall, and this was used to secure the odour stimulus during an exposure. Six arenas were placed next to each other and were separated by white cardboard (35.5 x 60 cm). Each was situated on a white opaque Plexiglas floor and covered with a clear Plexiglas lid with ventilation holes.

4.2.3. Behavioural Testing

4.2.3.1. Behavioural Responses to Adolescent Stressor Exposure

The exposure period corresponds to the latter phase of adolescence (Hodes &

Shors, 2005; Spear, 2004). During the first (PND40) and last (PND48) adolescent exposure session (stress or control), ADOL-S and ADOL-C rats taking part in Experiment 1 were videotaped using two Sony 8 mm digital cameras (CCD-TRV65 or CCD-TRV108) suspended directly above the arenas. Behaviours were then quantified for the first seven min of exposure, with the aid of The Observer 5.0.31 software (Noldus, Netherlands). The adolescent odour exposure arenas were designed to provide a relatively confined, vulnerable environment, while the larger adult behavioural test arenas contained hide boxes and allowed measurement of a wider range of species-typical defensive responses to cues of predation threat. For the purposes of analysis, the exposure arena was divided into three compartments of equal area: termed, the odour area, the middle area, and the safe area. Movement from one area into another was scored as a line cross, and frequency of line crosses was taken as a measure of general activity. Rates of entry into each area were also scored, along with durations of time spent in each area. Finally, rates and durations of contact with cagemate (bodily contact excluding the tail; termed social contact) were scored for each animal (and thus scores were equal for the two members of each cagemate pair). Effects of the stressor on social contact were of interest, because it is unknown whether the increases in peer-directed behaviour typically displayed by adolescent rats are influenced by stressful conditions or whether or not such an interaction could play a role in mediating effects of adolescent stress on adult phenotype.

4.2.3.2. Behavioural Testing During Adulthood

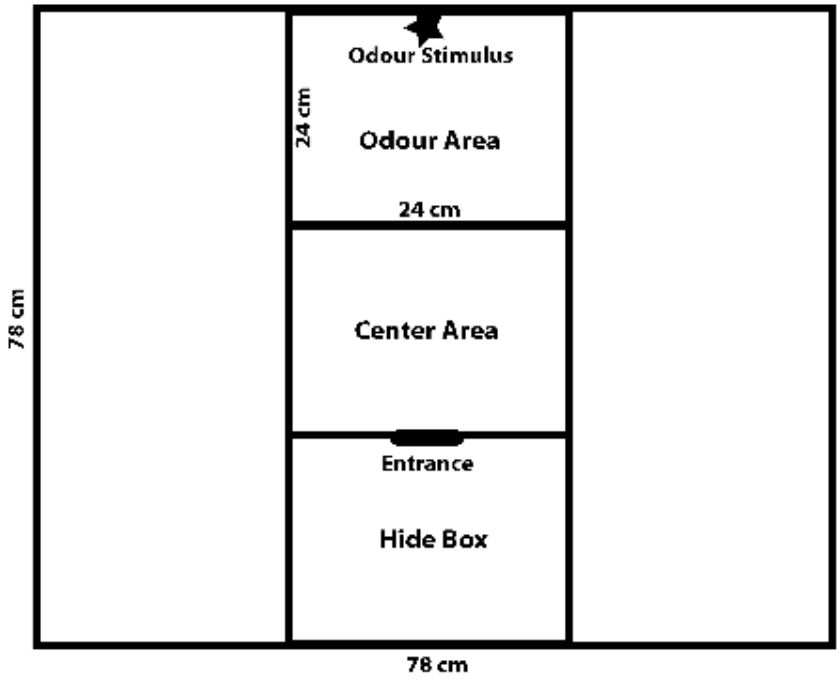
For ADOL-S and ADOL-C rats, adult testing began on PND60, which represents

early adulthood (Slawecki, Gilder, Roth, & Ehlers, 2003; Spear, 2004), and was comprised of two open field (OF) sessions, followed by a predator odour test (PT) session. Testing sessions (OF Day 1, OF Day 2, and PT) occurred across three consecutive days (20 min each for OF Day 1 and 2, 25 min for PT), and each session took place in the same apparatus. The behavioural apparatus measured 35.5 cm high, 79 cm wide, and 79 cm long and was made of 1/4-inch black Plexiglas (Fig. 4.2). A black Plexiglas hide box (22 cm high, 24 cm wide, and 21.5 cm long, with a 6 cm x 6 cm door at the front) was placed in the center of one wall. The behavioural apparatus was covered with a Plexiglas lid containing ventilation holes. For the PT test on Day 3, the odour stimulus was placed in the center of the wall opposite the hide box.

The first 20 min OF session (OF Day 1) was used to measure anxiety-related behaviours in response to a novel environment (Kalynchuk et al., 2004), while the second 20 min OF session (OF Day 2) permitted an assessment of habituation lasting 24 hr. On the third day, rats were administered the PT session, which began with habituation to the behavioural apparatus for 5 min, followed by presentation of CO for 10 min. This part of the PT session served as a within-subject control condition for assessing baseline activity and was administered to all animals. Following this, half of the ADOL-S group and half of the ADOL-C group were presented with the PO stimulus (CO-PO group), while the other half were presented with CO again (CO-CO group), for the final 10 min of the session (see Fig. 4.1). The order of CO and PO could not be counterbalanced for the CO-PO group, due to the rapid formation of contextual associations in rodents presented with cues of predation threat; (R. J. Blanchard et al., 2001; McGregor et al., 2002).

Behaviours elicited in the OF and PT sessions were recorded using a Sony 8 mm

Figure 4.2. Schematic illustration of the behavioural test apparatus employed for the open field (OF) exposures (Day 1 and Day 2) and predator odour test (PT) administered to rats in adulthood. The box contained a hide box across from the position of the odour stimulus (predator or control). For the purpose of analyzing space use, the box was divided into the hide box area, the center area, and the odour area. Locomotor activity was measured by analyzing movement from one area to another, including the areas outside of these 3 main areas.


Odour Stimulus
24 cm
Odour Area
24 cm
78 cm
Center Area
Entrance
Hide Box
78 cm

digital camera (CCD-TRV65 or CCD-TRV108) suspended above each test arena. Behaviours were later quantified with the aid of The Observer 5.0.31 software (Noldus, Netherlands), for the first 10 min of each 20 min OF session, and for the first 7 min of each of the 10 min PT sessions (CO and CO/PO). Rates and durations were quantified for the following non-defensive and defensive behaviours:

Rearing (exploratory, non-defensive) – Front legs leave contact with the floor of the arena. They may rest on a wall during rearing.

Grooming Bout (non-defensive) – A series of uninterrupted motions using the mouth and/or paws to clean any part of the body (minimum 1 s duration).

Head-out (defensive) – The head of the rat, including the eyes, protrudes from the hide box. A head-out ends when half the body is beyond the opening or when the head retreats into the hide box. The area midway between the forelimbs and hindlimbs is considered to represent the halfway point along the rostro-caudal body axis.

Odour Stimulus Contact (exploratory) – Bodily contact (excluding tail) with the odour source.

Space Use - Amount of time spent in, and rate of entry into the hide box, the center area, and the odour area (see Fig. 4.2).

Locomotion – Amount of time spent in, and rate of entry into any of the virtual regions (see Fig. 4.2).

4.2.4. Assessment of Plasma Corticosterone Levels

4.2.4.1. Corticosterone Levels Following Stress Testing in Adulthood

Immediately following the completion of the PT session, animals were

anesthetized with CO_2 gas and decapitated. Trunk blood was collected into 600 μl Microtainer plasma separator tubes containing lithium heparin (Becton Dickinson, United States), and the samples were then centrifuged at 6000 x g for two minutes at 4 ˚C. Blood plasma was collected and aliquoted into three 80 μl samples, which were frozen at -80 ˚C until assay.

4.2.4.2. Corticosterone Levels Following Stressor Exposure in Adolescence

Changes in physiological baseline levels of cort were assessed across the adolescent period. Samples were taken on evenings prior to the first and last PPO exposures (PND39 and PND47), between 1800h and 2200h, at the beginning of the light cycle. Resting levels of cort secretion reach their circadian nadir at this time; thus, these samples served to measure a physiological baseline for each rat (Atkinson & Waddell, 1997). In addition, changes in cort secretion following PPO (or control) exposure were assessed across the exposure period. Blood samples were collected between 1000h and 1600h, from the saphenous vein, following the first (PND40) and last (PND48) 30 min adolescent exposure session (ADOL-S and ADOL-C), and these were normalized to the respective mean group baseline at each of the sample periods. Immediately following the completion of the 30 min exposure period, animals were returned to their home cage and transported to an alternate room for the blood sampling. Transport time was approximately 5 minutes. To collect a sample of blood, a hind leg was shaved, and a thumb was used to apply pressure to the saphenous vein, which was pricked with a 21-gauge needle (Becton Dickinson, United States). The vein was bled into 600 μl Microtainer plasma separator tubes and treated as above.

4.2.4.3. Corticosterone Assay

Samples were diluted 1:50 with assay buffer (tris-buffered saline with sodium azide preservative and addition of steroid displacement reagent) and assayed using the Correlate-EIA Corticosterone Enzyme Immunoassay Kit (Assay Designs, Michigan, USA). Each 96-well plate was assayed as per the manufacturer's instructions, with samples maintained on ice throughout. Intra- and inter-assay coefficients of variation for this kit range between 6.6 - 8.0% and 7.8 - 13.1%, respectively.

4.2.5. Expression of D1 and D2 Dopamine Receptors in Adult Prefrontal Cortex and Striatum

4.2.5.1. Tissue Collection

Immediately following the second 10-min CO session of the PT in adulthood, Experiment 1 rats designated to the CO-CO group (not exposed to PO in adulthood) were anesthetized in a CO_2 chamber, and brains were rapidly removed, flash-frozen, and stored at –80 ˚C until processing. Specimens were warmed to -4 ˚C, and sections (200 - 400 μm) between +3.20 to +2.20 relative to Bregma were taken using a cryostat with the knife temperature set at -10 ˚C. Sections were mounted on glass slides chilled on dry ice, and the PFC (infralimbic and dorsopeduncular subregions) and striatum were micropunched with an 18 gauge blunted needle attached to a syringe using a modified Palkovitz procedure (Perrot-Sinal, Davis, & McCarthy, 2001). The syringe was filled with air before obtaining the punches for each region from each brain, which permitted the punches to be expelled rapidly from the needle into a microtube. Tubes were placed

immediately on dry ice and stored at –80 ˚C until further processing, and the needle was cleaned with ethanol between samples.

4.2.5.2. Western Immunoblotting

Samples of each region from each animal were homogenized in 40 μl chilled lysis buffer consisting of 50 mM Tris-HCl, 0.25% Na-deoxycholate, 1% w/v Triton X-100, 150 mM NaCl, 1mM EDTA, 1 mM activated Na-orthovanadate, and a protease inhibitor cocktail (1 μg/ml aprotinin, leupeptin, and pepstatin; 1 mM phenylmethylsulfonyl fluoride). Samples were manually disrupted with a teflon pestle, followed by 10 min of ultrasonication in an ice-cold water bath. Total protein concentrations were determined using the colorimetric method of Bradford. Western blotting methods were similar to those described previously (Perrot-Sinal et al., 2001). Briefly, protein (30 μg) from each sample was loaded onto 10% SDS-polyacrylamide gels that were cast the preceding evening and stored covered by 0.1% SDS at 4 ˚C. Electrophoresis was conducted for 3 hours at 125 V, and separated protein was then transferred to a polyvinyl difluoride membrane (BioRad, Hercules, CA). Membranes were washed briefly in 0.1 M Tris-buffered saline containing 0.05% Triton X-100 (TTBS) and then blocked with constant agitation for one hour at room temperature in TTBS containing 4% non-fat dry milk. Membranes were then incubated with agitation in 1:1000 anti-D1 DA receptor (D1DR: Santa Cruz) and 1:20000 anti-glyceraldehyde-3-phosphate dehydrogenase (Gapdh; Chemicon; used as a loading control) overnight at 4 °C. The following day, membranes were washed three times for 5 min each in TTBS and then incubated in a goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG in TTBS with agitation for 30 min at room

temperature, followed by another three 5-min rinses in TTBS and 2 rinses in TBS. Immunoreactive bands were detected using an enhanced chemiluminescence kit (New England Biolabs, Inc., Beverly, MA) and membranes were exposed using Kodak Imagestation 440. Membranes were again washed three times for 5 min each in TTBS and incubated in an avidin HRP-conjugate IgG in TTBS (1:5000) with agitation for 30 min at room temperature, followed by another three 5-min rinses in TTBS and 2 rinses in TBS, which allowed detection of broad range molecular weight standards (Biorad, Hercules, CA) run in one lane on each blot. D1DR was detected as a single band at the expected molecular weight of 74 kDa and was analyzed relative to intensity of the Gapdh band at 36 kDa. The following day, blots were stripped and reincubated with anti-D2 dopamine receptor (D2DR) antibodies (1:1000). Blots were visualized as above and the band corresponding to a molecular weight of 51 kDa was analyzed, again relative to Gapdh intensity.

4.2.6. Statistical Analyses

4.2.6.1. Behaviour

For each of the following behavioural test sessions, separate statistical analyses were conducted: 1) adolescent responses to stressor exposure (first session (PND40) and last session (PND48)), 2) adult responses during the OF sessions (OF Day 1 and OF Day 2), and 3) adult responses to the PT session (CO-CO and CO-PO). For each analysis, a repeated measures ANOVA was conducted to analyze each dependent variable (see above). For analyses 1 and 2 above, Sex (male or female) and adolescent stress (ADOL-stress) treatment (ADOL-S and ADOL-C) were between-subjects factors. For analysis 1,

Session was the repeated measure, and for analysis 2, Day was the repeated measure. For analysis 3, there is an additional between-subjects factor (ADULT ODOUR), as only half the animals were exposed to PO. For this analysis, the group who had received only the control odour stimulus in adulthood (i.e., CO-CO) was analyzed separately from the group who experienced predator odour exposure (i.e., CO-PO), and patterns of results were compared between the two groups.

