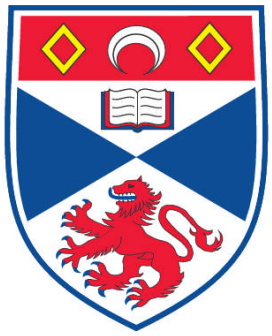
DEVELOPMENTAL AND SEX DIFFERENCES IN RESPONSES TO NOVEL OBJECTS: AN EXPLORATION OF ANIMAL MODELS OF SENSATION SEEKING BEHAVIOUR

De-Laine M. Cyrenne

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



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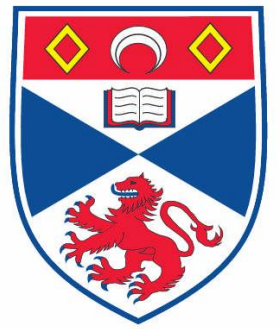

This thesis is submitted in partial fulfilment for the degree of PhD
at the
University of St Andrews

September 26, 2011

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Acknowledgements

Thanks to the School of Psychology at the University of St Andrews for the opportunity to learn from such a diverse and talented academic staff.

Especially, I would like to thank Dr. Gillian Brown, my supervisor and mentor, for her help and patience, and a shared laugh (and occasional glass of wine) or two along the way.

Thanks also to my co-supervisors for their support and guidance: Dr. Eric Bowman and Prof. Philip Winn.

Thanks also to the members of staff in the lab for their assistance: Wendy Taylor, Mike Kinnear, Steve Laing and the all the others over the time. A very heartfelt thank you goes to Mary Latimer for her support and advice – from that first meeting at SFN in Atlanta and throughout my stay at St Andrews.

Lin MacLean – Thank you!!!

And, finally, my partner Eric, who put up with the long-distance calls and the jet lag, knowing that, in the end, it was just one part of the journey. The best part of the adventure is yet to come – may it always be in front of us.

Abstract

Human adolescents exhibit higher levels of sensation seeking behaviour than younger or older individuals, and sensation seeking is higher in males than females from adolescence onwards. Data suggest that changes in gonadal hormone levels during adolescence and differences in the dopamine neurotransmitter system are the bases for why some people exhibit sensation seeking behaviour while others do not. However, causal relationships between physiology and behaviour have been difficult to establish in humans. In order to explore the physiological influences on novelty-seeking behaviour, we looked at response to novelty in a laboratory rodent. This research examined responses to novelty in the conditioned place preference (CPP) task and the novel object recognition (NOR) task in Lister-hooded rats, and assessed the benefits and limitations of each methodology. While the CPP task was not found to provide a reliable measure of response to novelty, the NOR task was more successful. In order to understand the ontogeny of sex differences in novelty responses, both males and females were tested from adolescence through to adulthood. While no sex difference was found in adults in the NOR test, mid-adolescent males exhibited higher novelty preference behaviour than either younger or older males, or females at each stage of development. Since gonadal hormones levels rise during adolescence, a pharmacological agent (a gonadotrophin-releasing hormone antagonist) was used to suppress gonadal hormone levels from early adolescence before again examining responses on the NOR test at mid-adolescence. Gonadal hormone suppression from early adolescence onwards eliminated the sex difference in the NOR test at mid-adolescence by reducing the male response to novelty, while no difference was measured in the female animals. These findings suggest that gonadal hormones play a significant role in the development of response to novelty, especially in males, and the implications for our understanding of human sensation-seeking behaviour are discussed.

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Chapter 1: Sensation seeking behaviour

1.0 Introduction

Sensation seeking is characterised by an attraction towards activities that are new or exciting. These activities could be new experiences, such as travel to exotic locales and taking the road less travelled, or daring adventures, such as skydiving and mountaineering. Typically, sensation seeking behaviours are higher in males than females, peak during adolescence, and decline with age (Ball, Farnhill & Wangeman, 1984; Zuckerman, Eysenck & Eysenck, 1978), but why is there a difference between the sexes and across age groups? The aim of this research is to take a closer look at the age and sex differences in sensation seeking and explore the physiological mechanisms that could lead to an understanding of why males, especially adolescents, show higher levels of this behaviour.

Some sensation seeking behaviours can be viewed as positive experiences, but can involve risks. For example, people who participate in sports considered high-risk (e.g. skydiving, rock climbing, skiing) were more likely to be classified as high sensation-seekers than non-participant controls (Blenner, 1993; Gomà-i-Freixanet, 1991; Malkin & Rabinowitz, 1998; Zaleski, 1984). However, not all activities associated with sensation seeking are risky or dangerous. High sensation seeking levels have been associated with playing chess (Joireman, Fick & Anderson, 2002), preferring rock music over slower tempo or classical music (Litle & Zuckerman, 1986; McNamara & Ballard, 1999), travelling to new places (Zuckerman, 1994), and enjoying modern abstract art over more representational art forms (Furnham & Avison, 1997; Rawlings, 2003). The association of sensation seeking with both high- and low-risk activities suggests the novelty of the experience, as well as a desire for

stimulation, drives behavioural choices in high sensation seekers rather than preferences for a specific activity.

Though some associated behaviours are considered more positive avenues for expression, others are more negative and have more potential for abuse. Several studies found relationships between high sensation seeking and increased levels of drugs and alcohol use, risky sexual behaviour, and gambling (Zuckerman, 1994). For example, male and females undergraduate students who scored high on a questionnaire measure of sensation seeking were more likely to consume greater quantities of alcohol per occasion than low scorers (e.g. Earleywine and Finn, 1991; Johnson & Cropsey, 2000), more likely to try illicit drugs of all classes (e.g. marijuana, hallucinogens, and amphetamines) than low sensation seekers (Kish and Donnenwerth, 1972; Kumar, Pekala & Cummings, 1993), and more likely to exhibit increased frequency of unprotected sex and a greater number of partners (Arnold, Fletcher & Farrow, 2002; Zuckerman et al., 1976). Sensation seekers have reported feeling a 'natural high' or arousal when participating in stimulating activities, therefore the same neural regions may be affected as drugs of abuse, particularly the dopaminergic system (Zuckerman, 1994). Understanding the mechanisms underlying sensation seeking therefore has potential implications for a range of mental and physical health risks.

Adolescence is period of transition, with both physiological changes occurring at puberty, and emotional changes while moving toward independence. Risk taking behaviour increases at this age, theoretically because *perceived* risk declines, which, combined with increased sensation seeking and impulsiveness, leads to increased rates of drug use, recklessness and other risky behaviour (Benthin, Slovic & Severson, 1993). From a developmental neurobiological perspective, risk taking behaviour

could be a result of increased desire for stimulation and change, along with greater peer influences, that precedes mature cognitive control (Berndt, 1979; Steinberg, 2004). Dahl (2001) compared the process to starting the engine without a skilled driver behind the wheel. Risk taking, however, is not the same as sensation seeking, though they share many of the same behavioural characteristics (Arnett, 1992; Jonah, 1997; Martin et al., 2002; Zuckerman, 1994). So while the definition of sensation seeking trait includes 'the willingness to take risks', it is not an essential aspect of the trait (Zuckerman, 1994). Therefore, measures of behaviours associated with risk taking (e.g. drug abuse) may not be generalizable to understanding the motivations or physiological correlates of sensation seeking.

The activational effects of gonadal hormones trigger the onset of puberty, leading not only to the acquisition of secondary sexual characteristics, but also to behavioural changes (Kuhn et al., 2010; Spear, 2000). Since sensation seeking and risk taking is elevated in adolescent males, changing levels of gonadal hormones are a likely mechanism affecting the expression of these behaviours at different levels in males and females. Studies of hormone related disorders suggest that the gonadal hormones could be the mechanism for the pubertal changes in the dopaminergic pathways and structures (Hier & Crowley, 1982; Morse, Scheff & DeKosky, 1986; Murphy & DeCarli, 1993). Sexually dimorphic dopaminergic system changes during adolescence support the hypothesis that an overactive dopaminergic system without corresponding changes in the prefrontal cortex leads to increased risk taking behaviour (Bramen et al., 2010; Giedd, 2004; Giedd et al., 1997; Steinberg, 2004, 2010). However, as sexual differentiation occurs early in the gestational process, the organizational effects of gonadal hormones *in utero* could lead to systemic changes that are not apparent until the onset of puberty.

Although the data suggest physiological differences between humans are the bases for why some people exhibit sensation seeking behaviour while others do not, cause and effect between physiology and behaviour is difficult to determine in humans. Much of the understanding of the physiological influences of sensation seeking in humans is dependent on correlational studies, comparing responses on self-report questionnaires about behaviour to measureable levels of circulating gonadal hormones and dopamine metabolites, or, more recently, fMRI readings and genotypes. Prenatal influences are harder to measure, as are the organizational effects of gonadal hormones during adolescence, though some relationships can be assessed through either clinical patients or postmortem tissue analysis. In each case, it can be difficult to establish cause and effect between physiology and behaviour.

Behavioural experimentation in animals provides a way to determine what variables, (e.g. gonadal hormones, dopamine) are responsible for a given behaviour in that species. However, a similarity of behaviour between animals and humans is not enough to establish that the motivations or mechanisms producing the behaviour are equivalent (Zuckerman, 1984b). Clark (1984) gave a logical argument indicating what must be considered for a comparative approach to sensation seeking: if animals exhibit behaviour similar to sensation seeking, if both then correlate with the same biological factors in each species, and, finally, if the same physiological mechanisms produce the same behaviour in both animals and humans, then direct comparisons can be made.

Though many tests have been designed to measure sensation seeking in animals, some tasks are better at eliciting intrinsic exploration, including those where animals are 'free' to choose between novel an familiar stimuli, rather than 'forced' tests in which fear may be motivating behaviour (Hughes, 1997). Therefore, this

research will examine animal responses to novelty in two designs, the conditioned place preference test (CPP) and the novel object recognition test (NOR), and assess each for benefits and limitations. Although the terms 'sensation seeking' and 'novelty seeking' have been used interchangeably in the literature, for this thesis, sensation seeking will be used to describe the behaviour in humans, and novelty seeking will be used with animal behaviour. Additionally, most studies of novelty preferences in rodents examine this behaviour in male, adult animals. In order to understand the ontogeny of sex differences in responses to novelty, both males and females will be tested from adolescence through adulthood. Through the use of animal models, it is possible to get a better understanding of the physiological mechanisms that *may* be influencing age and sex differences in sensation seeking in humans.

1.1 Sensation seeking in humans

Sensation seeking is a trait defined by the "seeking of varied, novel, complex, and intense sensations and experiences, and the willingness to take physical, social, legal, and financial risks for the sake of such experience" (Zuckerman, 1994, p.27). Marvin Zuckerman and colleagues (1964) created the first self-report questionnaire designed to measure individual differences in levels of sensation seeking behaviour, the Sensation Seeking Scale (SSS). According to Zuckerman and colleagues (1964), the development of this test was influenced by the previous works of Hebb and Thompson (1954), Berlyne (1960) and Fiske and Maddi (1961), all of which explored the concept of an optimal level of stimulation, where some individuals crave stimulus reduction while others seek out stimulation to attain their optimal level (Zuckerman, Kolin, Price and Zoob, 1964). Early research into this area by Zuckerman and colleagues (1962) explored responses to perceptual isolation, finding large differences

between subjects, thus leading to the search for a general factor of sensation seeking. Questions in the early SSS forced test-takers to choose between two contrasting preferences between sensation extremes, familiar or unfamiliar situations, routine or irregularity and enjoyment of thrilling or dangerous activities.