Wherever significant interaction effects were indicated, appropriate simple effects analyses were conducted, and statistics for these are reported when they revealed a significant contribution.

The criterion for considering findings significant was set at $\alpha = 0.05$.

4.2.6.2. Corticosterone Levels

For the adult samples, a 3-factor ANOVA was conducted, with Sex (male or female), ADOL-stress (ADOL-S or ADOL-C), and ADULT ODOUR (CO-CO or CO-PO) as between-subjects factors.

For the adolescent samples, data for the two sexes were analyzed separately. The well-established sex differences in the set-up of the adult HPA axis emerge over the adolescent period (McCormick & Mathews, 2007); however, details of this process are still unclear. ADOL-stress (ADOL-S or ADOL-C) was treated as the between-subjects factor, and there were two levels of the repeated measure, Sampling Period 1 (experimental sample on PND40 normalized to baseline sample on PND39) and Sampling Period 2 (experimental sample on PND48 normalized to baseline sample on PND47).

4.3. Results

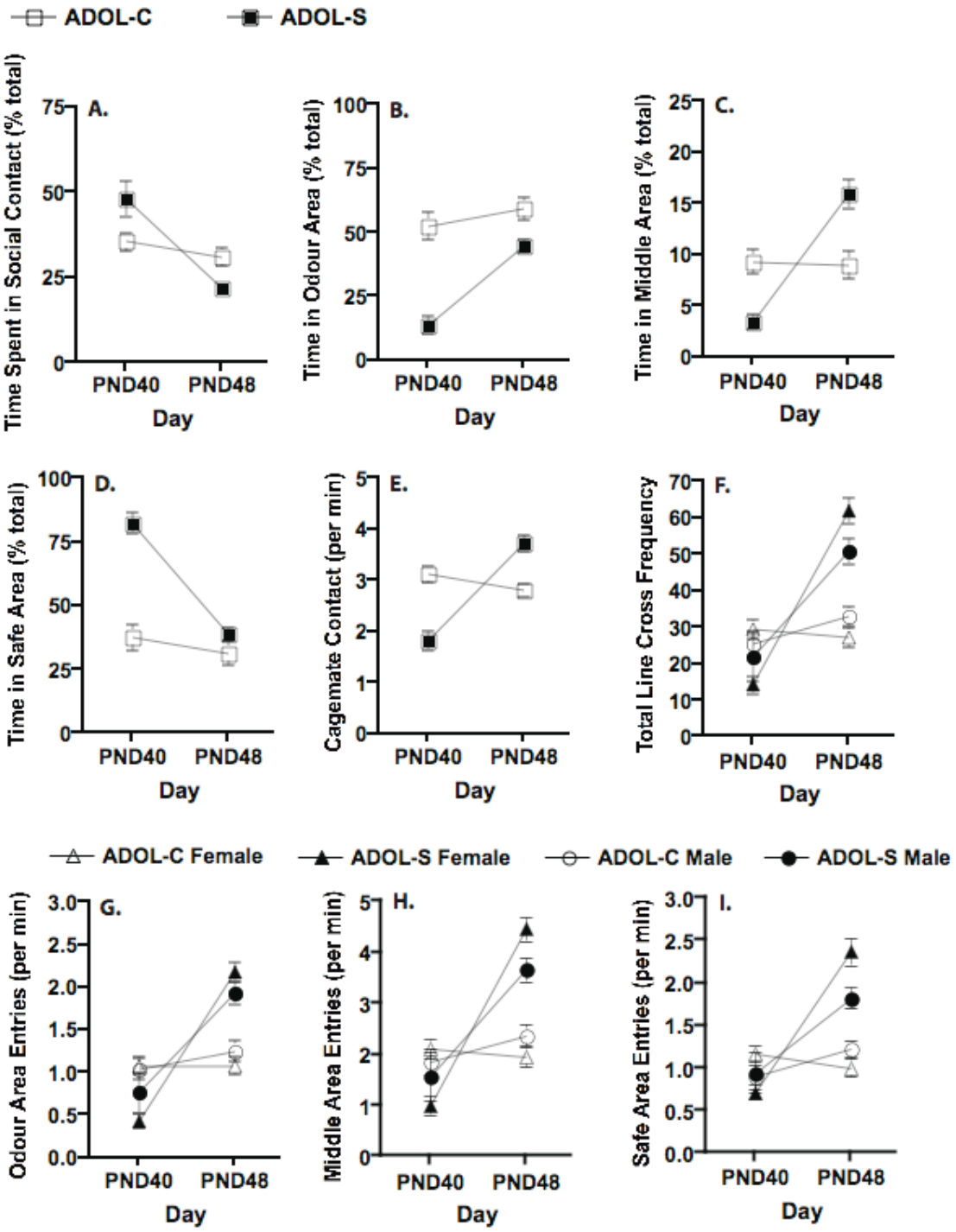
4.3.1. Behavioural Responses to Adolescent Exposures

Several measures of general activity were quantified for the first seven minutes of the first (PND40) and last (PND48) adolescent exposure in ADOL-S and ADOL-C groups. ADOL-S rats showed a robust response to the initial cat odour exposure, in the form of inhibition of activity, avoidance of the odour source, and increased huddling with the cagemate (manifest as increased social contact). Across repeated exposures, however, they increased several measures of activity in the exposure environment to levels significantly higher than ADOL-C.

4.3.1.1. Avoidance of Cat Odour During Adolescence

Repeated measures ANOVA indicated significant Session x ADOL-stress interaction effects for durations spent in all regions, as well as duration of social contact (*p's* < 0.01). Simple effects analyses were conducted for each of the first (PND40) and last (PND48) adolescent exposure sessions. Upon initial odour exposure on PND40, ADOL-S rats spent more time in contact with the cagemate (Fig. 4.3A), less time in the odour area (Fig. 4.3B) and middle area (Fig. 4.3C), but more time in the safe area (Fig. 4.3D), relative to ADOL-C (*p*'s < 0.01). By the final exposure on PND48, ADOL-S rats were still spending less time in the odour area relative to ADOL-C (Fig. 4.3B; $p < 0.05$). However, they had *increased* their time in the middle area to levels significantly

Figure 4.3. Behaviours exhibited by rats in the first seven min of predator odour (ADOL-S) or control (ADOL-C) exposure during the first (post natal day (PND) 40) and last (PND48) of five sessions administered during the adolescent period. ADOL-S rats initially showed robust inhibitory responses to cat odour exposure; however, repeated exposures induced hyperactivity in these animals. ADOL-S rats spent more time in social contact on PND40, but less time on PND48, relative to ADOL-C (A). They spent less time in the odour area on both days (B) and less time in the middle area on PND40, but more time on PND48 (C). ADOL-S rats spent more time in the safe area on PND40, relative to ADOL-C (D). Rates of cagemate contact were higher in ADOL-C rats relative to ADOL-S on PND40 (E). Finally, adolescent stress (ADOL-stress) increased rates of line crossing (F), as well as odour area (G), middle area (H), and safe area entrance rates (I), and females showed more pronounced effects in all of these activity measures. (All p's < 0.05).


ADOL-C
ADOL-S
A.
Time Spent in Social Contact (% total)
B.
Time in Odour Area (% total)
C.
Time in Middle Area (% total)
D.
Time in Safe Area (% total)
E.
Cagemate Contact (per min)
F.
Total Line Cross Frequency
PND40
PND48
Day
ADOL-C Female
ADOL-S Female
ADOL-C Male
ADOL-S Male
G.
Odour Area Entries (per min)
H.
Middle Area Entries (per min)
I.
Safe Area Entries (per min)

above ADOL-C (Fig. 4.3C), and correspondingly *decreased* their time spent in social contact (Fig. 4.3A; p's < 0.01). An avoidance response to cat odour presentation was further supported by significant main effects of ADOL-stress on overall durations of time spent in the odour area and in the safe area, respectively (p's < 0.001), whereby ADOL-S rats spent more time out of the odour source area and retreating within the safe area, relative to ADOL-C.

4.3.1.2. Cat Odour-induced Changes in Activity Rates During Adolescence

Repeated measures ANOVA indicated a Session x ADOL-stress interaction effect for rates of social contact (Fig. 4.3E), and simple effects analysis revealed that, during the first exposure session on PND40, ADOL-S rats showed reduced contact rates relative to ADOL-C rats (p < 0.05). Moreover, a number of Session x ADOL-stress x Sex interaction effects emerged, and these are depicted by separation of the sexes for the appropriate measures in Figure 4.3. Simple effects analyses conducted for the first and last exposure sessions revealed that, during the first exposure on PND40, ADOL-S rats showed reduced rates of line crossing (Fig. 4.3F), odour area entrances (Fig. 4.3G), and middle area entrances (Fig. 4.3H), relative to ADOL-C rats (p's < 0.05), which is consistent with the typical adult pattern of behavioural inhibition displayed in response to predation threat cues. However, by the fifth adolescent exposure session on PND48, ADOL-S rats were showing *increased* rates of line cross (Fig. 4.3F), entrance into all three regions (Fig. 4.3G-I), and social contact (Fig. 4.3E; p's < 0.001). Simple effects analyses for each sex indicated that these effects were driven primarily by the behaviour of the females for rates of line cross (Fig. 4.3F) and middle area entrance (Fig. 4.3H; p's

< 0.01 in females but > 0.05 in males), and a significant Session x ADOL-stress interaction (Fig. 4.3I; $p < 0.001$ in females but > 0.05 in males) for safe area entrance rate. In all cases, ADOL-S females had become hyperactive (relative to ADOL-C) in response to the final odour exposure on PND48. The three-way interaction for rate of odour area entries was not further resolved by separation of the sexes in simple effects analyses.

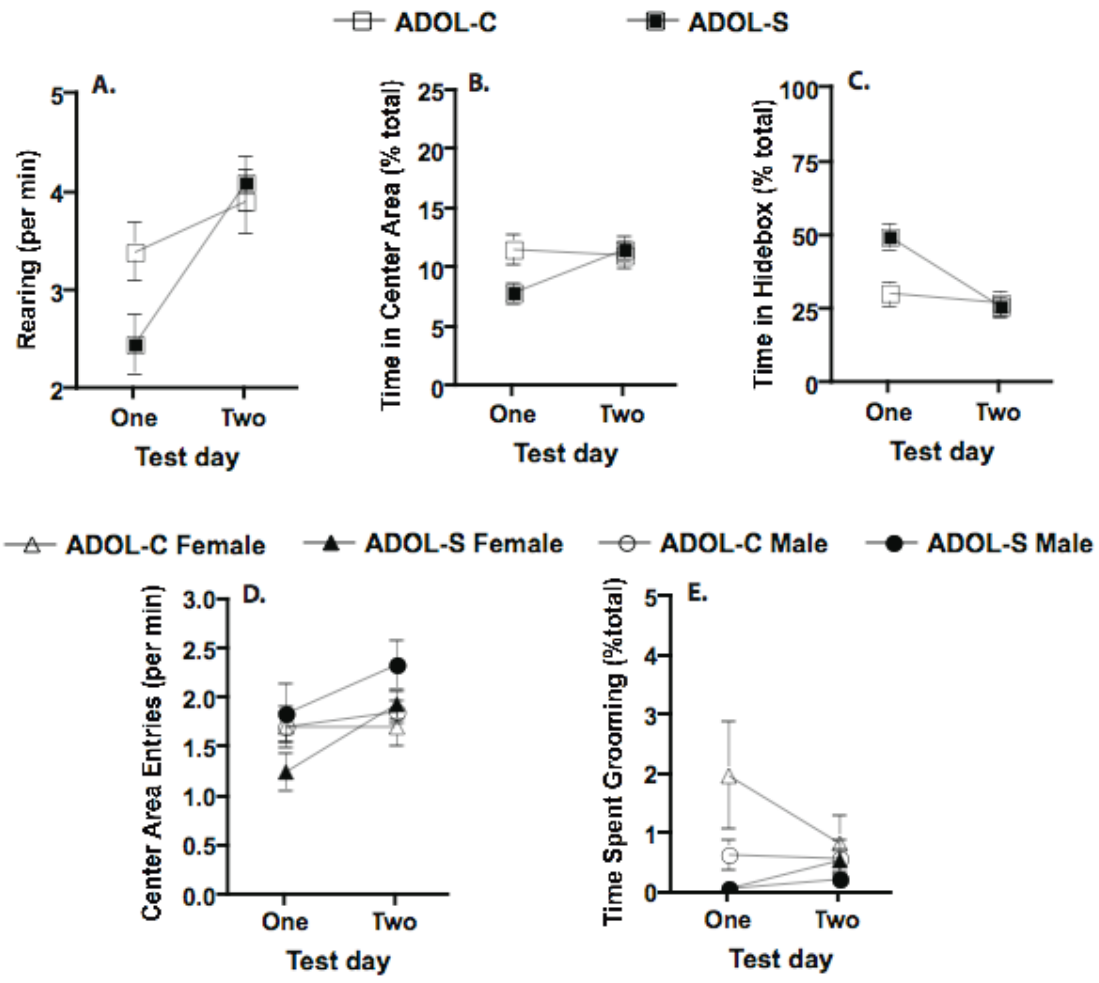
In addition, main effects of ADOL-stress were observed for rates of line crossing (Fig. 4.3F; $p < 0.01$), as well as odour area (Fig. 4.3G; $p < 0.05$), middle area (Fig. 4.3H; $p < 0.01$), and safe area (Fig. 4.3I; $p < 0.001$) entrance, with ADOL-S rats showing higher overall movement between regions relative to ADOL-C.