Following further iterations of the sensation seeking scale (SSS-II, SSS-III), Zuckerman (1971) moved from the notion of a single general sensation seeking factor to the revised SSS-IV, which defined four subscales: 'thrill and adventure-seeking' (TAS; e.g. 'I would like to try parachute jumping'), 'boredom susceptibility' (BS; e.g. 'I get very restless staying at home'), 'disinhibition' (Dis; e.g. 'I like wild parties') and 'experience-seeking' (ES; e.g. 'I like to try new foods'). Zuckerman's original definition of sensation seeking behaviour, along with the four subscales, has become one of the more widely used in studies that have looked at the association between personality traits and behaviour. For example, while both those in high physical risk occupations (e.g. fire-fighting or mountain rescue) and those in high risk sports (e.g. parachutists, mountain climbers, race car drivers) showed higher levels of Dis than age-matched controls, only the high risk sport participants scored higher on the TAS subscale (Zaleski, 1984).

1.1.0 Sensation seeking measures

Zuckerman's SSS has gone through several versions, refining questions to be more culturally and gender neutral as well as less colloquial for the modern test-taker. The current version, SSS-V, was initially constructed in 1978 based on responses from both American and English population samples (Zuckerman, Eysenck, & Eysenck, 1978), with subsequent updates to the language published in 1994 (Zuckerman, 1994). Although the SSS-VI has been developed (Zuckerman, 1984a), which has a modified

format to avoid forced-choice questions and is more resistant to cultural changes, it measures only the TAS and Dis subscales and, thus, the SSS-V remains the more used measure of sensation seeking (Zuckerman, 1994).

However, several authors have criticized Zuckerman's SSS and the ability of the test to accurately assess sensation seeking; for example, Gray and Wilson (2007) criticised the SSS-V for the forced-choice format and suggested removal of several dated items as well as changing to a Likert type response format. A response by Zuckerman (2007) noted that several of the items they used were from the 1978 version of the SSS-V, and did not include the 1994 updates. Although Gilchrist and colleagues (1995) had a similar concern with the 1978 version of the SSS-V, that several items required updating, the authors concluded that the scale was a reliable measure of sensation seeking. An additional critique of the SSS-V was made by Jackson and Maraun (1996) regarding the validity of the scale, questioning if the SSS actually measures sensation seeking. Zuckerman defended the validity of both the construct and the content, indicating that the use of factor analysis during development of the SSS allowed for the construct of sensation seeking and the subscales to be defined by the data (Zuckerman, 1996).

In an attempt to explore components of sensation seeking and address some of the criticisms of the SSS-V, Jeffrey Arnett (1994) developed a new scale, the Arnett Inventory of Sensation Seeking (AISS), designed to isolate responses to the novelty and intensity of a stimulus apart from its risk-taking aspects. However, analysis of both the AISS and the SSS-V using structural equation modelling suggests that there is little distinction between the scales, with the SSS-V showing higher reliability (Ferrando & Chico, 2001). In contrast to Zuckerman and Arnett, an assessment based on needs rather than activities was developed by Roth and colleagues (2007) during

their investigation of age and sex differences in sensation seeking. Roth and colleagues were concerned that the activities listed in the questions of both the SSS-V and the AISS could be construed as too youthful, resulting in an inaccurate age-related decline in sensation seeking. Rather than ask about the desire to have a specific experience, the Need Inventory of Sensation Seeking (NISS) measured responses to more general questions such as "I prefer strong and impressive experiences." However, the researchers found that the NISS confirmed the findings of prior studies using the SSS-V and the AISS, and concluded that an age-related decline in sensation seeking was robust and perhaps due to a decreased need for stimulation (Roth et al., 2007).

Several alternate, comprehensive tests have included subscales associated with sensation seeking in addition to other dimensions of personality. Among these tests are the Eysenck Personality Questionnaire – Revised (EPQ-R; Eysenck, Eysenck & Barrett, 1985), the I_7 Impulsiveness Questionnaire (I_7; Eysenck, Pearson, Easting & Allsopp, 1985), the Temperament and Character Inventory (TCI; Cloninger, Przybeck, Svrakic & Wetzel, 1994), and Zuckerman and Kuhlman's Personality Questionnaire (ZKPQ; Zuckerman, Kuhlman, Joireman, Teta & Kraft, 1993). Positive correlations have been found between the SSS-V total score and both the TCI-NS (novelty seeking) and the ZKPQ-ImpSS (impulsive sensation seeking) subscales (Zuckerman & Cloninger, 1996; Zuckerman et al., 1993). Associations were also indicated between the Dis, BS and ES subscales of the SSS-V with the L (lie) and P (psychoticism) subscales of the EPQ-R, which the authors termed Impulsive Unsocialized Sensation Seeking (ImpUSS) (Glicksohn & Abulafia, 1998). Studies examining sensation seeking can therefore be compared even though the

specific test used to assess the behaviour may differ based on researcher preferences and the nature of the research question.

Sensation seeking has been measured in humans mainly through the use of self-report questionnaires, though reports of experiences and behavioural observation have been used in some studies (Zuckerman, 1984). However, questionnaires can have limitations that need to be considered when interpreting results in correlational studies. Self-report responses can be affected by social desirability (SD), which is the possibility that respondents may answer questions in a way that is more socially desirable than truthful, thereby affecting the validity of the results. A way to check if SD is problematic with a particular scale is to administer the test along with instructions to answer the questions in a way to give socially desirable responses, and compare the results to scores with unbiased instructions. Farley and Haubrich (1974) administered the SSS-IV to a group of college students instructing them to respond so as to give a "good impression" or a "bad impression" to others, and found no difference in scores between the groups or against their baseline scores. In a separate study, however, when the test-takers were instructed to give the best possible image to someone they found attractive, males scored higher than baseline on the TAS and BS subscales, while females scored higher in all the scores (Rowland & Heatherton, 1987). These data suggest that responses on questionnaire measures of sensation seeking are not entirely robust.

1.1.1 Age and sex differences

In human studies, sensation seeking levels were generally highest during the adolescent years than at younger or older ages (Arnett, 1992; Kelley, Schochet, & Landry, 2004; Roth, Schumacher & Brähler, 2005; Spear 2000; Steinberg & Morris,

2001; Zuckerman, 2006). In addition, males were reported to engage in more sensation seeking behaviours than females across all age categories, while both males and females showed decreased sensation seeking as they age (Ball, Farnhill & Wangeman, 1984; Magaro, Smith, Cionini & Velicogna, 1979; Roth et al., 2005; Trimpop, 1998; Zuckerman, Eysenck & Eysenck, 1978). These data collectively suggest that age and sex differences in sensation-seeking are relatively robust.

Several studies of adolescents and juveniles, with ages tested ranging from 7 to 19 years old, reported males as higher than females in total sensation seeking, risk-taking or venturesomeness, whereas females were typically rated higher in empathy or experience-seeking during these ages (Arnett, 1992; Butković & Bratko, 2003; Pérez, Ortet, Plá & Simó, 1986; Randhawa, De Lacey & Saklofske, 1986; Zuckerman, Eysenck & Eysenck, 1978). Studies of juveniles, however, are limited, perhaps due to the nature of the questions on the various measures (Zuckerman, 1994). A children's version of the SSS was developed by Russo and colleagues (1991, revised 1993), which showed increased total sensation seeking from ages 7 to 12 years of age and from 9 to 14 years of age, with males higher than females in TAS and total scores. Similar finding were reported in an earlier study by Kafry (1982), which indicated an increase between 6 and 10 years of age, although males were higher than females only in the older age groups. Additional research is needed, however, to understand the pattern of sensation seeking expression from early childhood through adolescence.

Adolescence is the period of transition from childhood to adulthood, generally between the ages of 12 to 18 years, but up to the age of 25 years has been considered as late-adolescence (Spear, 2000). Higher proportions of risk taking, reckless and antisocial behaviours, such as drunk driving, illegal drug use, fighting or sex without contraception, were exhibited by adolescents than by younger or older age groups

(Arnett, 1992; Jonah, 1997; Steinberg, 2004). During the adolescent period of development, one theory for increased risk taking is that the perception of risk declines while the desire for change, or sensation seeking, increases (Benthin, Slovic & Severson, 1993; Irwin, 1993; Steinberg, 2007). Additionally, Rolison and Scherman (2002) found sensation seeking and lowered perceived risk were better predictors of risk taking behaviour than perceived benefits. Lowered risk perception combined with the need for additional stimulation by adolescents could result in increased participation in activities with potentially negative outcomes.

Yet, while drug use or risky driving in adolescents has been associated with both high sensation seeking and risk taking, the two behavioural traits are not synonymous (Andrucci, Archer, Pancoast & Gordon, 1989; Arnett, 1992; Jonah, 1997; Martin et al., 2002). For example, high sensation seeking was associated with risky sexual behaviours such as increased frequency of unprotected sex and a greater number of partners (Arnold, Fletcher & Farrow, 2002; Horvath & Zuckerman, 1993; Zuckerman et al., 1976). However, while White and Johnson (1988) found increased sexual activity in adolescents who were high in disinhibition and impulsivity, the authors did not find a difference in the use of contraception between the high and low rated groups. The adolescents exhibited a high level of sensation seeking, but not increased risk (White & Johnson, 1988). Therefore, Steinberg (2004, 2007, 2010) proposed that developmental neuroscience was a better approach to study risk taking in adolescents rather than assessment of risk perception. An increased desire for stimulation and change (sensation seeking), when combined with greater peer influences before mature cognitive control, leads to risk taking behaviours (Steinberg, 2004).

Although total sensation seeking decreases across the lifespan, age and sex interactions are evident on the subscales of the SSS-V, suggesting the pattern of decline differs between males and females. In an English sample, decreased scores from age group 16-19 years through age group 60+ years were found only on the TAS and Dis subscales in males, with little difference in the BS and ES subscales, whereas females decreased in all four subscales (Zuckerman et al., 1978). In addition to Total SS, males were higher than females in the TAS, Dis and BS subscales, but not in ES. Although similar results were found by Ball and colleagues (1984) in an Australian sample, a sharper decrease was observed in male ES and Dis scores than females in the 30-39 age group, and thus female scores were somewhat higher than males at this age, particularly on the ES subscale. While examining cross-cultural validation of the SSS-V, two additional studies found no age decline or sex difference on the ES subscale (Canada: Ridgeway & Russell, 1980; China: Wang et al., 2000), suggesting both males and females remain open to new experiences as they age. Alternately, Zuckerman (1994) suggested that the ES subscale was the most influenced by educational and cultural differences, thereby reflecting generational changes in attitudes, especially in women. Thus, due to the cross-sectional rather than longitudinal design of the research, age and sex differences found in measures of sensation seeking, including the other subscales of the SSS-V may also be influenced by generational changes (Roth et al., 2005; Zuckerman, 1994).

In a recent meta-analysis of sex differences in impulsivity measures, Cross and colleagues (2011) evaluated the effect sizes of sex differences in sensation seeking and risk taking among age groups ranging from 11-15 years to greater than 40 years. On the subscales of the SSS, TAS and Dis showed the greatest sex differences in effect size, while the BS subscale had a moderate difference, males higher than

females in each case, and no sex difference was indicated on the ES subscale. Additional analysis indicated that sex differences remained consistent through the different age groups for the SSS-Total and subscales. However, on the Venturesomeness scale of the I_7 (Eysenck et al., 1985), the sex difference, males higher than females, was greatest in the adolescent and young adult age groups, 15-18 and 18-21 years of age (Cross, Copping & Campbell, 2011). Although the articles selected for inclusion in the meta-analysis were limited to English language studies published between 1980 and 2008, the sensation seeking measures included the SSS-II (Zuckerman et al., 1964) through the SSS-VI (Zuckerman, 1984) as well as the newer I_7 (Eysenck et al., 1985). Therefore, age related changes in sex differences on the SSS may have been more difficult to distinguish due to potential generational differences between cohort groups.

Evidence suggests that sensation seeking behaviours are higher in males than females, peak in adolescence, and decline with age. Longitudinal studies may refine knowledge of age and sex difference patterns, but changes in cohort attitudes and opportunities, especially regarding male and female roles, could continue to affect the detection of differences using existing questionnaires. Yet, since sex differences across the lifespan appear relatively robust and have been replicated cross-culturally in a growing number of studies, biological factors may influence individual and group differences in sensation seeking behaviour.

1.1.2 Physiological influences

There are two approaches to examining the physiological aspects of sensation seeking behaviour. One way is to look at the basis for the behaviour, or why some people choose jump out of airplanes, for example, and others do not. Do some individuals

have a predisposition toward sensation seeking? The second approach coincides with the first by asking what physiological responses may be occurring in those who do score high in sensation seeking compared to those who score low. As males generally score higher than females and sensation seeking levels decline with age, examining physiological influences can also help with understanding sex and age differences.

Gonadal hormones

Levels of circulating gonadal hormones (oestrogens, progesterones and androgens), differ between males and females and also vary greatly during the lifespan. During puberty, the circulation of gonadotrophin-releasing hormone (GnRH) stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), leading to the production of testosterone in males and oestrogen in females (Sisk & Foster, 2004). Hormones in adults can affect behaviour rapidly, since sex steroids can act similar to neurotransmitters, or more slowly, via genomic actions (Ball & Balthazart, 2006; Balthazart & Ball, 2006; Sisk & Foster, 2004). Age and sex differences in sensation seeking suggest that gonadal hormone levels may influence sensation seeking behaviours.

An early study found positive relationships between the Dis subscale of the SSS-IV and both androgens and oestrogens in young adult males (age 17-23, $N = 76$), and with oestrogens in a small sample of females (mean age 19, $N = 7$) (Daitzman, Zuckerman, Sammelwitz & Ganjam, 1978). A subsequent study by Daitzman and Zuckerman (1980) compared the gonadal hormone levels of males (age 17-20, $N = 40$) who scored at the high and low extremes on the Dis subscale. Positive relationships were found between Dis scores and levels of the androgen testosterone, and the oestrogens 17-β estradiol and estrone (Daitzman & Zuckerman, 1980). More

recent studies of males have had mixed results. Several researchers reported a positive correlation between testosterone and various measures of sensation seeking (Aluja & Torrubia, 2004; Bogaert & Fisher, 1995; Gerra et al., 1999), while others indicated no significant relationship (Dabbs, Hopper & Jurkovic, 1990; Rosenblitt, Soler, Johnson & Quadango, 2001). The results of studies examining the relationship between hormones and females were also mixed. Balada and colleagues (1993) found the lowest TAS scores in females with the highest oestrogen levels, in agreement with the earlier study by Daitzmen (1978), but did not replicate the positive association Daitzmen found with the Dis subscale in the more recent study (1980). Additionally, no relationship was indicated between sensation seeking and testosterone in women (Rosenblitt et al., 2001). In each of these cited studies examining the relationship between gonadal hormones and sensation seeking levels, the participants' levels of circulating gonadal hormones at the time of the study were measured and compared to responses on sensation seeking questionnaires. However, as sex differentiation begins *in utero*, gonadal hormones could influence sensation seeking at an earlier stage in development.

Several reviewers have detailed early studies of the prenatal organizational role of steroidal hormones and their influence beyond anatomical differentiation of males and females (Arnold & Gorski, 1984; McCarthy, 2004; Young, Goy & Phoenix, 1964). Recently, researchers have been examining sex differences as a result of epigenetic effects of steroidal hormones during development and how these changes affect behaviour throughout the lifespan (McCarthy et al., 2009; Pfaff et al., 1992). In particular, testosterone appears to be responsible for most physical and behavioural sex differences by masculinizing both the brain and the body (Morris, Jordan & Breedlove, 2004). For example, females who were exposed to abnormally

high levels of testosterone prenatally (as the result of a condition known as congenital adrenal hyperplasia) exhibited increased male-typical play behaviour and had more male playmates during childhood (Servin, Nordenström, Larsson, & Bohlin, 2003). In a study of adult males with androgen deficits, both hypogonadal and eugonadal, no effect of androgen administration on sensation seeking scores was found within each group (O'Carroll, 1984). However, the same study found that the males with adult acquired deficiencies scored higher in sensation seeking than the hypogonadal males (O'Carroll, 1984). Yet another argument is that other genes not related to steroidal hormones, such as SRY, can influence sex differences during development (Arnold, 1996). However, as sexual differentiation occurs early in the gestational process, the organizational effects of gonadal hormones *in utero* could lead to systemic changes that are not apparent until the onset of puberty.

With regards to sensation seeking, few researchers have examined the association between physical measures linked with developing levels of steroidal hormones and sensation seeking behaviour. Based on animal studies, females in opposite-sex dizygotic twins are thought to experience increased levels of testosterone *in utero* compared to same-sex twins, and, therefore, exhibit more "masculinized" behaviours (Miller, 1994). Using this hypothesis, a reanalysis of the sample of twins from earlier studies (Fulker et al., 1980; Zuckerman et al., 1978) was completed to examine the differences in sensation seeking between same-sex and opposite-sex dizygotic twins, and increased sensation seeking levels in females of opposite-sex pairs were indicated (Resnick, Gottesman & McGue, 1993). However, a different study failed to find a similar relationship in opposite-sex twins, perhaps a result of a smaller sample size and younger subjects (age 13 years), although opposite-sex twin

males exhibited higher sensation seeking, particularly Dis, than same-sex twin females (Cohen-Bendahan et al., 2005).

The ratio of the second to fourth digit length (2D:4D ratio) is thought to be an indicator of prenatal testosterone exposure, with males tending to have a lower ratio (4^{th} finger longer) than females (McFadden & Shubel, 2002; Manning, Scutt, Wilson & Lewis-Jones, 1998). However, no association was found between digit ratio and sensation seeking in a meta-analysis of the small number of studies examining the relationship between the 2D:4D ratio and sensation seeking (Voracek, Tran & Dressler, 2009). The difficulty in measuring prenatal steroidal hormone levels in humans along with the lack of conclusive associations between hypothesized measures and sensation seeking, suggest additional mechanisms may be mediating sex differences in sensation seeking.

Dopamine

The standard definition of sensation seeking refers to actively seeking sensations and experiences with the idea that stimulation leads to an optimal level of arousal; therefore, the rewarding aspects of novel stimuli may be affecting the same neurological areas as drugs of abuse (Zuckerman, 1994). Chronic drug users have been known to experience feelings of anhedonia when not under the influence of drugs, which correlates with a reduction in dopamine receptor densities (Volkow, Fowler, Wang & Goldstein, 2002; Koob & Le Moal, 2001; Wise, 1982, 2008). Feelings of anhedonia were also found in a sample of skydivers, suggesting similar neurological mechanisms affect both sensation seeking and addiction (Franken, Zijlstra & Muris, 2006). Since increased drug use is associated with the Dis subscale of sensations seeking, differences in the dopaminergic system may also influence why

some people exhibit increased sensation seeking behaviour and others do not (Bardo, Donohew & Harrington, 1996; Zuckerman, 1994). As adolescents are thought to be more sensitive to the rewarding properties of drugs and sex differences are found in patterns of drug use, dopaminergic responses could also contribute to the age and sex differences found in sensation seeking (Becker, 2009; Doremus-Fitzwater, Varlinskaya & Spear, 2010; Spear, 2000).

Dopamine is involved in the processing of reward via the mesolimbic pathway, which originates in the ventral tegmental area (VTA) and acts on the nucleus accumbens (NAc), and through interconnection with the mesocortical pathway, which projects to the amygdala, hippocampus, and prefrontal cortex (PFC) (Berridge, 2004; Kelley & Berridge, 2002; Wise & Rompre, 1989). In MRI studies, researchers have found gray matter thickening, thought to be a sign of synaptic growth during cortical reorganization, coincides with puberty and typically peaks a year earlier in females than in males before decreasing again to adult levels (Bramen et al., 2010; Giedd et al., 1999; Huttenlocher & Dabholkar, 1997; Sisk & Zehr, 2005). For both sexes, the peak volumes of gray matter occurs earlier in the frontal and parietal lobes, between 10 and 12 years of age, than in the temporal lobe, which extends to mid-adolescence, or the prefrontal cortex, which does not mature until the early 20's (Giedd, 2004; Giedd et al., 1999; Huttenlocher & Dabholkar, 1997).

Within the dopaminergic pathways, sexually dimorphic enlargement of hippocampal (in males) and amygdala (in females) volumes has been associated with puberty, which typically occurs earlier in females (Bramen et al., 2010; Giedd, 2004; Giedd et al., 1997). Additional evidence of sexual dimorphism within the hippocampus can be found in the area of spatial abilities. In an f-MRI study, young adult males had higher hippocampal activation in spatial tasks than females, who had

higher activation in the parietal and prefrontal cortices, suggesting developmental differences in this area (Grön et al., 2000). Generally, hippocampal activation has also been linked with novelty detection, while hippocampal volume has been positively associated with the ES subscale of the SSS-V, the desire for novel experiences (Kumaran & Maguire, 2009; Martin et al., 2007).

In humans, females with Turner's syndrome, a gonadal hormone deficiency, had reduced hippocampal volume, similar to hormone dependent decreases in hippocampal projections observed in gonadectomised female rats (Morse, Scheff & DeKosky, 1986; Murphy & DeCarli, 1993). Hypogonadal males with untreated androgen deficits before puberty showed reduced spatial abilities, which have been associated with hippocampal functioning, compared to either males treated during puberty or to eugonadal males who acquired deficits as adults (Hier & Crowley, 1982). Together, these findings suggest the hormonal influence on hippocampal development during adolescence is time-sensitive and sex-specific, thereby influencing age and sex differences in the dopaminergic system.