4.3.2. Behavioural Responses in Adulthood

4.3.2.1. Open Field Behaviour

There was a significant Day x ADOL-stress interaction for rates of rearing (Fig. 4.4A), as well as for durations of time spent in the center area (Fig. 4.4B) and in the hide box (Fig. 4.4C; p's < 0.05). These effects are suggestive of a greater response in the ADOL-S animals to the novelty of the open field, as ADOL-S animals reared less frequently and spent less time in the center but more time in the hide-box on Day 1, though these differences did not reach statistical significance when simple effects analyses were conducted for Day 1 and Day 2. There was also a main effect of ADOL-stress on rates of rearing (Fig. 4.4A; $p < 0.05$), with ADOL-C rats showing higher overall rates compared with ADOL-S.

Figure 4.4. Rats exposed to cat odour during adolescence (ADOL-S) showed enhanced responses to novelty during the first of two 20 min open field (OF) exposures administered in early adulthood on post natal day (PND) 60 and 61). This was manifest as reduced rates of rearing (A) and time in the center area (B), as well as increased time in the hide box in OF Day 1. There were also significant Sex x Day x ADOL-stress interaction effects for rates of center area entries (D) and durations of time spent grooming (E). (All p's < 0.05).


ADOL-C
ADOL-S
A.
Rearing (per min)
5
4
3
2
One
Two
Test day
B.
Time in Center Area (% total)
25
20
15
10
5
0
One
Two
Test day
C.
Time in Hidebox (% total)
100
75
50
25
0
One
Two
Test day
ADOL-C Female
ADOL-S Female
ADOL-C Male
ADOL-S Male
D.
Center Area Entries (per min)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
One
Two
Test day
E.
Time Spent Grooming (%total)
5
4
3
2
1
0
One
Two
Test day

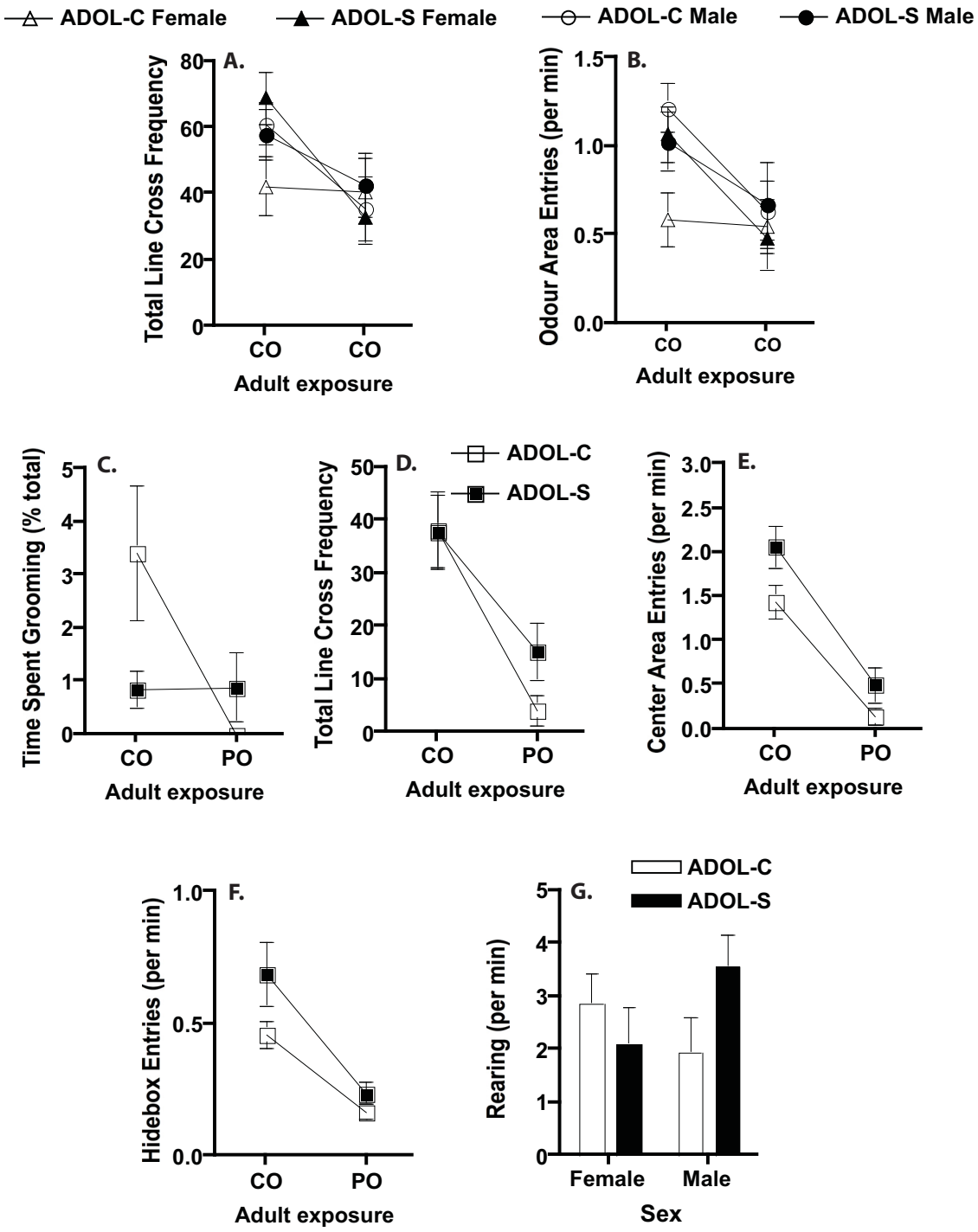
In addition, there were significant Sex x Day x ADOL-stress interaction effects for rates of center area entries and duration of time spent grooming (Fig. 4.4D-E; p's < 0.05); however, these complex interactions were not further resolved by simple effects analyses.

4.3.2.2. Responses to Cat Odour Exposure

There were significant Sex x Day x ADOL-stress interaction effects in the CO-CO group for line cross frequency (Fig. 4.5A) and for rates of odour area entry (Fig. 4.5B; p's < 0.05), and these appear to be driven by a tendency toward less activity in the ADOL-C females during the first control odour exposure, though simple effects analyses did not provide statistical confirmation at the accepted level of significance.

For the CO-PO group, there was a significant Sex x ADOL-stress interaction for rates of rearing (Fig. 4.5G; $p < 0.05$), such that ADOL-S appeared to display increased rates relative to ADOL-C only in males, though this difference in males did not reach the criterion for statistical significance in simple effects analyses for each sex. There were significant main effects of ADOL-stress for time spent grooming (Fig. 4.5C), as well as for rates of line cross (Fig. 4.5D) and center area (Fig. 4.5E) and hide-box entry (Fig. 4.5F; p's < 0.05). ADOL-S rats showed increased activity but spent less time grooming, relative to ADOL-C, further suggesting that the effects of adolescent stressor exposure endured into adulthood.

Figure 4.5. Behaviours exhibited by adult rats during the first 7 min in each condition of a predator odour test (PT) administered on post natal day (PND) 62**.** Half of the ADOL-C and ADOL-S rats were exposed only to control odour (CO) during two 10 min sessions (CO-CO), and for this group there were significant Sex x Day x ADOL-stress interaction effects for line cross frequency (A) and for rates of odour area entry (B), with ADOL-C females showing less activity during the first CO exposure. The other half of ADOL-C and ADOL-S rats received predator odour (PO) exposure for the second 10 min session (CO-PO); ADOL-S rats showed increased activity but spent less time grooming, relative to ADOL-C, across both sessions. They spent less time grooming (C), crossed more lines (D), and entered the center area (E) and hide-box (F) more frequently (G). In addition, ADOL-S males, but not females, showed increased rates of rearing (H). (All p's < 0.05).


ADOL-C Female
ADOL-S Female
ADOL-C Male
ADOL-S Male
A.
Total Line Cross Frequency
80
60
40
20
0
CO
CO
Adult exposure
B.
Odour Area Entries (per min)
1.5
1.0
0.5
0.0
CO
CO
Adult exposure
C.
Time Spent Grooming (% total)
5
4
3
2
1
0
CO
PO
Adult exposure
D.
ADOL-C
ADOL-S
Total Line Cross Frequency
50
40
30
20
10
0
CO
PO
Adult exposure
E.
Center Area Entries (per min)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CO
PO
Adult exposure
F.
Hidebox Entries (per min)
1.0
0.5
0.0
CO
PO
Adult exposure
G.
ADOL-C
ADOL-S
Rearing (per min)
5
4
3
2
1
0
Female
Male
Sex

4.3.3. Corticosterone Levels Following Cat or Control Odour Exposure in Adulthood

Only a sex difference was statistically demonstrated in these particular samples (see Methods for sample description). Females had higher circulating cort levels relative to males ($p < 0.05$).

4.3.4. Expression of D1 and D2 Dopamine Receptors in Adulthood

Expression of D2 DA receptors in mPFC was significantly decreased in ADOL-S rats, relative to ADOL-C ($p < 0.01$; Fig. 4.6). Levels of D1 DA receptors were also observed to be lower in this brain region relative to ADOL-C; however, this reduction was not statistically significant ($p = 0.223$; Fig. 4.6), nor was there a group difference in dorsal striatal expression of either D1 or D2 (data not shown).

4.3.5. Corticosterone Levels in Response to Adolescent Exposures

There was no effect of ADOL-stress treatment on male cort levels (Fig. 4.7A); however, there was a significant ADOL-stress x Sampling Period interaction in the females ($p < 0.05$; Fig. 4.7B). Simple effects analyses conducted for each sampling period indicated lower normalized cort levels in ADOL-S females, relative to ADOL-C, at Sampling Period 2 ($p < 0.05$), following repeated stressor exposures. An ad-hoc examination of raw values suggested this effect might have been due to higher baseline cort levels in the females at Sampling Period 2.

Figure 4.6. Expression levels of D1 and D2 dopamine (DA) receptors in dorsal striatum and medial prefrontal (infralimbic and dorsopeduncular) cortices (mPFC) were analyzed using western immunoblotting on samples collected from the control group who did not receive predator odour exposure in adulthood. ADOL-S rats showed decreased expression of the D2 DA receptor in mPFC, relative to ADOL-C rats (*$p = 0.019$). Two representative samples are shown for each group. The bar graph illustrates data that were generated by analyzing the intensity of the band corresponding to D2 (at ~ 51 kDa), relative to the band corresponding to the loading control, Gapdh (at ~ 37 kDa).

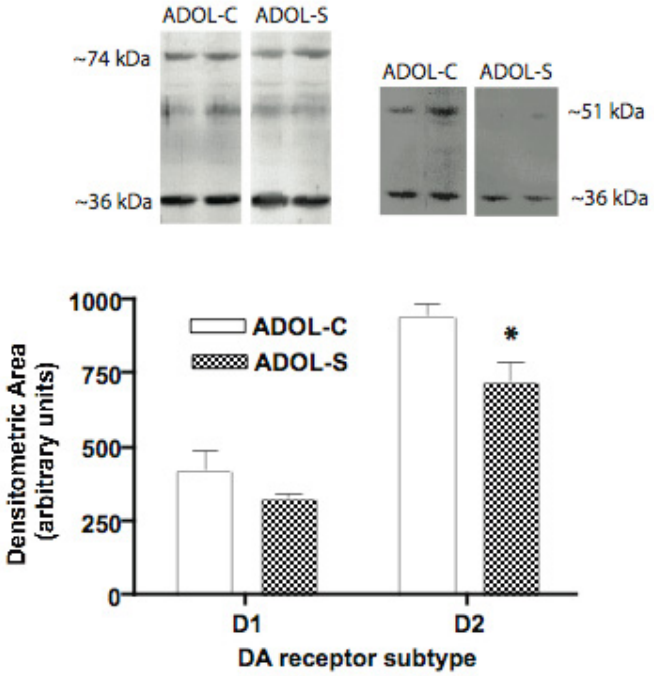
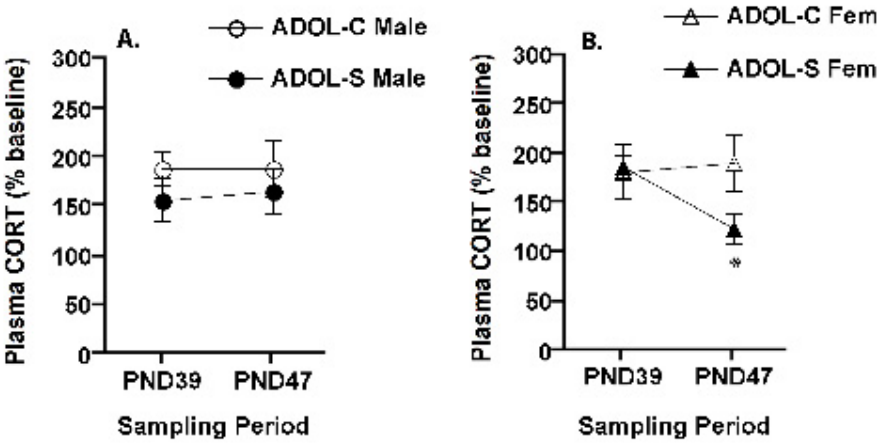

ADOL-C
ADOL-S
~74 kDa
~36 kDa
ADOL-C
ADOL-S
~51 kDa
~36 kDa
1000
750
500
250
0
Densitometric Area
(arbitrary units)
ADOL-C
ADOL-S
*
D1
D2
DA receptor subtype

Figure 4.7. Plasma corticosterone (cort) levels in male (A) and female (B) rats exposed repeatedly to cat odour (ADOL-S; see Methods) or a control condition (ADOL-C) in an adolescent stress paradigm (ADOL-stress). A physiological baseline of cort secretion was assessed on the evening prior to the first (post natal day (PND) 40) and last (PND48) stressor exposure, and experimental samples were taken following each of the stressor exposures. Data are presented as experimental sample normalized to baseline for each day. There were no statistically significant effects of ADOL-stress in males; however, in females, normalized cort levels were lower in ADOL-S relative to ADOL-C rats at Sampling Period 2 ($*p < 0.05$).