Apart from the general neurological structures associated with the dopaminergic system, differences in dopamine receptor levels or functioning could contribute to differences in sensation seeking levels. The five types of dopamine receptors, D1 – D5, can be further classified into two groups based on their biochemical and pharmacological properties. The D1-like group includes the D1 and D5 receptors, found postsynaptically in the caudate putamen, nucleus accumbens, cerebral cortex and amygdala. The D2-like group, which includes the D2, D3 and D4 types, is primarily found presynaptically in areas such as the VTA and the substantia nigra (SN), and give rise to the dopaminergic pathways. Among the D2-like receptors, the D4 type is highly expressed in the frontal cortex, the hippocampus and

the amygdala (Khan et al., 1998; Vallone, Picetti & Borrelli, 2000). An inverse relationship has been found between D2-like receptors in the midbrain and sensation seeking as measured on the TPQ (Zald et al., 2008). Zald and colleagues suggested that the lower level of midbrain D2-like receptors, which are presynaptic, results in increased availability of dopamine in high sensation seekers, as there is decreased inhibition of dopamine release by the autoreceptors. This hypothesis is consistent with the increased activation found in the hippocampus of high sensation seekers, which, along with the increased hippocampal volume in adolescent males, could contribute to the age and sex differences found in sensation seeking (Giedd et al., 1997; Kumaran & Maguire, 2009; Martin et al., 2007). The dopaminergic midbrain changes, along with the delayed development of the prefrontal cortices, may lead to the increased risk taking behaviour seen during adolescence, especially in males (Steinberg, 2004, 2010). However, there is still the question as to why high sensations seekers have lower levels of midbrain D2-like receptors.

Several genetic polymorphisms that affect aspects the dopaminergic system have been investigated for links to sensation seeking. These include two on the D4 receptor gene, D4 VNTR (variable number of tandem repeats) on exon III, and -521 C/T found in the promoter region. Although the 7-repeat allele of the D4 VNTR and the -521 C/C polymorphism have been associated with higher sensation seeking, the findings have been inconsistent (Kluger, Siegfried & Ebstein, 2002; Munafò, Yalcin, Willis-Owen & Flint, 2008; Schinka, Letsch & Crawford, 2002). An additional genetic polymorphism that has been associated with sex differences in sensation seeking is in the gene for catechol-O-methytransferase (COMT), an enzyme which degrades both catecholamines and catechol-estrogens, particularly in the prefrontal cortex (Chen et al., 2004). Baseline COMT activity was found to be sexually

dimorphic, with females exhibiting 17% lower COMT activity than males, while the Val158Met polymorphism has been associated with 35-50% higher COMT activity resulting in lower levels of dopamine in the prefrontal cortex (Chen et al., 2004; Jiang, Xie, Ramsden & Ho, 2003; Xie, Ho & Ramsden, 1999). Several studies have examined the link between the Val158Met polymorphism and sensation seeking levels, and, although the results were inconclusive, there was a sex-by-genotype interaction, suggesting variations in oestrogens could be affecting the availability of COMT (Golimbet, Alfimova, Gritsenko, & Ebstein, 2007; Lang, Bajbouj, Sander, & Gallinat, 2007; Reuter & Hennig, 2005; Strobel, Lesch, Jatzke, Paetzold, & Brocke, 2003).

Few experimental studies have been completed in humans to link responses on the sensation seeking questionnaires to behavioural or physiological responses. Measures of the hormone prolactin during dopamine challenge tests, involving the administration of various levels of dopaminergic agonist and antagonists, were able to discriminate between high and low sensation seekers, possibly demonstrating a link between dopaminergic functioning and personality (Netter, 2006). During a functional magnetic resonance imaging (fMRI) study, Wittmann and colleagues (2008) found differences in striatal activity related to behaviour on a decision making task with a novelty component (Wittmann, Daw, Seymour & Dolan, 2008). Increased striatal and midbrain areas activation as a result of the novelty aspect of the task were then linked to the novelty-seeking subscale of the TPQ, suggesting a dopaminergic link to novelty seeking (Wittmann et al., 2008). In another study, differences between high- and low-scoring sensation seeking humans were found on event-related potentials (ERPs) in responses to novel stimuli (Zheng et al., 2010). While these experimental studies show links between the sensation seeking personality and

physiology influences of behaviour, cause and effect relationships may be more practical to establish through the use of animal models.

1.2 Novelty preference in animals

While it is not known whether non-human animals possess a trait that directly resembles human sensation-seeking behaviour, studies of animals can potentially provide information about the biological bases of some aspects of this trait. Accordingly, an animal model of sensation seeking would examine behaviours similar to those associated with human sensation seeking measures. Several studies have indicated strong positive relationships between various self-reported measures of novelty seeking and total sensation seeking score, as well as the ES and TAS subscales (McCourt, Gurrera, & Cutter, 1993; Zuckerman, 1994; Zuckerman and Cloninger, 1996). Although novelty seeking may be associated only with the TAS and ES aspects of human sensation seeking measures, novelty seeking is also a characteristic of the general sensation seeking trait. Additionally, studies comparing rodents with high and low responses to novelty (HR vs. LR) have found differences between the groups of animals which are analogous to those found between high and low sensation seeking humans, including increased sensitivity to the reinforcing properties of drugs in the HR animals (Dellu, Piazza, Mayo, Le Moal & Simon, 1996; Pawlak, Ho, & Schwarting, 2008). Since animal responses to novelty can be measured and compared to human responses to novelty, the behaviour of animals in a novel task can potentially model aspects of sensation seeking.

While sensation seeking in humans examines the desire for new experiences and a willingness to take risks, usually for enjoyment, the motivation for animals to explore novel stimuli is not as easily determined. Rather than measuring behavioural

responses to food or drug rewards, novelty seeking in animals examines neotic preferences, characterized by both approach and avoidance behaviours (Montgomery, 1955; Montgomery & Monkman, 1955; Welker, 1957). Exploratory behaviours are signified by the animal approaching novel stimuli, whereas behaviours avoiding novel objects or environments are thought to signify a fear response to the stimuli, which may be influenced by the degree of novelty. Intense novelty may elicit an avoidance response, whereas mild novelty may elicit further exploration and approach behaviours. Therefore, novelty seeking in animals can be seen as a balance between expressions of neophilia and neophobia (Barnett, 1958; Berlyne, 1950).

Sensation seeking in humans is thought to be intrinsically motivated, seeking experiences out of curiosity rather than a means to alleviate basic needs such as hunger (Berlyne, 1954). Several theories of what motivates animals to display exploratory behaviours have been proposed in order to distinguish between behaviours that are novelty seeking rather than goal driven during testing (Hughes, 1997). In animals, it is more difficult to distinguish between intrinsic and extrinsic motivations for exploratory behaviours as both can be present simultaneously, as in exploring previously blocked paths of a familiar maze by non-deprived (e.g. food or water) rats (Cohen & Stettner, 1968; Hughes, 1997). The theory of an exploratory motive can be demonstrated in animals that show habituation to familiar stimuli, as evidenced by a decrease in exploratory response. When novel or unfamiliar stimuli are introduced to animal, the novelty and complexity of stimuli compared to the familiar can elicit increased exploration (Glanzer, 1953). However, the nature of the novel stimulus should also be considered, as both very high degrees of novelty or too little novelty, determined by previous exposure or similarity to the familiar stimulus,

would reduce exploratory behaviour due to fear or disinterest (Hughes, 1997; Montgomery, 1955).

1.2.0 Novelty preference measures

Behavioural assessments of exploratory behaviour can be divided into two basic categories: 'forced' tasks that require entry to a novel environment or measure interaction with a single, novel object, and 'free' tests of exploration that provide the animal a 'choice' of novel versus familiar objects or environments (Welker, 1957). The open field test is one example of a 'forced' task where an animal is placed into an unfamiliar environment from which there is no escape, while locomotion and behaviours such as rearing and sniffing are recorded to evaluate exploration (Hall, 1934; Hughes, 1997, 2008; Renner, 1990). While the animal may move around the environment, it would be difficult to ascertain if the exploratory behaviour was a result of intrinsic motivation or just looking for a means to escape due to fear (Hughes, 1997). Similar reservations have been applied to the use of the elevated plus-maze or the T-maze as measures of exploratory behaviour, as avoidance of previously unexplored arms could be fear motivated (Hughes, 1997). Although 'forced' novelty tests may be useful measures of locomotion, they do not provide an accurate assessment of a preference for novel stimuli over the familiar.

Many different 'free' tests have been used to measure novelty preferences during exploration, including light/dark boxes, hole-board apparatus, arenas containing novel objects, and place preference tests (Brown & Nemes, 2008; Hughes, 1997; Renner, 1990). In each test, animals actively respond to a given stimulus or environment that differs in novelty, complexity or location from a familiar stimulus or environment. Generally, time spent interacting with the novel stimulus is compared

to time spent with the familiar stimulus, generating novelty preference ratios that can be analysed for individual or group differences in response to novelty. Although preference tests are a better measure of novelty seeking, the decision of which test to use depends partly on the nature of the research question. Among the more widely used tests are those where animals can display location preferences, object preferences or learning for exploratory rewards (Hughes, 1997).

Light/dark boxes and emergence tests are examples of location preference tests that are based on a study by Fehrer (1956), where animals were confined to one box for 24 hours before being allowed free access to an identical, but novel environment. Generally, an animal is familiarized to one area of a testing apparatus before being given access to both the familiar and a novel area, and allowed to move freely between the two areas. The amount of time spent exploring the novel area, latencies to enter the novel area, and the number of entries can then be compared between groups. As the exploration of the novel area is relatively independent of the amount of locomotion, the location preference test is useful in drug or lesion studies, when locomotion may be impaired (Hughes, 1997, 2007; Kelley, 1993). Although searching for a means to escape could still act as an extrinsic motivator to explore the novel area, when the only difference between the novel and familiar areas is sensory (e.g., differences in brightness), the motivation to explore is more likely intrinsic (Hughes, 1997).

Tests of object exploration involve introducing one or more novel objects into a familiar environment then measuring the amount of time interacting with the objects. Although a single novel object may elicit exploratory behaviour, the absence of contact with the novel object could also be a neophobic response to the object. One way to reduce this possibility is to first expose an animal to one or two objects in

a familiar environment, which then also become familiar. In the final testing session, a novel object is introduced at the same time as a familiar object and the interaction with both objects is compared to measure preferences (Hughes, 2007). This version of an object exploration test is commonly referred to as the novel object recognition (NOR) task (Berlyne, 1950; Ennaceur & Delacour, 1988). In addition to assessing responses to novelty, the NOR task is also used to examine recognition and spatial memory through variation of the inter-trial intervals or placement of the objects within the environment (Dix & Aggleton, 1999; Ennaceur & Delacour, 1988).

Although reinforcers in classical conditioning can be extrinsic, as with food and water, rats have demonstrated learning in order to have access to novelty which indicates intrinsic exploration (Hughes, 1997; Myers & Miller, 1954). Based on the observation that rats can acquire a preference for a location previously paired with a rewarding experience (Carr et al., 1989; Rossi & Reid, 1976), Bevins and Bardo (1999) were the first to use conditioned place preference (CPP) tests to examine novelty preference in rats. The CPP task aims to pair an unconditioned stimulus (US), in this case a novel object, with an otherwise neutral environment over a series of conditioning trials. If the novel object was sufficiently rewarding to the animal, the environment where the object was present can become the conditioned stimulus (CS), and the animal will show a preference for that environment over one that was empty during conditioning. Conditioned tasks are considered to be a stronger indicator of the appetitive properties of novelty preference, similar to the rewarding aspects of drugs of abuse leading to addiction (Bevins & Bardo, 1999). However, while the CPP task can elicit a preference for an area paired with drugs of abuse after a single pairing, conditioning and testing for a novelty preference can take six or more days (Bardo & Bevins, 2000; Bevins & Bardo, 1999; Tzschentke, 1998, 2007).

1.2.1 Age and sex differences

As in humans, rodents exhibit both physiological and behavioural changes as they transition through developmental stages from birth to adulthood. Rat pups remain with their lactating dam during a period of infancy until weaning, which typically occurs when they reach the age of 21 days (postnatal day, *pnd*, 21). The adolescent period ranges from weaning at pnd 21 through early adulthood at pnd 60, with puberty occurring between pnd 28-42, earlier in females than males (Spear, 2000; Tirelli, Laviola, & Adriani, 2003). Studies of novelty preference behaviour in weanlings (pnd 18-23) have encountered problems with animals failing to explore, suggesting extrinsic motivations may be stronger at this age (Anderson et al, 2004; Reger, Hovda, & Giza, 2009). Additionally, since sex differences in human sensation seeking become apparent in adolescence, animal studies of age and sex differences in novelty seeking typically begin at this stage of development.

In both forced exploratory tests and location preference tests, adolescent rodents reportedly locomote more than adults and also spend more time than adults in the potentially aversive areas of novel environments, such as the centre of an open field (Andrade et al., 2003; Arakawa, 2005; Elliott, Faraday, Phillips & Grunberg, 2004; Schochet, Kelley & Landry, 2004). In contrast, other studies have reported that adolescent rodents exhibit lower levels of locomotion and spend less time in relatively aversive areas of novel environments than adults (Adriani & Laviola, 2000; Candland & Campbell, 1962; Renner, Bennett & White, 1992; Slawecki, 2005). Those studies that have investigated sex differences in locomotor response to novel environments during adolescence have generally either reported that female adolescents locomote more, and spend more time in aversive areas, than males (Estanislau & Morato, 2006;

Fraňková & Barnes, 1968; Lynn & Brown, 2010) or reported no adolescent sex difference in response to novel environments (Masur, Schutz & Boerngen, 1980; Slob, Huizer & Van der Werff ten Bosch, 1986).

Few previous studies have compared the performance of adolescent and adult rodents of both sexes in object preference tests. Ricerri and colleagues (2000) examined the ontogeny of spatial and object discrimination in the NOR task and found that mice spent more time exploring the novel object compared to the familiar objects at pnd 28, 46 and 90, but not at pnd 18. No significant sex differences were found in any of the age groups. Although comparisons between age groups were not reported, the response to the novel object was elevated at pnd 46 and 90 compared to pnd 18 and 26 (Ricceri, Colozza & Calamandrei, 2000). However, in the same task, Calamandrei and colleagues (2002) did not find any age (pnd 28, 45 or 70) or sex differences in the control animals of a drug study, although all the groups exhibited a preference for the novel object (Calamandrei, Rufini, Valanzano & Puopolo, 2002). Both studies used a 7-sesssion design, with object novelty not tested until the last session, and a decrease in locomotion and total object exploration due to habituation or fatigue may have contributed to differences in novel object preferences.

Even in adult rodents, relatively few studies have examined sex differences in novelty preference. Most studies use male rodents and mainly examine spatial and non-spatial memory processes. In two studies of adult rodents, no sex differences were found in novel object preference on the NOR task between males and females when short inter-trial-intervals were used, although females continued to show a preference for the novel object at longer intervals than males (Ghi, Orsetti, Gamalero & Ferretti, 1999; Sutcliffe, Marshall & Neill, 2007). In contrast, two other studies with the NOR task reported that adult males outperformed females after short or

longer interval (Frick & Gresack, 2003; Kosten, Lee & Kim, 2007). Kosten and colleagues (2007) attributed discrepancies in findings between studies to differences in handling, strains, ages and testing protocols. Frick and Gresack (2003) agreed that strain differences could contribute to different findings.

Only one study has examined sex differences in the CPP test while also examining effects of age (adolescent vs. adult) and housing conditions (isolation vs. group) in rats (Douglas, Varlinskaya & Spear, 2003). After five days of conditioning, during which the animals were alternately exposed to one side of a testing apparatus that was paired with a novel object or to a second side that remained empty, the animals were tested on day six and allowed free access to both, now empty, sides. Douglas and colleagues (2003) found not only an overall effect of conditioning, that animals exposed to novel objects had a higher preference coefficient than control animals, but also interactions with age, sex and housing condition. In adolescent males, both group housed and isolated animals displayed a higher preference coefficient than controls; in adults, only the isolated animals were higher than the controls. Females, in contrast, exhibited a higher preference coefficient than controls only in the group housed condition, for both adolescents and adults, and not in the isolated condition in either age group. The authors suggested that isolation enhanced conditioning in adult males, but suppressed it in adult females (Douglas, Varlinskaya & Spear, 2003).

1.2.2 Physiological influences

Behavioural experimentation in animals provides a way to determine what specific physiological mechanisms (e.g. gonadal hormones, dopamine) are potentially affecting a given behaviour in that species. For example, inferences can be made

about what systems and behaviours are being affected during adolescence as well as influencing sex differences by varying the amounts of hormones through gonadectomy and replacement. Agonists and antagonists can be administered in various dosages to understand how drugs act systemically. Since animals, especially rodents, reach adulthood in weeks instead of years, the effect of prenatal, juvenile or adolescent manipulations on adult systems and behaviours can be studied in ways that are not feasible in humans.

When sex differences have been examined in object preference tests, the research has been discussed in the context of differences in memory (Frick & Gresack, 2003; Ghi, Orsetti, Gamalero & Ferretti, 1999; Kosten, Lee & Kim, 2007; Sutcliffe, Marshall & Neill, 2007). While there have been several studies comparing the performance of adolescents and adults, few have examined sex differences (Calamandrei, Rufini, Valanzano & Puopolo, 2002; Reger, Hovda, & Giza, 2009; Ricceri, Colozza & Calamandrei, 2000). Since the novel object recognition test is used in both studies of memory and novelty-seeking, in order to understand the interaction of age and sex differences, the results from both areas of study are considered.

Although there is a component of memory in object preference tasks, additional neural actions may mediate novelty preferences (Hughes, 2007). As with humans, the role of gonadal hormones and dopamine are of interest in novelty seeking behaviour in rodents. There is evidence for sexually dimorphic differences in the dopaminergic system during development, though studies examining the effect of these differences on novelty seeking behaviour are limited (reviewed in Becker, 2009; Spear, 2000; Wahlstrom, White & Luciana, 2009). Therefore, this section will primarily focus on the existing literature from studies with a behavioural component,

especially in performance in the CPP and NOR tests. Based on the review of the various exploratory tests, both 'forced' and 'free', the CPP and NOR tests may be better at eliciting behaviour in animals that is comparable to sensation seeking in humans.

Memory

The NOR task has been adapted to study both working and episodic memory function by changing objects (recognition), object locations (spatial), and contextual elements (Dix & Aggleton, 1999; Eacott & Norman, 2004; Ennaceur & Delacour, 1988; Langston & Wood, 2010). Some of the neural areas involved in recognition memory are the perirhinal and entorhinal cortices, the hippocampus, and the prefrontal cortex (Aggleton & Brown, 2006; Brown & Aggleton, 2001). As changes to the hippocampus and prefrontal cortex occur during adolescence in humans and have been associated with sensation seeking, these neural areas may be of interest when looking at differences in novelty seeking (Kumaran & Maguire, 2009; Martin et al., 2007)

Lesions to the hippocampus, as well as to various limbic areas with connections to the hippocampus, generally have little effect on preference for the novel object in the NOR task (Ainge et al., 2006). Deficits are reported in spatial and contextual memory performance in animals with hippocampal or fornix lesions, suggesting that object recognition alone utilizes different neural areas (Ainge et al., 2006; Eacott & Gaffan, 2005; Ennaceur, Neave & Aggleton, 1997; Mumby, 2001; Mumby et al., 2002). Lesions to the parahippocampal region, specifically the perirhinal cortex, do cause deficits in object recognition when there is high object ambiguity (Brown & Aggleton, 2001; Mumby & Pinel, 1994). However, when the

novel stimulus is more visually distinct from the familiar, animals with perirhinal lesions continue to show a preference for the novel object in the object recognition task (Bartko et al., 2007; Norman & Eacott, 2004). Therefore, while memory may be a component of novelty preference as measured by behaviour on the NOR task, responses to novelty also may be mediated by other neural actions (Hughes, 2007).

Gonadal hormones

Several rodent studies have examined the link between gonadal hormone levels during adolescence and exploratory behaviours (e.g. Beatty, 1979; Broida & Svare, 1984; Lipska & Weinberger, 1994; Palanza et al., 2001), however, this section will primarily examine the activational effects of gonadal hormones on the NOR task. The possible mechanisms of organizational and activational effects of gonadal hormones on the dopaminergic system during development will be discussed in the next section.

Data from adult rodents suggest that gonadal hormones influence NOR performance. Studies that have examined the association of gonadal hormones to novelty preference as measured in the NOR task have been done primarily in ovariectomized (OVX) adult females rats while investigating the effect of hormones on memory (Frye, Llaneza & Walf, 2009; Inagaki, Gautreaux & Luine, 2010; Jacome et al., 2010; Luine, Jacome & MacLusky, 2003; Walf, Rhodes & Frye, 2006; Wallace, Luine, Arellanos & Frankfurt, 2006). While using a 4-hour inter-trial interval, these studies reported that OVX animals have a lower preference for the novel object in the NOR task than control subjects (Wallace et al., 2006) and that novelty preference is enhanced by administration of ovarian hormones (e.g.; Frye et al., 2009; Inagaki et al., 2010; Jacome et al., 2010; Luine et al., 2003; Walf et al., 2006). These studies of adult females used a relatively long inter-trial interval (i.e. 4hrs). However, in the

studies discussed, only the effects of oestrogen and progesterone replacement, not androgen, were considered, and neither performance in castrated males nor sex differences were examined. In males, adult gonadectomized rats given testosterone spent more time with the novel object in a NOR task compared with those given estradiol supplements, while those without replacement hormones have shown deficits in spatial memory and extra-dimensional set-shifting on operant tasks testing cognitive function (Aubele, Kaufman, Montalmant, & Kritzer, 2008; Kritzer et al., 2007).