A.
ADOL-C Male
ADOL-S Male
Plasma CORT (% baseline)
300
250
200
150
100
50
0
PND39
PND47
Sampling Period
B.
ADOL-C Fem
ADOL-S Fem
Plasma CORT (% baseline)
300
250
200
150
100
50
0
*
PND39
PND47
Sampling Period

4.4. Discussion

This is the first study to use cat odour exposure as a paradigm for evaluating long-term outcomes of an ethologically relevant, psychological stressor experienced repeatedly within the adolescent period. Rodents innately display species- and context-specific behavioural responses to cues of predation threat; thus, this model will be very useful in investigating mechanisms that underlie late developmental programming of the stress response system.

Hubbard *et al*. (2004) previously examined juveniles' acute responses to cat odour cues and reported that locomotor suppression and risk-assessment behaviours begin to emerge between PND18 and 26. We, therefore, expected that the older adolescents (PND40-48) used in our study would recognize and respond to the threat; however, it was unknown whether the responses would change across repeated exposures, and/or whether the adult phenotype would be impacted. It was also unknown whether there would be sex differences in any observed effects. Sex differences were predicted, given their preponderance in studies examining adult stress responding (Beiko et al., 2004; Bland et al., 2005; Bowman et al., 2003; Perrot-Sinal et al., 2004), and also given interactions in HPA and hypothalamic-pituitary-gonadal development during puberty (Alves, Akbari, Anderson, Azmitia, McEwen, & Strand, 1997; Raap et al., 2000).

As expected, adolescent rats showed robust responses to the initial cat odour exposure on PND40, characterized by locomotor inhibition and avoidance of the odour source. They also spent more time in social contact (physically touching the cagemate with whom they were exposed). Of great interest, however, their responses to the stressor were altered with repeated exposure. By the final cat odour stressor exposure on

PND48, ADOL-S rats were displaying hyperactivity and spending less time in social contact, relative to ADOL-C animals. Furthermore, the odour avoidance response initially observed was attenuated, and ADOL-S rats were actually approaching the odour source *more* often than ADOL-C rats. On PND48, ADOL-S rats entered all regions of the exposure apparatus approximately twice as often as ADOL-C, whose behaviour, in contrast, remained consistent across the exposure period. Interestingly, the behavioural profile of the ADOL-S rats is consistent with a profile induced by nicotine exposure during adolescence, characterized by hyperactivity and an increase in anxiogenic behaviour in adulthood (Slawecki et al., 2003).

Sex differences in adolescent behaviours were limited to measures of activity rates, including total line crosses, with females showing a more dramatic alteration through the extremes of locomotor inhibition on PND40 and hyperactivity on PND48. Consistent with these sexually dimorphic findings, Douglas *et al*. (2004) used the conditioned place preference paradigm to demonstrate sex differences in the rewarding properties of adolescent social interaction, and McCormick *et al*. (2005) have shown that adolescent females are particularly susceptible to social stress administered during puberty.

In general, basal physiological levels of circulating cort vary widely among individuals, as does cort output in response to stress. ADOL-S female rats in Experiment 2 showed decreased stress-induced cort output, relative to ADOL-C females, following the final adolescent exposure, and this appears to have been precipitated by an increase in their physiological baseline cort levels across the exposure period. This is interesting, given the pronounced behavioural hyperactivity of ADOL-S females (Experiment 1) that

occurred across the same repeated stressor period. Our results suggest a possible relationship between hyperactive behaviour and increased basal levels of circulating cort in females, although a more thorough examination of the time course of HPA output across periadolescent stressor exposure will be required in order to evaluate this hypothesis.

A potential limitation of the cort data obtained in this study is that blood sampling involved transporting animals to a collection area at a significant distance from the initial site (~ 2 min travel time), leading to inflated cort values for baseline samples and a potential masking of any pre-existing differences between stressed and control groups for experimental samples. For example, the average baseline cort value on the evening prior to the first adolescent exposure was 170.1 ng/mL in the present study but only 7.7 ng/mL for data presented in Chapter 2. This confirms that animal transport rapidly increases circulating stress hormones and indicates that blood sampling should occur at a distance that can be traveled in 30 s or less following stressor exposure, in order to circumvent ceiling effects.

Increased locomotor activity was produced by our adolescent stressor treatment, and this persisted into early adulthood, along with alterations in anxiety-like behaviour. Specifically, ADOL-S adults showed an enhanced response to the novel open field (OF Day 1), manifest as lower rearing rates, less time spent in the center, and more time spent in the hide box. These effects were not observed during the second OF exposure (OF Day 2), suggesting that adult ADOL-S rats were particularly sensitive to the stress associated with a novel environment and exhibited an anxiogenic profile specifically in response to this situation. Moreover, during the PT session on the third day of adult

behaviour testing, ADOL-S rats exposed to cat odour during the final 10 min (CO-PO group) showed hyperactivity reminiscent of their behavioural response to the final stress exposure during adolescence. They spent less time grooming, but crossed more lines and entered the center area and hide box more frequently, and males also exhibited higher rates of rearing. These findings indicate an attenuation of the behavioural inhibition usually induced in adult rats by cat odour exposure. Despite this, there were no changes in odour avoidance and risk-assessment behaviours (odour area entry, time spent in the odour area, time spent in the hide box, and head-out frequency and duration), indicating that the ADOL-S group retained the ability to recognize the significance of the threat stimulus, despite their hyperactivity and lack of behavioural focus.

At sacrifice, adult females (both ADOL-C and ADOL-S) had higher circulating levels of cort, relative to ADOL-C/ADOL-S males; such a sex difference is consistent with the literature (Critchlow et al., 1963). We had also anticipated that the adolescent stressor paradigm might affect levels of cort in adulthood in response to the predator odour stress test. However, we did not observe any differences in adult cort between ADOL-S and ADOL-C rats. Others have recently found alterations in HPA output following chronic restraint stress administered to prepubertal, but not adult, males, demonstrating an effect of pubertal development on HPA plasticity (Romeo et al., 2006a). Our lack of effect could have been due to ceiling effects in cort levels induced by the transport of animals from a room in which the PT test took place to an alternate room for blood collection. Recently, we have identified transport time to be a significant stressor in rats (unpublished data).

Therefore, taken together, adult ADOL-S rats showed enhanced responses to novelty and alterations in some aspects of the response to a predator odour test. These long-term effects of adolescent stress are similar to the results of a study in which adolescent nicotine exposure induced hyperactivity (Slawecki et al., 2003) and altered adult expression of anxiety- and depressive-like behaviours, together with alterations in output of mesocortical stress peptides (Slawecki, Thorsell, El Khoury, Mathe, & Ehlers, 2005). In the present study, D2 dopamine receptors were significantly decreased in the mPFC of ADOL-S rats in adulthood, relative to ADOL-C rats. Dopamine receptors were examined in animals that were not exposed to cat odour in adulthood (CO-CO group); therefore, one can assume that the data represent static adult levels of receptors, suggesting a permanent effect of the adolescent stressor on mesocortical DA function. This effect was specific to the D2 receptor subtype, with no significant alterations being found in the D1 receptor subtype in this region. The effect was also specific to the mPFC, as no differences in either receptor subtype were noted between ADOL-S and ADOL-C rats in the dorsal striatum, a region with high density of DA receptors (Xu, Ling, Sahr, & Neal-Beliveau, 2005).

The long-term alterations in DA D2 receptors in mPFC noted in the present study were not unexpected, as the mPFC is innervated by dopaminergic neurons highly responsive to psychological stress (Spencer et al., 2005; Sullivan, 2004; Wall, Blanchard, Markham, Yang, & Blanchard, 2004; Wall, Blanchard, Yang, & Blanchard, 2003). Since DA neurons in this region are already highly responsive to stress prior to PND40 (Elsworth et al., 2001), this suggests an activity-dependent down-regulation. The potential implications of this finding are broad, because DA neurons innervating PFC are

integral not only in stress responding, but also for working memory function and behavioural flexibility (Chudasama & Robbins, 2004; Floresco & Magyar, 2006; Zahrt, Taylor, Mathew, & Arnsten, 1997). Dopaminergic drive in PFC plays a role in integrating internal and external cues in the regulation of goal-directed behaviour (Wright, Hebert, & Perrot-Sinal, 2007); therefore, a permanent adolescent stress-induced increase in D2 DA receptor pruning (resulting in decreased expression) specific to the PFC, which seems to be indicated by the western blot analysis of DA receptors in the CO-CO group, could have consequences for many attention-related behavioural parameters.

In conclusion, exposure of rats to a natural, psychological cat odour stressor during adolescence induced hyperactivity that endured into adulthood, especially in females. There was also evidence for effects of this adolescent stressor on adult anxiety-related responses and DA D2 receptor down-regulation in the mPFC. These results suggest that adolescence may be a sensitive period for programming adult stress responding, especially with respect to hyperactivity, anxiety-related responses, and mesocortical DA function.

CHAPTER 5

GENERAL DISCUSSION

Lisa Dawn Wright

5.1. Overview

The overall objectives for the present thesis as outlined in Chapter 1 were: 1) to characterize behavioural and endocrine responses of adolescent male and female rats across repeated exposure to cat odour stimuli, 2) to examine long-term changes in defensive behaviours and corticosterone (cort) levels in adult rats exposed to stress as adolescents, and 3) to examine adult levels of DA receptors following repeated adolescent stressor exposure. In general, these experiments demonstrate that adolescent rats of both sexes show robust defensive responses to cat odour stimuli; indeed, adolescent animals were more sensitive than adults to the behavioural effects of the stressors. The collar stimulus produced the most profound behavioural effects, as defensive responding did not habituate across the adolescent exposure period, and collar-exposed animals showed increased levels of defensiveness lasting into adulthood. In terms of stress-induced changes in adult levels of DA receptors, stressor-exposed animals showed lower levels of the D2 DA receptor in prefrontal but not striatal brain regions, relative to control-exposed animals, and no differences in levels of the D1 DA receptor.

In each of Chapters 2, 3, and 4, datasets were derived from cohorts of animals that were bred and raised in-house. Thus, a high degree of control was afforded, in terms of ensuring consistent breeding and housing conditions among experiments. For example, temperature, lighting, and humidity conditions were regulated in the same way across experiments, as were feeding regimens and handling of pups during the pre-weaning period. The experiments were designed as partial replications of one another, with each adding a novel component.

Because these studies were so complex and involved a large number of

behavioural and physiological variables, some minor methodological variations did arise from study to study, sometimes as an effort to improve the study design and sometimes to explore different versions of an experimental manipulation. Some of these methodological variations may have contributed to differences in results among studies; nonetheless, some results remained consistent from experiment to experiment, and from these, general conclusions may be drawn.

Before describing the overall conclusions that can be drawn from these thesis experiments, the differences in methodologies among studies will be outlined. All experiments involved exposing male and female rats to a predator odour stressor stimulus during the adolescent period, examining behavioural and/or physiological variables related to defense strategies during that period, and in most instances raising the animals to adulthood to examine long-term alterations in the same kinds of behavioural and physiological variables.

5.2. Methodological Variations Among Studies

In Chapter 2, animals were exposed on five occasions either to a J-cloth stimulus, a collar stimulus, or a control stimulus, every second day between postnatal day PND38 and PND46. Behaviour was monitored during the first 10 min of the first and final exposure sessions, and blood was drawn to measure baseline levels of circulating cort on PND37 and stress-induced levels on PND46. An identical protocol was used for Chapter 3, except only the collar and control stimuli were used. The novel component to this study involved the inclusion of a group that received the exposure manipulations during early adulthood, and the purpose was to verify whether or not effects of the manipulation

were specific to having been exposed during the adolescent period. In Chapter 4, animals were exposed on five occasions to either J-cloth or control stimuli during a slightly later phase of adolescent development (PND40, 41, 44, 47, and 48), designed to coincide with protracted changes in levels of prefrontal dopamine receptors during adolescent development, as this study involved examination of prefrontal dopamine receptor levels in adulthood. Accordingly, blood was drawn on PND39 to measure baseline levels of circulating cort and on PND48 to measure circulating levels. Since cort levels are inherently changing in a sex-dependent manner across the adolescent period, this may have been a source of variation in values among studies. Also, in this study, behaviour was monitored for only the first seven min of the first and final exposure sessions, which may have skewed some of the values, relative to the other studies, since behaviour changes across time within a single exposure to a J-cloth stimulus (Mashoodh et al., 2008).

In addition to these differences, there were minor variations in the adult open field test protocol. In general, behaviour was monitored in the open field for 10 min on a novel day, again for 10 min the following day, and then for 10 min in the presence of a predator odour stimulus on the third day. In Chapter 2, animals were exposed to a J-cloth stimulus in the open field on the third day, and a fourth open field day was added to the protocol, during which animals were exposed to a collar stimulus. Blood was drawn the evening prior to the first open field day to measure baseline levels of circulating cort and immediately following predator odour stress tests to measure stress-induced levels. However, animals were exposed to the open field for a total of 12 min on each predator odour stress test day in Chapters 2 and 3, but for 25 min in Chapter 4, with exposure to

the odour condition occurring in only half the animals during the final 10 min of exposure. These differences may have been a source of variation in stress-induced cort levels among studies. Furthermore, adolescent exposure sessions each lasted for a total duration of 30 min, adding a potential source of variation between adolescent and adult stress-induced cort levels.

A further source of variation between studies in values obtained for circulating cort levels is the addition of steroid displacement reagent during the cort assay procedure. For the studies described in Chapter 4, steroid displacement reagent was used, which resulted in cort values for this chapter approximating a measure of total cort (free plus protein-bound cort), whereas values for Chapters 2 and 3 approximated free cort levels. This difference likely contributed to the much higher baseline cort levels observed in Chapter 4, relative to Chapters 2 and 3.