Similar to humans, gonadotrophin-releasing hormone (GnRH) in rodents stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland, leading to the production of testosterone in males and oestrogen in females during puberty (Becú-Villalobos et al., 1997). GnRH then continues to be released cyclically in females and tonically in males (Dorner, 1981). In addition to the sex difference in gonadally intact animals, a sexually dimorphic response to gonadectomy has been observed (Gay & Midgley, 1969). In males, LH and FSH levels are affected within the first 24 hours after removal of the testes, and reach a plateau after 4 days. However, in females, a similar change in LH levels does not occur until 72 hours after ovariectomy, and does not match male levels until 10 days post-gonadectomy (Gay & Midgley, 1969; Yamamoto, Diebel, & Bogdanove, 1970). A similar pattern of sexually dimorphic post-gonadectomy LH levels was also measured in prepubertal animals (Yamamoto et al., 1970),

Gonadal hormones affect neural development not only prenatally in rats, but also during critical maturation periods, such as the first 10 days after birth, and during adolescence (see Becú-Villalobos et al., 1997; Spear, 2000). In early adolescence, concurrent with the onset of puberty, FSH and LH levels and pituitary sensitivity to

GnRH decrease in females, but increase in males, perhaps due to hypothalamic organization (Debeljuk, Arimura, & Schally, 1972a, 1972b).The period just prior to birth through pnd 10 is a critical period for sexual differentiation of the brain where varying gonadal hormone levels can lead to either ’defeminization’ or ‘masculinization’ in female rats, and either ‘demasculinization’ or ‘feminization’ of males rats (Becú-Villalobos et al., 1997; McCarthy, 2004).

Dopamine

Though several studies have indicated that dopamine acts to mediate the acquisition of place preference due to the rewarding qualities of drugs of abuse, few have examined the effect of dopamine in the CPP task with novel objects (Acquas & Di Chiara, 1994; Leone & Di Chiara, 1987; Morency & Beninger, 1986; Shippenberg & Herz, 1988). Besheer and colleagues (1999) examined the influence of dopamine receptor antagonists on the acquisition of conditioned place preference with novel objects as well as on performance in an NOR test. The responses during D1 antagonism on the CPP test were similar to those found with drugs of abuse; animals did not change their preference for the non-preferred side due to novel objects. However, D2/D3 antagonism continued to elicit a conditioned response. In the NOR test, none of the antagonists affected performance (Besheer et al, 1999). Since the D1 antagonist blocked the conditioned response in the CPP task but not the recognition of novelty in the object recognition task, the different responses could be due to difficulty associating the novel objects with the environmental cues rather than a decrease in the rewarding aspects of the novel objects. Besheer and colleagues (1999) suggested the findings may help with understanding the differences between sensation seeking and drug abuse.

The dopaminergic system has also been linked with animal performance in 'free choice' tasks (Dellu et al., 1996; Dulawa, Grandy, Low, Paulus, & Geyer, 1999; Woolley, Marsden, Sleight & Fone, 2003). In an examination of recognition memory, animals given raclopride, a D2 receptor antagonist, did not show a preference for the novel object in the NOR task (Woolley et al., 2003). Dellu and colleagues (1996) separated rats into high responder (HR) and low responder (LR) groups on the basis of locomotor responses, and found HR rats explored a novel arm of a Y-maze more often than LR rats. Additionally, the HR rats were more sensitive to reinforcement from amphetamines and food, had higher dopaminergic activity in the nucleus accumbens and lower dopaminergic activity in the cortex than LR animals (Dellu et al., 1996). Adult transgenic DRD4 knockout mice (i.e., mice that have been genetically altered to lack the D4 receptor) spent less time exploring novel objects than wild-type mice (Dulawa et al., 1999).

Developmental changes to the dopaminergic system in rodents may contribute to different responses between adolescents and adults in the NOR task (Dere, Huston & De Souza Silva, 2007; Wahlstrom, White & Luciana, 2009). Andersen and Teicher (2000) found developmental differences in striatal D2 receptor density in Sprague Dawley rats, with males, but not females, increasing 144 ± 26% between pnd 25 and 40, followed by an elimination of 55% by adulthood (pnd 120). Adult (pnd 120) D2 receptor densities were similar for males and females. A similar D2 receptor density spike was seen in males at pnd 40 in the prefrontal cortex, with a decrease to adult levels by pnd 80 (Andersen et al., 2000). An increase in dopamine D4 receptors has also been reported in male rats, with peaks reached at pnd 28 in the nucleus accumbens and caudate putamen, and peaks in the frontal cortex and hippocampus at pnd 60 (Nair & Mishra, 1995; Tarazi and Baldessarini, 2000). Although, to date,

developmental sex differences in receptor density have not been studied in the D4 receptor, the D4 receptor subtype is pharmacologically and structurally similar to the D2-like receptor (Van Tol et al., 1991, 1992). Behaviourally, rats with neonatal dopamine forebrain pathway lesions, which caused them to exhibit hyperactivity during early adolescence, displayed dose-dependent reversal of this activity when given D4 antagonists (Tarazi, Zhang, Davids & Baldessarini, 2002).

However, there is evidence that the effects of developmental hormone levels on the dopaminergic system may not emerge until after puberty, though perhaps not directly. In a different study of rats with neonatal medial prefrontal cortex lesions, locomotor activity in lesioned animals was the same as controls at pnd 35, but significantly higher than control animals at pnd 56 (Flores, Wood, Liang, Quirion & Srivastava, 1996). Increased levels of D2 receptors were seen in the striatal and limbic areas of lesioned animals at pnd 60 (Flores et al., 1996). Similar increased postpubertal locomotion was seen in animals with neonatal hippocampal lesions, which, additionally, was not mediated by castration at pnd 21 (Lipska & Weinberger, 1994).

Interactions between the developing gonadal hormone system and dopamine neurotransmitter system could potentially underlie the sex and age differences in novelty-preference. As catechol-o-methyltransferase (COMT), an enzyme necessary for the breakdown of dopamine, is down-regulated by estradiol, this could contribute to an increased availability of dopamine in females (Jiang, Xie, Ramsden, & Ho, 2003). COMT knockout mice have shown sexually dimorphic differences in exploratory behaviours, with homozygous females showing increased latency to emerge into the light in a light/dark open-field task (Gogos et al., 1998). However, Andersen and colleagues (2002) found that gonadectomy did not affect the

overproduction and subsequent pruning in males, and ovariectomy did not increase receptor production in female rats, suggesting receptor density changes were independent of changing hormone levels in adolescence (Andersen, Thompson, Krenzel & Teicher, 2002).

Given that gonadal hormone receptors are present in dopaminergic neurons, gonadal hormones could directly mediate dopaminergic processes (Ernst, Romeo & Andersen, 2009; Kritzer & Creutz, 2009; Kuhn et al., 2010). For example, within the amygdala, nonhuman primate studies have found a higher number of androgen receptors and a smaller number of oestrogen receptors, while a higher number of oestrogen receptors were found in the hippocampus (Clark, MacLusky & Goldman-Rakic, 1988; Morse, Scheff & DeKosky, 1986; Sholl & Kim, 1989). Since sexually dimorphic changes during adolescence have been found in both of these structures, the amygdala and the hippocampus, the combined organization and activational influences of gonadal hormones could lead to the emergence of sex and age differences in novelty seeking in rodents and sensation seeking in humans.

1.3 Thesis outline

In this chapter I have given an overview of the existing research on age and sex differences in sensation seeking behaviour in humans and novelty seeking behaviour in animals. The background includes discussion on the ways these behaviours are measured in both humans and animals along with the limitations of each. Additionally, the possible physiological mechanisms, including gonadal hormones and dopamine, contributing to age and sex behavioural differences were considered. The next four chapters report the results of research aimed at exploring age and sex differences in novelty seeking in rats and gaining an understanding of the role of

gonadal hormones in the development of the behaviour. **Chapter 2** looks at the results of a study on adult sex differences in the conditioned place preference test. Methodological issues of the task are discussed along with assessment of the suitability of the test to address the research issue in further studies. **Chapter 3** examines the responses of adult rats in the novel object recognition (NOR) test. Methodology is discussed to ensure that novelty seeking is being measured, and any influence of sex differences in memory performance is minimized. **Chapter 4** considers the ontogeny of sex differences in the NOR task by testing both male and females rats at early adolescence (pnd, 28), mid-adolescence (pnd 40), or early adulthood (pnd 80). After finding a sex difference in mid-adolescent animals, in **Chapter 5** the influence of gonadal hormones is assessed. Gonadal hormones were pharmacologically suppressed through GnRH antagonism to remove the influence of gonadal hormones in early adolescence before examining the impact on sex differences in the NOR task at mid-adolescence. Finally, in **Chapter 6**, the research findings are summarized and discussed along with future areas of interest.

Chapter 2: Conditioned place preference

2.0 Introduction

The aim of the preliminary studies was to determine the most robust test to use in examining adult sex differences in normal rats. Both unconditioned novel object preference tests and conditioned place preference tests are designed to differentiate animals with higher novelty-seeking tendencies by quantifying approach behaviours to novel stimuli and places (Belzung & Le Pape, 1994; Bevins et al., 2002; Hughes, 2007). However, since prior authors have considered conditioned tasks as a stronger indicator of the appetitive properties of novelty preference, similar to the rewarding aspects of drugs of abuse leading to addiction (Bevins & Bardo, 1999), the conditioned place preference (CPP) task was selected as the first test to administer to adult animals.

Generally, the CPP task aims to pair an unconditioned stimulus (US) with an otherwise neutral environment over a series of conditioning trials. If the properties of the US are sufficiently rewarding (or aversive) to the animal, the environment where the US was present can become the conditioned stimulus (CS), eliciting approach (or avoidance) behaviours to the previously neutral location. In the CPP task, over several days an animal is alternately introduced to each side of a two-chambered, partitioned apparatus, one side of which is always paired with a stimulus that is predicted to be rewarding while the other is always unpaired. Several cues, which can include the markings on the chamber walls, texture of the floor surface or composition of the litter material, vary between the two sides but are kept constant during the conditioning period. The intent is for the animal to pair one set of cues with the presence of a reward (e.g. novel object, drug). On test day, with no rewarding

stimulus present, the partitions are removed and the animal is able to freely interact with both chambers. Although no stimulus is present, an animal is said to have developed a conditioned preference if it spends a larger percent of the total time in the chamber previously paired with the stimulus.

Early work with a variant of the CPP task was conducted by Rossi and Reid (1976) while examining the ability of morphine to elicit a positive affective state. Rossi and Reid found that male Sprague-Dawley rats spent more time in the chamber that was previously paired with morphine than control animals for which it was paired with saline. Since then, an increasing number of drug studies have shown the CPP task effective at eliciting a preference for an area paired with the conditioning stimulus, among which are drugs of abuse. Included in these studies are stimulants (e.g. amphetamines, cocaine, nicotine, caffeine), opiates (e.g. morphine, heroin), as well as ethanol and, in some studies, cannabanoids (Bardo & Bevins, 2000; Tzschentke, 1998, 2007). Two advantages of CPP, that it is effective after a single drug pairing and that it is sensitive to low drug doses, could be contributing to the increased use of CPP in assessing drug reward responses (Bardo & Bevins, 2000; Tzschentke, 1998, 2007). In addition to drugs, natural reinforcers such as social interaction, sexual interaction and food have also been effective at producing a conditioned place preference in rodents (Tzschentke, 1998, 2007).