5.3. Conclusions Regarding Effects of Stressor Exposure During Adolescence

5.3.1. Efficacy of the Stressor Manipulation

In general, the stressor manipulations used during the adolescent period were effective in eliciting an inhibitory response, whereby animals reduced overall frequencies of movement in the presence of predator odour. Some aspects of the inhibitory response, such as linecross rates, appear to be more sensitive to predator odour exposure in adolescents, relative to adults (Mashoodh et al., 2008). In the study by Mashoodh *et al.* (2008), adults showed reduced linecross rates upon acute predator odour exposure, but this inhibited activity was not sustained across repeated daily exposures. In the studies presented herein, however, adolescents showed sustained inhibition of activity across

repeated exposure sessions. These findings suggest that adolescents and adults use different behavioural strategies for dealing with repeated exposure to cues of predation threat. Adolescents respond by minimizing activity and by demonstrating thigmotaxis-like behaviour (see below). While these strategies limit the ability of a predator to detect the presence of the adolescents, they suggest an approach that is not focused on the actual threatening stimulus. Adults rely less heavily on these tactics, perhaps because they have developed the ability to isolate the source of threat and to employ active defense strategies that more efficiently limit their detection by potential predators, such as risk assessment behaviour (Hubbard et al., 2004).

Stressor exposure reduced linecross rates across the exposure period, relative to control exposure, for the adolescents used as subjects in Chapter 2 (J-cloth stimulus or collar stimulus exposure) and Chapter 3 (collar stimulus exposure). However, there was no difference between stressor and control-exposed adults in Chapter 3, and in Chapter 4, linecross rates were reduced in stressor-exposed adolescents relative to control during the first exposure session only.

A reduction in adolescent rearing rates in collar-exposed relative to control-exposed animals did not emerge until the final exposure session in Chapter 2, although it was present across the exposure period in Chapter 3. Stressor- and control-exposed adults (Chapter 3) did not show this difference.

In Chapter 2, only control animals increased linecross and rearing rates across the exposure period, suggesting an inhibitory response to the novelty of the exposure context during the initial session that subsequently diminished over time. However, in Chapter 3, these effects were seen across all groups. Grooming rates increased across exposure

sessions for all groups in both Chapters 2 and 3. In Chapters 2 and 3, stressor exposure (J-cloth or collar) reduced stimulus area entrance rates in adolescents during both the first and last exposure sessions and in Chapter 4 during the first exposure session only, but this effect was not seen in adults (Chapter 3).

Altogether, these findings suggest that, in adolescents, some aspects of the inhibitory response to predator odour habituate across repeated exposures, whereas this response seems to remain more stable during the initial 10 min of repeated exposure sessions in adults (Mashoodh et al., 2008).

Because the inhibitory response to predator odour was prominent in adolescents, a 'freezing' response (complete lack of movement, except to breathe) was examined in Chapter 3. Freezing was found to be particularly important for the response of adolescents to an initial predator odour exposure. On average, they spent over a third of their time freezing, whereas predator odour-exposed adults spent less than 10% of their time freezing during an initial exposure session. Furthermore, this freezing response in adolescent stressor-exposed animals was found to be negatively associated with stimulus contact rates during the predator odour stress test in adulthood, suggesting it is predictive of adult stressor avoidance behaviour.

Predator odour avoidance did not emerge as prominently in these studies, relative to prior work (Hubbard et al., 2004). In Chapter 4, stressor-exposed adolescents spent less time overall in the odour area during the first and final exposure sessions, relative to control-exposed adolescents; however, this aspect of the odour avoidance response did not emerge in Chapters 2 or 3. Relative to control-exposed animals, time spent in the safe area was increased in stressor-exposed animals in both age groups in Chapter 3 and in

adolescents during the first exposure in Chapter 4, but this effect did not emerge in Chapter 2.

A thigmotaxis-like response was observed more consistently. Relative to control exposure, stressor exposure (either J-cloth or collar stimuli) reduced the time adolescents spent in the middle of the exposure arena in Chapter 2. This effect also emerged for rats in either age group (adolescents or adults) exposed to the collar in Chapter 3. In Chapter 4, exposure to the J-cloth reduced time spent in the middle during the first exposure session only, relative to control exposure.

Social behaviour is deemed to be a particularly important aspect of adolescent development (Spear, 2000); however, stressor exposure did not appear to have a profound impact on durations animals spent in direct physical contact. More complex patterns of adolescent social interaction, such as rough-and-tumble play, were not displayed prominently within the exposure environment and thus were not a focus of these studies. A more detailed analysis of the types of social behaviours typically exhibited in adolescents, such as play fighting (Pellis et al., 1992), may be necessary to determine whether or not stressor exposure changes the dynamic of interactions between individuals and to explore how these potential changes might be related to defensive responses. Relative to control exposure, stressor exposure increased durations spent in contact with the cagemate during the first exposure session in Chapter 4 only. Collar exposure reduced cagemate contact rates in both Chapters 2 and 3, relative to control exposure. Associations among individual levels of social behaviour, cort levels, and defensive behaviours were examined for significant correlations; however, patterns of findings were mainly study-specific, precluding their generalization to all individuals.

In addition to an inhibitory response, adolescents generally showed elevated circulating cort levels after repeated exposure to predator odour stimuli, relative to control exposure. In Chapter 2, the adolescent group exposed acutely to a stressor stimulus on PND38 did not show an elevation in circulating cort levels above those exhibited by adolescents exposed to a control stimulus. The novelty of the exposure arena likely resulted in elevated cort levels in the control group that mitigated any potential differences in levels between stressor- and control-exposed adolescents at this time point. This is supported by a visual inspection of the data (see Fig. 2.4), which shows that, at least in males, levels of cort were lower in control-exposed adolescents after a fifth stimulus exposure, relative to an acute exposure, a pattern consistent with novelty (which would be greater after an acute exposure). These data were not directly compared statistically, since they were derived from different cohorts of animals. The apparent difference was not observed in females; however, the data are confounded by a shift in the sex difference in circulating cort levels that occurs between PND38 and adulthood (see below).

After repeated stimulus exposures, adolescents exposed to the J-cloth (Chapter 2) showed elevated cort levels, relative to control-exposed adolescents. It was surprising that a similar effect did not occur in the collar-exposed group in Chapter 2. In Chapter 3, however, adolescents and adults repeatedly exposed to the collar showed an elevation in circulating cort after the final exposure, relative to control exposure.

5.3.2. Sex Differences During Adolescence

Overall, there were a number of interesting findings with regard to sex differences

during adolescence. The thesis data provide evidence that adult sex differences in cort levels emerge at this time, and this may involve organizational effects of pubertal gonadal hormone secretions. Early in adolescence, males showed lower levels of circulating testosterone, relative to adult males (Chapter 3), and also did not yet show a sex difference in baseline circulating levels of cort, relative to adolescent females (Chapters 2 and 3). Females, on the other hand, may have already experienced increases in circulating levels of ovarian hormones by the time they were exposed to the adolescent stressor manipulation. Therefore, sex differences in the timing of pubertal gonadal hormone secretions likely contributed to the sex differences observed in adolescent cort levels and behaviour and also to any enduring effects of the stressor manipulation, due to the potential for organizational effects of gonadal hormones in the females but not the males.

A very interesting and novel finding from Chapter 2 was that males were circulating higher levels of cort after an acute predator stimulus exposure on PND38, relative to females. Adult females consistently circulate higher baseline and stress-induced cort levels than males (Mashoodh et al., 2008; McCormick, Mathews, Thomas, & Waters), although higher cort levels have been observed on PND30 in males, relative to females, following acute exposure to social isolation and pairing with a new cage mate (McCormick et al., 2007). One possibility for this finding is that introduction of a strange cage mate is more stress provoking in adolescent males than in adolescent females. In the present thesis work, the cat odour-exposed adolescent males were circulating between 100 and 200 ng/mL immediately after an acute stimulus exposure, whereas the levels for cat odour-exposed females were below 100 ng/mL. By the final exposure on PND46,

this sex difference had been reversed, with females circulating higher cort levels than males. In Chapter 3, the sex difference at the end of the exposure phase did not quite reach significance, suggesting that the shift in overall circulating cort levels between males and females occurs around this age and may not be fully established by PND46. Indeed, in studies employing the mixed isolation/social stress adolescent stressor paradigm, cort levels following an acute swim stress administered on PND45 or 46 were similar in males and females (Mathews et al., 2008b; McCormick, Smith, & Mathews, 2008). Together, these findings add significantly to what we know about sex differences in cort release during adolescence.

5.4. Conclusions Regarding Long-Term Effects of Stressor Exposure

5.4.1. Efficacy of Stressor Stimuli in the Open Field

In Chapter 2, open field linecross rates, grooming rates, and rearing rates were lower during both predator odour test sessions (J-cloth stimulus and collar stimulus), relative to Days 1 and 2, suggesting an inhibition of activity in the presence of the odour stimuli. The centre and stimulus areas were entered less frequently during the predator odour test sessions, relative to Days 1 and 2, providing further support of anxiogenic behaviour in the presence of either stressor stimulus. Furthermore, animals spent longer in the safety of the hide box during the stress test sessions, relative to Days 1 and 2. Together, these findings provide validation for the use of the adult predator odour test as a stress-provoking challenge.

5.4.2. Effects of Prior Stressor Exposure in Adult Open Field Tests

In Chapter 2, it was found that prior repeated exposure to either the J-cloth or the collar stimulus during adolescence reduced linecross rates relative to the control group when analyzing general open field behaviour across Days 1 and 2 only. This finding is interesting, because it shows that, although all animals responded to the predator odour stress tests with inhibited activity, those exposed to the stressors during adolescence showed a more generalized anxiogenic behavioural profile in adulthood. In Chapter 3, this finding was replicated in females exposed to the collar stimulus during adolescence, and in Chapter 4, animals exposed to J-cloth stimuli during adolescence reared less frequently during Days 1 and 2 in the open field, relative to those that were control-exposed. Chapter 3 also revealed that animals exposed during adolescence (either stressor or control exposure) were more inhibited, entered the odour area less frequently, and spent more time in the hide box as adults in the open field, relative to those exposed during early adulthood. This suggests that adolescent manipulation alone (repeated handling and exposure to the stressor context) may be enough to contribute to an adult anxiogenic profile.

Repeated adolescent stressor exposure enhanced adult avoidance of predator odour stimuli in the open field, and this effect appeared to be specific to having received the exposure during adolescence. In Chapter 2, when extra analyses were conducted to examine the predator odour stress test session separately from the other open field sessions, females exposed to the stressor stimuli during adolescence spent less time investigating the stressors, relative to females exposed to control stimuli during adolescence. In Chapter 3, both males and females exposed to the collar during

adolescence investigated it less frequently during the adult stress test, relative to those exposed to control stimuli during adolescence. They also spent less total time investigating the collar during the adult stress test, relative to animals exposed to control stimuli during adolescence or to collar stimuli during adulthood. In Chapter 2, animals exposed repeatedly to the collar stimulus as adolescents entered the centre less frequently across all open field test sessions. However, this effect did not emerge in Chapter 3.

Interestingly, animals investigated the J-cloth stimulus in the open field for significantly longer than they investigated the empty alligator clip on both Days 1 and 2. This finding is intriguing and suggests that further work should be done in regard to comparing the effects of different predator odour stressor stimuli and in sorting out the sources of variation in effects. An eloquent study design would involve replicating the experiments conducted in Chapter 2, except stressor stimuli would be produced by having cats wear J-cloths around their necks and by rubbing collars on the cats to coat them in hair and dander. This design could be used to test whether or not rats would also be willing to explore collars if they were coated in hair and dander, as opposed to being infused with odour through wear. Also, other differences in effects of the J-cloth and collar stimuli could be tested to determine if they arise from the source of the cat odour associated with each stimulus.

Repeated adolescent stressor exposure may increase baseline levels of circulating cort. In Chapter 3, repeated stressor exposure increased baseline levels of circulating cort from the exposure phase to the open field test phase in both adolescent and adult animals. This finding did not emerge in Chapter 2, perhaps because all animals were of adolescent age while undergoing exposure sessions, and adolescent baseline cort levels are generally

lower than adult baseline cort levels (see Chapter 3), making it more difficult to differentiate increases in baseline levels between stressor- and control-exposed animals from adolescence to adulthood. In other words, the stress-induced increase in baseline levels of circulating cort may be driven more strongly by data derived from animals exposed during adulthood.

Repeated adolescent exposure to the collar stimulus may alter future cort responses to predator odour stimuli within an individual animal. In Chapter 3, cort levels circulating after the final exposure session were significantly positively correlated with levels circulating after the open field stress test in all groups except those stressed during adolescence. Positive correlations were also observed for these measures in the adolescent control and J-cloth-exposed groups in Chapter 2, but not in the adolescent collar-exposed group, suggesting that repeated adolescent exposure to the collar, specifically, disrupts the correlation between exposure phase and test phase experimental cort levels.

5.4.3. Sex Differences in Open Field Tests

In general, adult females showed more evidence of anxiogenic and defensive behaviour and circulated higher levels of cort, relative to adult males. In Chapter 2, females increased hide box entrance rates during both adult stress test sessions, relative to Days 1 and 2 in the open field. They also spent longer in the head-out position during the stress tests, relative to Day 2, but these effects were not seen in males. Relative to males, these females entered the hide box more frequently, spent longer durations within the hide box, exhibited a head-out posture more frequently, and spent longer durations in a

head-out position during the adult predator odour stress test.

In both Chapters 2 and 3, adult females circulated higher baseline and stress-induced cort levels, relative to males, and in Chapter 4, females circulated higher normalized cort levels following predator odour exposure in the open field. In comparison, these sex differences had not fully emerged during adolescence.

5.4.4. Adult Dopamine Receptor Levels

In Chapter 4, adult D2 DA receptor levels were lower in PFC brain regions of rats exposed to stressors during the adolescent period, relative to control rats. Because D2 is an inhibitory receptor, its activation causes hyperpolarization of neurons on which it is found. The PFC has an overall inhibitory influence on HPA activity (Diorio, Viau, & Meaney, 1993; Herman et al., 2005; McDougall, Widdop, & Lawrence, 2004). Thus, D2 DA receptors in PFC may allow DA responses to stress to inhibit prefrontal activity, thereby enhancing HPA reactivity. An adolescent-stress induced decrease in adult levels of prefrontal D2 receptors may therefore allow for more cognitive control over HPA activity in adulthood, driven by an enhancement in inhibitory input from the PFC.

5.5. Overall Conclusions and Future Directions

Main findings reported in this thesis that are of significance to the field include: 1) Males were circulating higher stress-induced cort levels relative to females at the beginning of adolescence, 2) Adolescents showed a more pronounced defensive response to repeated predator odour exposure, relative to adults, 3) Repeated predator odour exposure, particularly during the adolescent period, enhanced avoidance of homo- and

heterotypic predator odour stimuli in adulthood, 4) Repeated predator odour exposure may increase baseline levels of circulating cort, and 5) Repeated predator odour exposure decreased adult levels of the D2 dopamine receptor in prefrontal cortex.

Future studies should be designed to further expand these findings, while incorporating the potential importance of adolescent social behaviour on defensive strategies. For example, adolescent social behaviours, such as aspects of play fighting, could be quantified in an environment that is relatively enriched, compared with the confined space of the exposure arena used for the present thesis experiments, in which play fighting did not typically occur. Animals could then be exposed repeatedly to multiple stressor stimuli within the enriched setting, to see how this would affect play behaviour and cort levels, and long-term outcomes could be examined in adulthood, particularly in regard to effects on defensive responding and prefrontal dopamine function.

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Appendix I. *P*-values for simple effects analyses and post-hoc tests performed following significant effects derived from analysis of variance (ANOVA) procedures performed on various measures obtained during adult testing in a novel open field (Day 1), a familiar open field (Day 2), in response to a J-cloth stimulus (J-cloth; cloth covered with hair/dander from a cat), and in response to a collar stimulus (Collar; collar previously worn by a cat) in male and female rats exposed repeatedly (5 exposures) during adolescence to a control stimulus (C), the J-cloth stimulus (J), or the collar stimulus (Col). **Abbreviations:** LC – line crosses; SI – stimulus investigation; HB – hide box entrances; HO – head-outs from within hide box; R – rate; D – duration.

Measure	**Day 1 vs Day 2**	**Day 1 vs J-cloth test**	**Day 1 vs Collar test**	**Day 2 vs J-cloth test**	**Day 2 vs Collar test**	**J-cloth test vs Collar test**	**Group effect for Day 1 vs Day 2 only?**	**Group effect for J-cloth vs Collar tests only?**
LC (R)	NS	<0.001	<0.001	<0.001	<0.001	NS	Group 0.022 J<C 0.034 Col<C 0.040	NS
Groom (R)	NS	0.013	0.002	<0.001	0.001	NS	NS	NS
Female groom (D)	0.026	0.022	0.043	0.001	0.004	NS	NS	NS
Rear (R)	NS	0.005	0.011	<0.001	<0.001	NS	NS	NS
Centre area entrance (R)	NS	0.001	0.005	0.015	0.002	NS	Group 0.005 Col<C 0.005	NS
Female centre (D)	NS	NS	NS	NS	NS	NS	NS	NS
Stimulus area entrance (R)	NS	0.006	<0.001	0.001	<0.001	0.019	NS	NS
Stimulus area (D)	NS	0.001	NS	0.003	NS	NS	NS	NS
SI (R)	NS	<0.001	<0.001	<0.001	0.001	0.001	NS	NS
Female SI (D)	NS	0.001	0.007	<0.001	0.043	0.028	NS	Main 0.034 J<C 0.038 Col<C 0.044
Male SI (D)	NS	0.001	0.005	0.001	0.009	0.010	NS	NS
Female HB entrance (R)	NS	0.027	0.001	NS	NS	NS	NS	Main 0.048 Col<C 0.040
Female HB (D)	NS	0.013	0.002	0.003	<0.001	NS	NS	NS
Fem HO (D)	NS	NS	0.051	0.019	0.003	NS	NS	NS

Appendix II. Significant correlations among measures from adolescence (ADOL) and adulthood (ADUL) in male and female rats exposed repeatedly (5 exposures) during adolescence to a control stimulus (ADOL-Control), a J-cloth stimulus (ADOL-J-cloth; cloth covered with hair/dander from a cat), or a collar stimulus (ADOL-Collar; collar previously worn by a cat) and tested in adulthood in a novel open field (Day 1), a familiar open field (Day 2), in response to the J-cloth stimulus (J-cloth), and in response to the collar stimulus (Collar). See Figure 2.1 for a timeline. Abbreviations: ADOLBAS – baseline corticosterone (cort) levels measured on the night prior to the first adolescent exposure (~PND37); SOCCON1 – social contact exhibited during the first adolescent exposure (~PND38); SOCCON5 – social contact exhibited during the fifth adolescent exposure (~PND46); OA1 – odour area occupation during the first adolescent exposure (~PND38); OA5 – odour area occupation during the fifth adolescent exposure (~PND46); GROOM1 – grooming exhibited during the first adolescent exposure (~PND38); GROOM5 – grooming exhibited during the fifth adolescent exposure (~PND46); ADOLACT1 – line crosses (activity) during the first adolescent exposure; ADOLACT5 – line crosses during the fifth adolescent exposure; ADOLEXPT – cort levels measured immediately following the fifth adolescent exposure; ADULBAS – baseline cort levels measured on the night prior to J-cloth exposure in adulthood; ADULJcloth - cort levels measured after exposure to the J-cloth stimulus in adulthood; ADULCollar - cort levels measured after exposure to the collar stimulus in adulthood; OAJcloth/OACollar – odour area occupation measured during J-cloth/collar tests in adulthood; SIJcloth/SICollar – stimulus investigation measured during J-cloth/collar tests in adulthood; CENTJcloth/CENTCollar – centre time measured during J-cloth/collar tests in adulthood; GROOMJcloth/GROOMCollar – grooming measured during J-cloth/collar tests in adulthood; ACTJcloth/ACTCollar – line crosses (activity) measured during J-cloth/collar tests in adulthood; HBJcloth/HBCollar – hide box usage measured during J-cloth/collar tests in adulthood; HOJcloth/HOCollar – head-out postures made during hide box usage measured during J-cloth/Collar tests in adulthood; D – duration; R – rate. *P*-values are in parentheses. *** Statistically significant correlation ($p < 0.05$) and contained in Table 1 (Chapter 2).

Factor 1	Factor 2	ADOL-Control	ADOL-J-cloth	ADOL-Collar
OA1 (R)	GROOMCollar (R)	**0.524 (0.045)**		
OA1 (D)	ADOLACT1	**0.510 (0.044)**		
OA1 (D)	ADOLACT5	**0.658 (0.006)**		
SOCCON1 (R)	OAJcloth (R)	**-0.597 (0.040)**		
ADOLBAS	SOCCON5 (R)	**0.706 (0.002) *****		
ADOLBAS	SOCCON5 (D)	**0.730 (0.001) *****		
SOCCON1 (D)	HBJcloth (D)	**0.690 (0.013)**		
SOCCON1 (D)	ACTJcloth	**-0.705 (0.010)**		
SOCCON1 (D)	GROOMJcloth (D)	**-0.585 (0.045)**		
SOCCON5 (R)	ADOLEXPT	**0.538 (0.032) *****		
ADOLACT1	GROOMCollar (R)	**0.577 (0.024)**		
ADOLACT1	HBCollar (D)	**-0.535 (0.040)**		
ADOLACT5	HBCollar (D)	**-0.657 (0.008)**		
ADOLEXPT	ADULBAS	**0.729 (0.001) *****		
ADOLEXPT	HOCollar (R)	**0.538 (0.039)**		
ADULTBAS	HOCollar (R)	**0.571 (0.026)**		
GROOMJcloth (R)	OAJcloth (R)	**0.581 (0.048)**		
GROOMJcloth (R)	OACollar (R)	**0.791 (0.004)**		
GROOMJcloth (R)	ACTJcloth	**0.704 (0.011)**		
GROOMJcloth (R)	ACTCollar	**0.783 (0.004)**		
GROOMJcloth (D)	ACTJcloth	**0.592 (0.043)**		
GROOMJcloth (D)	ACTCollar	**0.650 (0.031)**		
OAJcloth (D)	CENTJcloth (D)	**0.645 (0.023)**		
OAJcloth (D)	ACTJcloth	**0.806 (0.002)**		
OACollar (R)	ADULJcloth	**0.562 (0.029)**		
HBJcloth (D)	CENTJcloth (D)	**-0.696 (0.012)**		
SIJcloth (D)	CENTJcloth (D)	**0.723 (0.008)**		
SIJcloth (D)	ACTJcloth	**0.755 (0.005)**		
CENTJcloth (D)	ACTJcloth	**0.592 (0.043)**		
SIJcloth (D)	HOCollar (D)	**0.641 (0.033)**		
CENTJcloth (D)	HOCollar (D)	**0.633 (0.037)**		
ACTJcloth	SICollar (R)	**0.625 (0.040)**		
CENTCollar (D)	ACTCollar	**0.626 (0.013)**		
SICollar (R)	HBCollar (R)	**0.539 (0.038)**		
HOCollar (R)	ADULCollar	**0.632 (0.012)**		
ADULJcloth	HBCollar (D)	**-0.575 (0.025)**		
HBCollar (R)	CENTCollar (R)	**0.664 (0.007)**		
OA1 (R)	OA5 (R)		**0.871 (0.001)**	
OA1 (R)	SOCCON1 (R)		**0.854 (0.002)**	
OA1 (R)	SOCCON5 (R)		**0.640 (0.046)**	
OA1 (D)	OA5 (D)		**0.890 (0.001)**	
OA1 (D)	ADOLEXPT		**-0.778 (0.014)**	
OA1 (D)	SIJcloth (D)		**0.825 (0.006)**	
OA1 (D)	SICollar (D)		**0.860 (0.001)**	
OA1 (D)	CENTCollar (D)		**0.842 (0.002)**	
OA1 (D)	ACTCollar		**0.775 (0.009)**	
OA5 (R)	ADOLACT5		**0.987 (<0.001)**	
OA5 (R)	ADOLEXPT		**-0.581 (0.037)**	
OA5 (R)	OACollar (R)		**0.750 (0.002)**	

OA5 (R)	CENTJcloth (R)		**0.609 (0.047)**	
OA5 (R)	CENTCollar (R)		**0.803 (0.001)**	
OA5 (R)	ACTCollar		**0.793 (0.001)**	
OA5 (D)	ADOLACT5		**0.687 (0.007)**	
OA5 (D)	SIJcloth (D)		**0.730 (0.011)**	
OA5 (D)	SICollar (D)		**0.640 (0.014)**	
OA5 (D)	CENTCollar (D)		**0.693 (0.006)**	
OA5 (D)	ACTJcloth		**0.625 (0.040)**	
OA5 (D)	ACTCollar		**0.665 (0.010)**	
SOCCON1 (R)	SOCCON5 (R)		**0.753 (0.012)**	
SOCCON1 (R)	ADOLACT1		**0.853 (0.002)**	
SOCCON1 (R)	ADOLACT5		**0.752 (0.012)**	
SOCCON1 (R)	OA5 (R)		**0.781 (0.008)**	
SOCCON5 (R)	ADOLACT5		**0.758 (0.002)**	
OA1 (R)	HBCollar (R)		**0.913 (<0.001)**	
OA1 (R)	CENTCollar (R)		**0.863 (0.001)**	
SOCCON1 (R)	CENTJcloth (R)		**0.692 (0.039)**	
SOCCON1 (R)	ACTJcloth		**0.700 (0.036)**	
SOCCON1 (R)	CENTCollar (R)		**0.715 (0.020)**	
SOCCON1 (R)	HBCollar (R)		**0.741 (0.014)**	
SOCCON1 (D)	HOJcloth (D)		**0.691 (0.039)**	
ADOLACT1	OA5 (R)		**0.778 (0.008)**	
ADOLACT1	HBCollar (R)		**0.907 (<0.001)**	
ADOLACT5	OACollar (R)		**0.755 (0.002)**	
ADOLACT5	CENTCollar (R)		**0.784 (0.001)**	
ADOLACT5	CENTCollar (D)		**0.577 (0.031)**	
ADOLACT5	ACTCollar		**0.790 (0.001)**	
ADOLEXPT	HOJcloth (D)		**0.692 (0.027)**	
OAJcloth (R)	ADULCollar		**-0.701 (0.016)**	
OAJcloth (D)	ADULJcloth		**-0.635 (0.036)**	
OAJcloth (D)	HOJcloth (D)		**-0.706 (0.015)**	
HBJcloth (R)	CENTJcloth (R)		**0.794 (0.004)**	
HBJcloth (R)	ACTJcloth		**0.661 (0.027)**	
HBJcloth (R)	HOJcloth (R)		**0.915 (<0.001)**	
HOJcloth (R)	CENTJcloth (R)		**0.648 (0.031)**	
HOJcloth (R)	HBCollar (R)		**0.667 (0.025)**	
SIJcloth (R)	SICollar (R)		**0.650 (0.030)**	
CENTJcloth (R)	HBCollar (R)		**0.765 (0.026)**	
ACTJcloth	HBCollar (R)		**0.621 (0.042)**	
GROOMJcloth (D)	CENTJcloth (D)		**0.631 (0.037)**	
SIJcloth (D)	SICollar (D)		**0.772 (0.005)**	
HBCollar (D)	HOCollar (D)		**0.737 (0.003)**	
SICollar (D)	CENTCollar (D)		**0.736 (0.003)**	
CENTCollar (D)	ACTCollar		**0.678 (0.008)**	
SOCCON1 (R)	GROOMCollar (R)			**0.606 (0.048)**
SOCCON1 (D)	ADOLACT1			**-0.638 (0.035)**
SOCCON1 (D)	ADOLACT5			**-0.696 (0.017)**
SOCCON1 (D)	OA5 (D)			**-0.823 (0.002)**
OA1 (R)	ADOLEXPT			**-0.871 (<0.001)**
SOCCON1 (D)	ADOLEXPT			**0.768 (0.006)**
OA1 (R)	OAJcloth (R)			**0.742 (0.014)**