Based on the work of Rossi and Reid (1976) and Carr and colleagues (1989), Bevins and Bardo (1999) were the first to use conditioned place preference tests to examine novelty preference in rats. Because of the association between drug use and novelty seeking behaviour in both human and non-human animals, Bevins and Bardo selected the CPP test to examine the rewarding aspects of novelty. While a number of subsequent studies have used novel objects in CPP tasks, most have been carried out

in this one research group (reviewed by Bevins & Besheer, 2005). Unlike testing responses to drugs, where one pairing can be enough to elicit a preference, CPP appears to require several days of conditioning trials to assess the rewarding aspects of novelty. Despite this, the presence or absence of habituation trials, the number of conditioning days, the time spent in each trial, as well as the statistical determination of acquisition of preference differs between studies. In the following section, I assess the evidence that rats form a conditioned place preference to novel objects and the evidence that the glutamatergic and dopaminergic neurotransmitter systems play a role in novel object place conditioning.

Novel object place conditioning

Bevins and Bardo (1999) used a design with only one 10-minute session per day while assessing the effectiveness of the CPP task with novel objects in singly housed, male Sprague-Dawley rats (n = 14 per group). These 10-minute sessions included two habituation trials to determine a side preference, followed by 8 days of conditioning alternating between exposure to the non-preferred side now paired with a novel object on one day, a different object each session, and the empty side on the next day (4 days each side, 40 minutes total per side), then, finally, a preference test on the day after conditioning, the 11th day. Conditioned place preference measures were based on a change in preference during the test trial for the previously non-preferred side from that same preference during the habituation trial. If an increase in preference for the non-preferred side occurred, then it was attributed to conditioning with the novel objects.

In this study, consisting of three experiments, Bevins and Bardo (1999) found an increase in preference for the non-preferred side of a chamber if that side had been

paired with a novel object during conditioning, but not in control animals that were not exposed to novel objects. This increase in preference was eliminated by administration of MK-801, an NMDA receptor antagonist (10-minutes prior to each conditioning trial), which had previously been shown to block place conditioning with cocaine and morphine (e.g. Cervo & Samanin, 1995; Del Pozo, Barrios & Baeyens, 1996). Interaction with the objects during the conditioning trials was not affected. The authors therefore suggested that MK-801 affected the association of the novel objects with the environment, but did not detract from the appetitive qualities of the novel objects. In the first of the experiments, one of the concerns of the authors was the use of a between-subjects design, comparing test session side preferences of the group previously paired with a novel object to those of the control group in order to determine if a preference was indicated. Thus, the last two experiments in the same study used a within-subjects design to determine if side preference changes were higher in the animals conditioned with novel objects.

A separate study from the same lab (Besheer, Jensen & Bevins, 1999) examined the influence of dopamine receptor antagonists on the acquisition of conditioned place preference with novel objects. Although previous studies indicated that dopamine acted to mediate the acquisition of place preference due to the rewarding qualities of drugs of abuse (e.g. Acquas & Di Chiara, 1994; Shippenberg & Herz, 1988), the effect of dopamine in the CPP task with novel objects was not known. Using the same procedure as previously described of 8 conditioning sessions with alternate day exposure to paired or unpaired chambers, singly housed, adult male Sprague Dawley rats (n = 9-10 per group) were tested in the CPP task with novel objects after having been administered either saline, varying doses of D1 receptor antagonist SCH-23390, or varying doses of the D2/D3 receptor antagonist eticlopride.

Injections were administered 30 minutes prior to each conditioning session. Besheer and colleagues (1999) found the responses during D1 antagonism were similar to those found with drugs of abuse; animals did not change their preference for the non-preferred side due to novel objects except when the doses also affected object interaction. In contrast, the researchers were unable to block the acquisition of a conditioned response with the D2/D3 antagonist unless the dosage was high enough to also impair object interaction. These animals continued to exhibit an increase in preference for the non-preferred side which was previously paired with novel objects.

In the same study, Besheer also conducted a novel object recognition test (methodology discussed in the next chapter), and found that none of the dopamine antagonists eliminated a preference for the novel object without also impairing object interaction (Besheer et al., 1999). Therefore, if object interaction was a measure of the appetitive nature of novelty, as suggested by Bevins and Bardo (1999), then the ability of the D1 antagonist to block the conditioned response in the CPP task, but not the recognition of novelty in the object recognition task, could be due to interference with the association of the novel objects with the environmental cues rather than a decrease in the rewarding aspects of the novel objects. Based on these findings, Besheer and colleagues (1999) suggested dissociating the *preference* for a novel stimulus from the *ability* of a novel stimulus to alter behaviour over time, leading to a better understanding of the differences between those who seek novelty and those who engage in risky behaviours, such as abuse of drugs.

Bevins and colleagues (2002) later refined the testing procedures to expose the animals to both the empty and paired compartments on the same day during the 8 conditioning days, with an interval of 1-hour between exposures, rather than the 24-hour period used initially, thereby doubling the time spent in each compartment

during conditioning (80 minutes total per side). The duration of the entire test remained at 11 days. In a series of experiments with singly housed, adult male Sprague Dawley rats (n = 8-13 per group), the researchers used this basic procedure to examine the effects of varying protocols on eliciting a place preference with novel objects. Although all sessions were 10 minutes in duration, the animals increased their preference for the non-preferred side if it was paired with a novel object during conditioning trials only when exposed to the novel object for the full 10 minutes, but not when exposure to the novel objects was reduced to either 5 or 2.5 minutes at the end of each 10 minute conditioning session. This finding suggests that a minimum amount of exposure to the novel object during each conditioning session, rather than a set number of conditioning sessions, is necessary to elicit a conditioned response. Additionally, when the objects used during the conditioning sessions were not novel each day, but instead were introduced to the animal in its home cage for an hour prior to the session, place preference conditioning did not occur and object interaction during the conditioning sessions was lower in comparison. Therefore, the authors suggest the rewarding aspects of the novel objects are driving the conditioned response, rather than the restoration of novelty to the previously paired side due to the absence of the novel object on test day (Bevins et al., 2002).

Sex differences in novel object place conditioning

Only one study has examined the performance of both males and females in novel object place conditioning while also examining effects of age (adolescent vs. adult) and housing conditions (isolation vs. pair-housed) (Douglas, Varlinskaya & Spear, 2003). In the isolated condition, animals were separated from their cage-mate and singly housed for 10 days prior to the start of the conditioning sessions, which began

at postnatal day (pnd) 33 for adolescents and pnd 65 for adults. In contrast to the previously described designs (Besheer et al., 1999; Bevins & Bardo, 1999; Bevins et al., 2002), Douglas and colleagues (2003) did not use a pre-conditioning habituation trial; on the same day (rather than alternate days) male and female Sprague Dawley rats (n = 8 per group) were exposed to both the paired and empty sides of the apparatus during 15 minute sessions (order of exposure counter-balanced, with a 1-hour interval) on each of 5 conditioning days (75 total minutes per side); followed by a 10 minute testing session on day six. Preference was determined by use of a preference coefficient, calculated as a percent of the time spent on the paired side compared to the time on the unpaired side as a ratio to the total time spent on both sides. The preference coefficient was then compared to that of a control group, which were subject to the same conditioning sessions but had no exposure to novel objects in the CPP apparatus. A higher preference coefficient for the paired group indicated that conditioning was effective when a novel object acted as the stimulus.

Douglas and colleagues (2003) found not only an overall effect of conditioning, that animals exposed to novel objects had a higher preference coefficient than control animals, but also interactions between age, sex and housing condition. Because of these interactions, data from males and females were then analyzed separately. In adolescent males, both pair-housed and isolated animals displayed a higher preference coefficient than controls (no object present during conditioning); in adults, only the singly housed animals were higher than the controls. Females, in contrast, exhibited a higher preference coefficient than controls only in the group housed condition, for both adolescents and adults, and not in the isolated condition in either age group. However, no direct comparison of the preference coefficients was reported by the authors between males and females in any of the

conditions. Despite a different testing design in the CPP task, the results of Douglas and colleagues (2003) support the findings of previous studies (Besheer et al., 1999; Bevins & Bardo, 1999; Bevins et al., 2002), all of which found novel objects were effective at producing a conditioned place preference in singly housed, adult male Sprague Dawley rats. The authors suggested that isolation enhanced conditioning in adult males, but suppressed it in adult females (Douglas et al., 2003; Harmer & Phillips, 1998).

The aim of the experiments described in this chapter was to examine sex differences in adult animals in the CPP task with novel objects. Based on prior research with CPP testing and the use of novel objects (i.e., Bevins & Bardo, 1999; Douglas et al., 2003), the expected findings were that CPP would demonstrate responses to novelty not only by measuring object interaction during the conditioning trials but also by eliciting a conditioned preference due to the novel objects during the test on the last day. Besides assessing sex differences in adult preferences for novelty, the testing design would be evaluated to determine the effectiveness of the CPP task for evaluating responses to novelty in future planned experiments.

2.1 Experiment 1

2.1.0 Methods

Subjects and housing

A total of 16 adult Listar-hooded rats, 8 males and 8 females, were tested in the first experiment. Rats were housed in same-sex pairs in cages (measuring 25cm x 45cm x 15cm) with food and water provided *ad libitum*. Housing rooms were controlled for temperature (20 ± 1ºC) and humidity (55 ± 5%), and maintained on a 12-hour light: dark cycle (lights on 7am).

All appropriate guidelines and requirements were adhered to, as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85-23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986.

Apparatus

The initial test of CPP used an existing apparatus, which had sides and back that were a dark grey metal, and a front made from clear polycarbonate. A black polycarbonate divider was slotted into centre grooves, separating the oblong apparatus into a right and left side, each measuring approximately 45 cm x 48 cm x 45 cm (l x w x h). White paper (polyethelyene-backed absorbent lab bench paper) lined the inside walls of the right side of the chamber, with the exception of the clear side to allow viewing of behaviour, while no change was made to the left side walls. Floor surfaces were varied to provide additional distinction between the two sides; the left side was a metal mesh floor with bedding scattered on a tray underneath, and the right side was a solid, white polycarbonate floor. Various items were used as novel objects during conditioning trials including plastic Lego-like blocks, rubber animal chew toys and configurations of clear plastic blocks. An external polycarbonate chamber with a lid, centred between the two sides, was part of the apparatus and was used as a start box for the preference test on the last day. In order to facilitate behavioural observation yet minimize observer interference, the testing apparatus was raised above the floor on a table, approximately 90 cm off the floor.

Experimental design

At the beginning of each session, a rat was brought to the testing room in a carrying box measuring 42cm x 26cm x 13cm (l x w x h). During conditioning, the rat was

then placed in one side of the divided apparatus for a period of 10 minutes. The animal was then returned to its home cage and the apparatus was cleaned with a 70% ethanol solution. After a one-hour interval, the same rat was placed in the other side of the apparatus for 10 minutes, then returned to the home cage and the apparatus was cleaned before the next use. This procedure was repeated over five days, for a total of 100 minutes of conditioning, 50 minutes spent on each side of the apparatus. A different novel object each day was placed consistently for each rat in the centre of one of these two sides with daily first exposure randomized to either the novel object present or no object condition. On the sixth day, with the dividers between both sides of the chamber raised approximately 15 cm from the floor to allow free access to either side, which were now both empty, the rat was placed in the start box and allowed to enter into either side of the main apparatus and move around for 10 minutes.