SOCCON1 (D)	CENTJcloth (D)			**-0.720 (0.019)**
SOCCON1 (D)	ACTCollar			**-0.653 (0.029)**
SOCCON1 (D)	GROOMCollar (D)			**-0.647 (0.031)**
ADOLACT1	ADOLEXPT			**-0.784 (0.004)**
ADOLACT1	OAJcloth (R)			**0.688 (0.028)**
OA1 (R)	SIJcloth (R)			**0.805 (0.005)**
ADOLACT1	SIJcloth (R)			**0.708 (0.022)**
ADOLACT1	SIJcloth (D)			**0.647 (0.043)**
OA5 (R)	ADULCollar			**0.642 (0.018)**
OA5 (D)	GROOMCollar (D)			**0.817 (0.001)**
ADOLACT5	CENTJcloth (D)			**0.822 (0.001)**
ADOLACT5	ADULJcloth			**0.558 (0.047)**
ADOLACT5	ADULCollar			**0.775 (0.002)**
SOCCON5 (D)	ADOLACT5			**-0.617 (0.025)**
SOCCON5 (D)	ADULCollar			**-0.579 (0.038) *****
ADOLEXPT	GROOMCollar (R)			**-0.596 (0.032)**
ADOLEXPT	OAJcloth (D)			**-0.591 (0.043)**
ADOLEXPT	HBJcloth (D)			**0.738 (0.006)**
ADOLEXPT	OACollar (D)			**-0.615 (0.025)**
ADOLEXPT	HBCollar (D)			**0.734 (0.004)**
ADULBAS	ADULJcloth			**0.557 (0.048)**
ADULTBAS	HBCollar (R)			**0.568 (0.043)**
ADULJcloth	ADULCollar			**0.740 (0.004) *****
HOJcloth (D)	ACTJcloth			**-0.585 (0.046)**
SIJcloth (R)	ACTCollar			**0.607 (0.036)**
HBJcloth (D)	ACTCollar			**-0.639 (0.025)**
HBJcloth (D)	HBCollar (D)			**0.959 (<0.001)**
HBJcloth (D)	HOCollar (D)			**0.648 (0.023)**
HOJcloth (D)	HOCollar (D)			**0.962 (<0.001)**
OAJcloth (R)	CENTCollar (R)			**0.694 (0.012)**
OAJcloth (R)	ACTCollar			**0.771 (0.003)**
OAJcloth (D)	HBCollar (D)			**-0.724 (0.008)**
OAJcloth (D)	CENTCollar (D)			**0.594 (0.042)**
OACollar (D)	SIJcloth (D)			**0.625 (0.030)**
OACollar (D)	HBJcloth (D)			**-0.598 (0.040)**
OACollar (D)	ACTJcloth			**0.615 (0.033)**
OACollar (D)	ACTCollar			**0.872 (<0.001)**
ACTJcloth	HBCollar (D)			**-0.595 (0.041)**
ACTJcloth	HOCollar (D)			**-0.660 (0.020)**
GROOMCollar (R)	OACollar (R)			**0.647 (0.017)**
GROOMCollar (R)	SICollar (R)			**0.820 (0.001)**
GROOMCollar (R)	CENTCollar (R)			**0.722 (0.005)**
GROOMCollar (D)	OACollar (D)			**0.628 (0.021)**
GROOMCollar (D)	SIJcloth (D)			**0.647 (0.023)**
GROOMCollar (D)	SICollar (D)			**0.594 (0.032)**
OA1 (R)	CENTJcloth (R)		**0.749 (0.020)**	**0.733 (0.016)**
OA1 (R)	ACTJcloth		**0.755 (0.019)**	**0.813 (0.004)**
OA5 (R)	SOCCON5 (R)		**0.724 (0.003)**	**0.563 (0.045)**
OA5 (D)	ADOLEXPT		**-0.555 (0.049)**	**-0.653 (0.015)**
ADOLACT1	OA5 (D)		**0.668 (0.035)**	**0.657 (0.028)**
ADOLACT1	CENTJcloth (R)		**0.715 (0.030)**	**0.780 (0.008)**

ADOLACT1	CENTCollar (R)		**0.790 (0.007)**	**0.683 (0.020)**
ADOLACT1	ACTJcloth		**0.692 (0.039)**	**0.777 (0.008)**
ADOLEXPT	SIJcloth (R)		**-0.869 (0.001)**	**-0.772 (0.003)**
ADOLEXPT	SICollar (R)		**-0.561 (0.046)**	**-0.556 (0.048)**
ADOLEXPT	CENTJcloth (R)		**-0.735 (0.016)**	**-0.637 (0.026)**
ADOLEXPT	CENTCollar (R)		**-0.648 (0.017)**	**-0.598 (0.031)**
ADOLEXPT	ACTJcloth		**-0.892 (0.001)**	**-0.804 (0.002)**
ADOLEXPT	ACTCollar		**-0.692 (0.009)**	**-0.684 (0.010)**
ADOLEXPT	SIJcloth (D)		**-0.727 (0.017)**	**-0.684 (0.014)**
OAJcloth (R)	ADOLEXPT		**-0.891 (0.001)**	**-0.823 (0.001)**
OAJcloth (R)	OACollar (R)		**0.711 (0.014)**	**0.737 (0.006)**
OAJcloth (R)	SICollar (R)		**0.643 (0.033)**	**0.603 (0.038)**
OACollar (R)	ADOLEXPT		**-0.704 (0.007)**	**-0.562 (0.046)**
OACollar (R)	SIJcloth (R)		**0.839 (0.001)**	**0.631 (0.028)**
OACollar (R)	CENTJcloth (R)		**0.651 (0.030)**	**0.790 (0.002)**
OACollar (D)	HBCollar (D)		**-0.590 (0.027)**	**-0.625 (0.022)**
CENTJcloth (R)	ACTCollar		**0.654 (0.029)**	**0.790 (0.002)**
ACTJcloth	CENTCollar (R)		**0.693 (0.018)**	**0.697 (0.012)**
HOCollar (D)	HBCollar (D)		**0.737 (0.003)**	**0.595 (0.032)**
HOJcloth (D)	HBJcloth (D)		**0.940 (<0.001)**	**0.650 (0.022)**
ADOLEXPT	ADULCollar	**0.652 (0.006)**	**0.578 (0.038)**	
ADOLACT1	ADOLACT5	**0.576 (0.020)**	**0.721 (0.019)**	
OA1 (R)	ADOLACT5	**0.570 (0.021)**	**0.812 (0.004)**	
OA1 (R)	ACTCollar	**0.524 (0.045)**	**0.762 (0.010)**	
ADOLACT1	GROOMCollar (D)	**0.524 (0.045)**		**0.721 (0.012)**
SOCCON1 (D)	SOCCON5 (D)	**0.520 (0.039)**		**0.881 (<0.001)**
OA5 (R)	ADOLACT5	**0.939 (<0.001)**		**0.954 (<0.001)**
OA5 (D)	SOCCON5 (D)	**-0.624 (0.010)**		**-0.711 (0.006)**
ADULTBAS	ADULCollar	**0.810 (<0.001)**		**0.583 (0.036)**
GROOMCollar (R)	ACTCollar	**0.705 (0.003)**		**0.735 (0.004)**
GROOMJcloth (D)	HBJcloth (D)	**-0.599 (0.040)**		**-0.633 (0.027)**
GROOMCollar (D)	ACTCollar	**0.582 (0.023)**		**0.610 (0.027)**
OAJcloth (D)	SIJcloth (D)	**0.935 (<0.001)**		**0.714 (0.009)**
OACollar (R)	SICollar (R)	**0.754 (0.001)**		**0.840 (<0.001)**
OACollar (D)	SICollar (D)	**0.698 (0.004)**		**0.875 (<0.001)**
HBJcloth (R)	HOCollar (R)	**0.690 (0.019)**		**0.745 (0.005)**
HBJcloth (R)	ADULCollar	**0.644 (0.024)**		**0.584 (0.046)**
HBJcloth (D)	ACTJcloth	**-0.946 (<0.001)**		**-0.634 (0.027)**
HBJcloth (D)	SIJcloth (D)	**-0.710 (0.010)**		**-0.626 (0.029)**
SICollar (R)	CENTCollar (R)	**0.826 (<0.001)**		**0.827 (<0.001)**
SICollar (R)	ACTCollar	**0.717 (0.003)**		**0.851 (<0.001)**
SICollar (D)	ACTCollar	**0.634 (0.011)**		**0.727 (0.005)**
HBCollar (R)	ADULCollar	**0.652 (0.008)**		**0.760 (0.003)**
HBCollar (D)	ACTCollar	**-0.813 (<0.001)**		**-0.659 (0.014)**
OA1 (R)	ADOLACT1	**0.968 (<0.001)**	**0.974 (<0.001)**	**0.967 (<0.001)**
ADOLACT1	ACTCollar	**0.534 (0.040)**	**0.655 (0.040)**	**0.642 (0.033)**
OAJcloth (R)	SIJcloth (R)	**0.880 (<0.001)**	**0.906 (<0.001)**	**0.809 (0.001)**

OAJcloth (R)	CENTJcloth (R)	**0.740 (0.006)**	**0.754 (0.007)**	**0.828 (0.001)**
OAJcloth (R)	ACTJcloth	**0.931 (<0.001)**	**0.881 (<0.001)**	**0.943 (<0.001)**
OAJcloth (D)	HBJcloth (D)	**-0.801 (0.002)**	**-0.678 (0.022)**	**-0.756 (0.004**
OACollar (R)	CENTCollar (R)	**0.532 (0.041)**	**0.758 (0.002)**	**0.929 (<0.001)**
OACollar (R)	ACTJcloth	**0.630 (0.038)**	**0.719 (0.013)**	**0.726 (0.008)**
OACollar (R)	ACTCollar	**0.765 (0.001)**	**0.875 (<0.001)**	**0.939 (<0.001)**
SIJcloth (R)	CENTJcloth (R)	**0.874 (<0.001)**	**0.763 (0.006)**	**0.725 (0.008)**
SIJcloth (R)	ACTJcloth	**0.889 (<0.001)**	**0.859 (0.001)**	**0.815 (0.001)**
CENTJcloth (R)	ACTJcloth	**0.900 (<0.001)**	**0.949 (<0.001)**	**0.933 (<0.001)**
HBJcloth (R)	HBCollar (R)	**0.790 (0.004)**	**0.681 (0.021)**	**0.848 (<0.001)**
ACTJcloth	ACTCollar	**0.647 (0.031)**	**0.654 (0.029)**	**0.775 (0.003)**
CENTJcloth (R)	CENTCollar (R)	**0.618 (0.043)**	**0.738 (0.009)**	**0.759 (0.004)**
HOCollar (R)	HBCollar (R)	**0.797 (<0.001)**	**0.799 (0.001)**	**0.874 (<0.001)**
CENTCollar (R)	ACTCollar	**0.819 (<0.001)**	**0.963 (<0.001)**	**0.981 (<0.001)**
HBCollar (D)	CENTCollar (D)	**-0.616 (0.014)**	**-0.536 (0.048)**	**-0.577 (0.039)**

Appendix III. Significant correlation coefficients (*p*-values in parentheses) calculated for relationships among behaviours and hormone levels in adolescents (ADOL) and adults, exposed to a collar stressor (Col) or a control stimulus (Con) administered during two exposure sessions (1 and 5 of 5) and exposed subsequently to a predator odour test (PT) in adulthood. Blood samples were taken to examine corticosterone (Cort) levels at the circadian nadir on the night prior to the first exposure (base) or following experimental manipulation (expt) during the 5th exposure session (exposure) or following the PT. Testosterone (Testost) was measured in blood samples of males only. Please see text for more details. Abbreviations/explanations: Cort – corticosterone; base – baseline; expt – experimental; D – duration; R – rate; ACT1 – activity (line crosses) exhibited during the first exposure session; ACT5 – activity (line crosses) exhibited during the fifth exposure session; OA1 – odour area occupation during the first exposure session; OA5 – odour area occupation during the fifth exposure session; SOCCON1 – social contact exhibited during the first exposure session; SOCCON5 – social contact exhibited during the fifth exposure session; FREEZE1 – Freezing exhibited during the first exposure session; FREEZE5 – Freezing exhibited during the fifth exposure session; GROOM1 – Grooming exhibited during the first exposure session; GROOM5 – Grooming exhibited during the fifth exposure session; ACT – activity; Testost – testosterone; SI – stimulus investigation; OA – odour area; HB – hide box; HO – head-out posture.

Factor 1	Factor 2	ADOL-Con	ADOL-Col	ADULT-Con	ADULT-Col
Cort base (exposure)	CENT (R; PT)	**0.647 (0.031)**			
Testost (exposure)	OA1 (R)	**0.904 (0.013)**			
Testost (exposure)	OA (R; PT)	**0.877 (0.022)**			
FREEZE1 (R)	SOCCON1 (D)	**0.767 (0.004)**			
FREEZE1 (R)	ACT5	**-0.691 (0.013)**			
FREEZE1 (R)	OA5 (R)	**-0.665 (0.018)**			
FREEZE1 (R)	SOCCON5 (D)	**0.647 (0.023)**			
FREEZE1 (R)	FREEZE5 (D)	**0.900 (<0.001)**			
SOCCON1 (D)	ACT5	**-0.779 (0.003)**			
SOCCON1 (D)	OA5 (D)	**-0.596 (0.041)**			
SOCCON1 (D)	FREEZE1 (D)	**0.824 (0.001)**			
SOCCON1 (D)	SOCCON5 (D)	**0.935 (<0.001)**			
SOCCON1 (D)	FREEZE5 (D)	**0.710 (0.010)**			
FREEZE1 (D)	ACT5	**-0.737 (0.006)**			
FREEZE1 (D)	SOCCON5 (D)	**0.671 (0.017)**			
FREEZE1 (D)	FREEZE5 (D)	**0.731 (0.007)**			
OA1 (D)	OA5 (D)	**0.630 (0.028)**			
OA1 (D)	FREEZE1 (D)	**-0.733 (0.007)**			
OA1 (D)	SOCCON5 (D)	**-0.724 (0.008)**			
OA1 (D)	FREEZE5 (D)	**-0.613 (0.034)**			
OA1 (D)	Cort base (PT)	**0.637 (0.026)**			
OA5 (D)	HB (D; PT)	**-0.603 (0.038)**			
OA5 (D)	HO (D; PT)	**-0.605 (0.037)**			
ACT5	OA (R; PT)	**0.586 (0.045)**			
ACT5	SOCCON5 (D)	**-0.612 (0.035)**			
ACT5	OA (D; PT)	**0.595 (0.041)**			
OA5 (R)	Cort base (PT)	**0.577 (0.049)**			
Cort base (PT)	CENT (R; PT)	**0.783 (0.003)**			
Cort base (PT)	OA (R; PT)	**0.583 (0.047)**			
SOCCON1 (D)	SI (D; PT)	**-0.644 (0.024)**			
SOCCON1 (D)	OA (R; PT)	**-0.676 (0.016)**			
SOCCON1 (D)	HB (D; PT)	**0.654 (0.021)**			
SOCCON1 (D)	HO (D; PT)	**0.755 (0.005)**			
SOCCON1 (D)	ACT (PT)	**-0.808 (0.001)**			
SOCCON5 (D)	Cort base (PT)	**-0.733 (0.007)**			
SOCCON5 (D)	SI (D; PT)	**-0.621 (0.031)**			
SOCCON5 (D)	OA (R; PT)	**-0.596 (0.041)**			
SOCCON5 (D)	CENT (D; PT)	**-0.615 (0.033)**			
SOCCON5 (D)	HB (D; PT)	**0.717 (0.009)**			
SOCCON5 (D)	HO (D; PT)	**0.752 (0.005)**			
SOCCON5 (D)	ACT (PT)	**-0.728 (0.007)**			
FREEZE5 (D)	HO (D; PT)	**0.684 (0.014)**			
Testost (exposure)	OA (R; PT)	**0.877 (0.022)**			
CENT (R; PT)	SI (R; PT)	**0.596 (0.041)**			
SI (D; PT)	OA (R; PT)	**0.659 (0.020)**			
SI (D; PT)	ACT (PT)	**0.709 (0.010)**			
Cort base (exposure)	FREEZE1 (R)			**-0.609 (0.047)**	
Cort base (exposure)	SOCCON1 (D)			**-0.719 (0.013)**	
Cort base (exposure)	FREEZE1 (D)			**-0.643 (0.033)**	

Cort base (exposure)	OA5 (D)			**-0.905 (0.005)**	
ACT1	Cort expt (exposure)			**0.685 (0.014)**	
ACT1	Cort expt (PT)			**0.657 (0.020)**	
OA1 (R)	Cort expt (PT)			**0.577 (0.049)**	
OA5 (D)	GROOM (D; PT)			**-0.873 (0.005)**	
OA5 (D)	ACT (PT)			**-0.717 (0.045)**	
SOCCON1 (R)	ACT5			**-0.711 (0.010)**	
SOCCON1 (R)	OA5 (R)			**-0.613 (0.034)**	
SOCCON1 (R)	CENT (R; PT)			**-0.699 (0.011)**	
SOCCON1 (R)	HO (R; PT)			**-0.773 (0.009)**	
FREEZE1 (D)	Cort expt (exposure)			**-0.603 (0.038)**	
Cort base (exposure)	GROOM (D; PT)			**0.698 (0.017)**	
Cort expt (exposure)	Cort base (PT)			**0.749 (0.008)**	
Cort expt (exposure)	SI (R; PT)			**-0.594 (0.042)**	
FREEZE1 (D)	Cort base (PT)			**-0.626 (0.039)**	
SOCCON1 (D)	Groom (D; PT)			**-0.671 (0.017)**	
SOCCON5 (D)	Groom (D; PT)			**0.771 (0.025)**	
SOCCON5 (R)	Cort expt (PT)			**-0.903 (0.002)**	
HO (R; PT)	Cort expt (PT)			**0.943 (0.005)**	
ACT (PT)	CENT (D; PT)			**0.622 (0.031)**	
ACT (PT)	GROOM (D; PT)			**0.613 (0.034)**	
ACT1	SOCCON5 (R)		**0.760 (0.004)**		
ACT1	SOCCON1 (D)		**-0.850 (<0.001)**		
ACT5	FREEZE5 (R)		**-0.773 (0.003)**		
OA1 (R)	SOCCON5 (R)		**0.680 (0.015)**		
OA1 (R)	Testost (PT)		**0.869 (0.025)**		
OA1 (D)	Cort expt (PT)		**0.770 (0.003)**		
OA1 (D)	Testost (PT)		**0.931 (0.007)**		
OA5 (R)	Testost (exposure)		**0.833 (0.040)**		
OA5 (D)	SOCCON5 (D)		**-0.725 (0.008)**		
OA5 (D)	FREEZE5 (D)		**-0.605 (0.037)**		
SOCCON1 (R)	FREEZE1 (R)		**-0.796 (0.002)**		
FREEZE1 (R)	SOCCON5 (R)		**-0.680 (0.015)**		
SOCCON1 (D)	SOCCON5 (R)		**-0.920 (<0.001)**		
SOCCON1 (D)	Testost (PT)		**-0.983 (<0.001)**		
FREEZE1 (D)	SI (R; PT)		**-0.607 (0.036)**		
SOCCON5 (R)	GROOM (D; PT)		**-0.631 (0.028)**		
SOCCON5 (R)	Testost (PT)		**0.991 (<0.001)**		
OA5 (R)	FREEZE5 (R)		**-0.769 (0.003)**		
Testost (exposure)	GROOM (D; PT)		**0.857 (0.029)**		
HO (R; PT)	GROOM (R; PT)		**-0.702 (0.011)**		
OA (R; PT)	GROOM (R; PT)		**0.759 (0.004)**		
HB (D; PT)	GROOM (D; PT)		**-0.611 (0.035)**		
Cort base (exposure)	Cort expt (exposure)				**0.888 (0.001)**
Cort base (exposure)	SOCCON5 (D)				**-0.810 (0.005)**
ACT1	Cort base (PT)				**-0.983 (0.017)**
ACT5	Cort expt (exposure)				**-0.939 (0.005)**
ACT5	Testost (PT)				**0.842 (0.035)**
ACT5	HO (R; PT)				**-0.643 (0.045)**

SOCCON5 (R)	Cort expt (exposure)				**-0.734 (0.016)**
SOCCON5 (D)	Cort expt (exposure)				**-0.894 (<0.001)**
SOCCON1 (R)	SOCCON1 (D)				**0.889 (0.003)**
SOCCON5 (R)	SOCCON5 (D)				**0.821 (0.004)**
SOCCON1 (R)	Cort expt (PT)				**0.875 (0.023)**
SOCCON1 (D)	Cort expt (PT)				**0.831 (0.040)**
OA5 (R)	Cort expt (exposure)				**-0.927 (0.008)**
OA5 (R)	HO (R; PT)				**-0.750 (0.012)**
OA5 (R)	Testost (PT)				**0.855 (0.030)**
OA5 (D)	SI (D; PT)				**-0.769 (0.026)**
FREEZE5 (R)	OA (R; PT)				**-0.774 (0.009)**
FREEZE5 (R)	HB (D; PT)				**0.693 (0.026)**
FREEZE5 (R)	OA (R; PT)				**-0.774 (0.009)**
Cort expt (exposure)	CENT (R; PT)				**-0.846 (0.034)**
Cort base (PT)	HO (D; PT)				**0.880 (0.021)**
OA (R; PT)	HB (R; PT)				**-0.705 (0.023)**
HO (D; PT)	Cort expt (PT)				**0.967 (0.002)**
OA1 (D)	SOCCON1 (D)	**-0.804 (0.002)**		**-0.571 (0.033)**	
Cort base (PT)	Testost (PT)		**0.821 (0.045)**		**-0.855 (0.030)**
HO (R; PT)	SI (R; PT)		**-0.655 (0.021)**		**-0.654 (0.040)**
ACT1	SOCCON1 (R)	**0.695 (0.012)**	**0.743 (0.006)**		
ACT5	OA5 (D)	**0.628 (0.029)**	**0.792 (0.002)**		
ACT5	FREEZE5 (D)	**-0.597 (0.041)**	**-0.633 (0.027)**		
SOCCON1 (R)	SOCCON5 (R)	**0.673 (0.017)**	**0.614 (0.034)**		
SOCCON5 (D)	FREEZE5 (D)	**0.681 (0.015)**	**0.586 (0.045)**		
CENT (R; PT)	OA (R; PT)	**0.871 (<0.001)**	**0.684 (0.014)**		
CENT (R; PT)	HB (R; PT)	**0.724 (0.008)**	**0.762 (0.004)**		
CENT (D; PT)	HB (D; PT)	**-0.697 (0.012)**	**-0.710 (0.010)**		
CENT (D; PT)	HO (D; PT)	**-0.603 (0.038)**	**-0.607 (0.036)**		
OA (D; PT)	ACT (PT)	**0.663 (0.019)**	**0.661 (0.019)**		
Cort base (exposure)	Cort expt (PT)			**0.632 (0.037)**	**0.797 (0.006)**
ACT1	FREEZE1 (D)			**-0.731 (0.003)**	**-0.908 (0.002)**
ACT5	SOCCON5 (R)			**0.662 (0.019)**	**0.697 (0.025)**
OA5 (R)	SOCCON5 (R)			**0.684 (0.014)**	**0.739 (0.015)**
FREEZE5 (D)	SI (R; PT)			**0.716 (0.046)**	**-0.650 (0.042)**
FREEZE5 (D)	OA (R; PT)			**0.728 (0.041)**	**-0.744 (0.014)**
OA (R; PT)	HO (R; PT)			**-0.681 (0.015)**	**-0.727 (0.017)**
HB (R; PT)	HO (R; PT)			**0.642 (0.025)**	**0.945 (<0.001)**
SI (R; PT)	HB (D; PT)			**-0.649 (0.022)**	**-0.685 (0.029)**
SI (D; PT)	HB (D; PT)			**-0.645 (0.024)**	**-0.720 (0.019)**
ACT5	SI (R; PT)	**0.664 (0.019)**			**0.739 (0.015)**
OA5 (R)	SI (R; PT)	**0.630 (0.028)**			**0.778 (0.008)**
FREEZE5 (D)	HB (D; PT)	**0.679 (0.015)**			**0.670 (0.034)**

Cort base (PT)	OA (R; PT)	**0.583 (0.047)**			**0.680 (0.044)**
Cort base (exposure)	Cort base (PT)		**0.754 (0.012)**	**0.677 (0.022)**	
CENT (D; PT)	Groom (D; PT)		**0.782 (0.003)**	**0.615 (0.033)**	
FREEZE1 (R)	FREEZE1 (D)	**0.866 (<0.001)**		**0.769 (0.001)**	**0.838 (0.009)**
Cort expt (exposure)	Cort expt (test)	**0.599 (0.040)**		**0.695 (0.012)**	**0.744 (0.014)**
SI (D; PT)	OA (D; PT)	**0.608 (0.036)**		**0.622 (0.031)**	**0.821 (0.004)**
SI (D; PT)	HO (D; PT)	**-0.668 (0.018)**		**-0.671 (0.017)**	**-0.765 (0.010)**
HB (D; PT)	ACT (PT)	**-0.596 (0.041)**		**-0.676 (0.016)**	**-0.764 (0.010)**
HO (D; PT)	ACT (PT)	**-0.760 (0.004)**		**-0.730 (0.007)**	**-0.753 (0.012)**
OA (D; PT)	HB (D; PT)	**-0.593 (0.042)**	**-0.705 (0.010)**	**-0.654 (0.021)**	
ACT1	FREEZE1 (R)		**-0.737 (0.006)**	**-0.699 (0.005)**	**-0.811 (0.015)**
Cort base (PT)	Cort expt (PT)		**0.688 (0.028)**	**0.813 (0.002)**	**0.828 (0.006)**
SOCCON1 (D)	Cort base (PT)	**-0.808 (0.001)**	**-0.791 (0.006)**		**0.907 (0.034)**
ACT1	OA1 (R)	**0.886 (<0.001)**	**0.918 (<0.001)**	**0.902 (<0.001)**	**0.983 (<0.001)**
ACT5	OA5 (R)	**0.974 (<0.001)**	**0.951 (<0.001)**	**0.914 (<0.001)**	**0.953 (<0.001)**
OA1 (R)	FREEZE1 (R)	**-0.677 (0.016)**	**-0.684 (0.014)**	**-0.789 (0.001)**	**-0.766 (0.027)**
FREEZE5 (R)	FREEZE5 (D)	**0.707 (0.010)**	**0.607 (0.036)**	**0.937 (0.001)**	**0.968 (<0.001)**
OA (R; PT)	SI (R; PT)	**0.788 (0.002)**	**0.726 (0.007)**	**0.764 (0.004)**	**0.742 (0.014)**
SI (R; PT)	SI (D; PT)	**0.680 (0.015)**	**0.694 (0.012)**	**0.785 (0.003)**	**0.744 (0.014)**
SI (R; PT)	OA (R; PT)	**0.788 (0.002)**	**0.726 (0.007)**	**0.764 (0.004)**	**0.742 (0.014)**
SI (R; PT)	OA (D; PT)	**0.868 (<0.001)**	**0.784 (0.003)**	**0.819 (0.001)**	**0.803 (0.005)**
OA (R; PT)	OA (D; PT)	**0.814 (0.001)**	**0.846 (0.001)**	**0.822 (0.001)**	**0.687 (0.028)**
OA (R; PT)	HB (D; PT)	**-0.639 (0.025)**	**-0.596 (0.041)**	**-0.734 (0.007)**	**-0.765 (0.010)**
OA (D: PT)	HO (D; PT)	**-0.692 (0.013)**	**-0.652 (0.022)**	**-0.633 (0.027)**	**-0.700 (0.024)**
HB (D; PT)	HO (D; PT)	**0.953 (<0.001)**	**0.941 (<0.001)**	**0.976 (<0.001)**	**0.974 (<0.001)**

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