The testing took place under dim, white light (approximately 25 lux), while the observer was seated approximately 90 cm away from the apparatus, at eye-level with the clear front side. All tests were conducted between 09:00-14:00 hours in the same testing room.

Behavioural recording

In each conditioning trial, time spent in forward locomotion, latency to approach novel object and time spent exploring the novel object were recorded for additional analysis. Object interaction was defined as time spent with the nose was in contact with the object, which excluded behaviours such as backing into the object, tail only contact, or time resting next to the object without direct interaction. During the preference test trial, the first side entered from the start box and time spent on each

side of the apparatus was recorded and analysed for preference. An animal was considered to have entered a side when both front paws were located on that side. Data during all trials were captured by the seated observer by means of real-time manual input using Observe software, previously developed in-house, onto a laptop computer. Various keystrokes were toggled to start or stop recording the duration of an activity on each side (e.g. locomotion, object contact), with the elapsed time before each key was depressed also captured (e.g. latency to contact).

Statistical Analysis

The effect of conditioning was analysed during the test trial, where it was defined as effective if animals spent a higher percentage of time on the side that was previously paired with a novel object during conditioning trials compared to the empty side. A preference coefficient was defined as [(Time spent on the paired side – Time spent on the unpaired side)/(Time spent on the paired side + Time on the unpaired side) * 100] (Douglas et al., 2003). During the conditioning trials, responses to the novelty were also assessed, based on time spent interacting with the novel objects.

One-sample *t*-tests were conducted to examine if animals showed a significant preference for the side previously paired with the novel objects. Analysis of variance (ANOVA) was used to examine if the preference coefficient, the dependent variable, differed by sex, the independent variable, as well as if preferences differed between each side of the apparatus. ANOVA and *t*-tests were also used to analyse locomotion and object contact, both reported in seconds, for sex and side differences. Additionally, locomotor activity was compared with preference coefficients by Pearson correlation. If a significant correlation was found, an analysis of covariance (ANCOVA) was carried out to examine whether sex and side differences were present

in the preference coefficients with locomotion as a covariate. Non-normally distributed data were analysed with Chi-Square tests as needed. A significant α of .05 was used for all comparisons, and Bonferroni pairwise or post hoc comparisons were used where appropriate.

All data were analysed using SPSS version 17.0 for Windows (2009) software package. Effect size (partial-eta squared, η_p^2) and power (β) values for ANOVAs were calculated by SPSS, and Cohen's *d* and power for *t*-tests were calculated with G*Power Version 3.0.8.

2.1.1 Results

Preference for novelty

During the preference test trial, animals did not display a significant preference for the side previously paired with a novel object (t_{15} = 1.19, p = .251). Analysis of males and females separately showed no significant preference for the side previously paired with novel objects for either sex ($ts_7 \leq 1.03$ $ps \geq .337$). The preference coefficient did not differ either by sex ($F_{1, 12}$ = .05, p =.827), by novel object side ($F_{1, 12}$ = 1.81, p =.203), or by the interaction of the two ($F_{1, 12}$ = .02, p =.879; **Figure 1**). Although the preference coefficient was higher when the novel object had been placed in the left chamber, even when that side was examined separately, the preference was not significant (t_7 = 1.52, p = .173). As total locomotion during the preference test did not show a significant correlation with the preference coefficient (r_{16} = -.07, p = .797), locomotion was not considered as a covariate in the analysis of the preference coefficient.

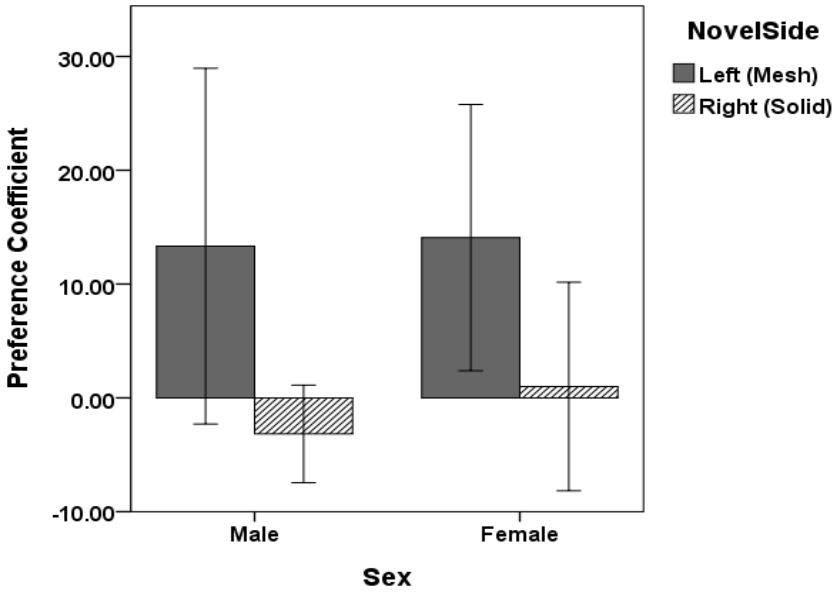

Figure 1. CPP 1 Mean preference coefficient during the preference test by sex and side previously paired with a novel object during conditioning trials. Error bars represent +/- 1 standard error of the mean.

Locomotion

Over the five conditioning days, both males and females exhibited higher locomotor activity, as defined by time spent in forward movement, when the novel object was present than when the chamber was empty ($ts_7 \geq 6.74$, $ps \leq .001$, $ds \geq .24$, $\beta s \geq .99$). There was a significant interaction between the sex of the animal, and which side was paired with the novel object in locomotion (right paired vs. left paired), on the right side of the apparatus ($F_{1, 12} = 7.85$, $p = .016$, $\eta_p^2 = .40$, $\beta = .73$), and similar trend on the left side ($F_{1, 12} = 3.82$, $p = .074$, $\eta_p^2 = .24$, $\beta = .44$). Males had higher locomotor activity in the right (solid floor) chamber (*M*s, *SD*s, in seconds: 262.28, 59.65 empty; 422.25, 44.40 with object) when an object was present compared to when no object was present ($p < .001$), and no difference in locomotion on the left side (metal mesh floor), ($p = .994$; *M*s, *SD*s, in seconds: 365.40, 68.58 empty; 365.14, 44.79 with object). Females, however, had higher activity in the left (*M*s, *SD*s, in seconds:

311.01, 36.90 empty; 404.28, 33.16 with object) when an object was present rather than when there was not an object (p = .017), and no difference on the right (p = .321; *M*s, *SD*s, in seconds: 323.94, 25.61 empty; 357.08, 44.89 with object). Males exhibited tendencies toward higher locomotion than females on the right side when a novel object was present (p = .065) and lower locomotion than females when it was empty (p = .078), but no differences on the left side, either empty or with an object (ps ≥ .134).

During the preference test, there were no sex differences in locomotion in either the chamber previously empty ($F_{1, 12}$ = .05, p = .826) or previously paired with an object ($F_{1, 12}$ = 1.35, p = .267), although females (*M*, 522.85; *SD*, 32.52, in seconds) displayed a tendency for higher total locomotion ($F_{1, 12}$ = 3.22, p = .098) than males (*M*, 485.99; *SD*, 42.95, in seconds). Overall, locomotion was significantly higher on the left side (metal mesh floor) of the apparatus (*M*, 282.62; *SD*, 56.17, in seconds) than the right side (solid floor) (*M*, 221.80; *SD*, 47.60, in seconds), (t_{15} = 2.55, p = .022, d = .64, β = .66), although there was not a difference based on whether a side was previously paired with a novel object (*M*, 260.91; *SD*, 57.76, in seconds) or was empty (*M*, 243.51; *SD*, 62.43, in seconds), (t_{15} = .62, p = .547). An ANOVA confirmed that this side difference in locomotion was not based on whether or not a side was previously paired with a novel object, since locomotion was higher on the left side of the apparatus both when it had been previously paired with a novel object ($F_{1, 12}$ = 5.19, p =.042, η_p^2 = .30, β = .55) and when it had been empty ($F_{1, 12}$ = 4.44, p =.057, η_p^2 = .27, β = .49).

There was no difference in the first side entered during the preference test based on the side previously paired with the novel object (χ^2 = .29, p = .590), nor was there a preference for the right or left side (χ^2 = 2.25, p = .134).

Object interaction

During the conditioning trials, males (*M*, 159.55; *SD*, 31.31, in seconds) spent more time interacting with the novel object than females (*M*, 124.41; *SD*, 19.10, in seconds), ($F_{1, 12}$ = 7.81, p =.016, η_p^2 = .39, β = .73). Although there was not a significant interaction between sex and side of the apparatus ($F_{1, 12}$ = 2.77, p =.122), the sex difference was primarily due to differences in object contact on the right side (solid floor) of the apparatus (p = .008; *M*s, *SD*s, in seconds: 172.18, 30.22 males; 116.11, 23.84 females). There were no sex or apparatus side differences in latency to approach the objects (Fs$_{1, 12} \leq$ 1.80, ps ≥.205).

2.1.2 Summary

The first test of novel object place preference used an existing apparatus that was modified as noted previously in order to provide different visual cues on each side. The subjects in this first study failed to exhibit a preference for the side paired with the novel object. The absence of conditioning was not due to the time spent in the conditioning trials, as the time in this study (50 minutes) was comparable to previous study (40 minutes) by Bevins and Bardo (1999).

There were several problems with the design of the apparatus which potentially influenced preference acquisition during the conditioning trials. In the course of testing it was observed that the paper liner used as a visual cue on the right side of the apparatus provided a distraction to the animals, especially the females, as they tended to interact with the paper rather than explore the environment and the novel object, if present. Although males displayed higher contact with the novel objects during the conditioning trials, there was a greater sex difference in contact on

the right side of the apparatus, with males showing higher interaction on that side, thus the distraction of the paper in the right chamber probably influenced preference acquisition. Also during observation of the conditioning trials, the heavier males seemed to have a more difficult time waking on the left side of the apparatus with metal mesh floor, yet spent more time inactive on the right side with the solid floor, which was confirmed by the significant interaction of sex and apparatus side in locomotion. Therefore, females may have exhibited decreased interaction and locomotion on the right due to the influence of the paper liner, while the males may have had a greater preference for the solid flooring on the right. Finally, as rodents generally prefer low light conditions (Crawley & Goodwin, 1980; Hascoët et al, 2001), it could be that the darker, left side of the apparatus elicited a preference. Each of these design problems could contribute to why the preference coefficient was higher when the novel object was placed on the left side of the testing apparatus during conditioning, even though males and females did not show a preference overall.

Since the design of the testing chambers probably influenced preference acquisition, a new CPP apparatus was built based on commercial designs and utilized for future tests. The apparatus was made out of clear polycarbonate, allowing the visual cues to be located on the outside of the apparatus, leaving the inside walls the same. The cues were also designed to have approximately equal amounts of light and dark areas, with the dark areas different shapes (triangles vs. squares), which should minimize light preferences. Since the solid floor seemed to encourage sedentary behaviour in the males, the metal mesh floor was retained, but the solid floor was replaced by one of equally spaced rods, in order for both floors to encourage exploration. The new apparatus was designed with three chambers, rather than an

external start box. This three chamber design had advantages over the apparatus used in the first test as it replicated commercial designs used in other research laboratories, background differences could be controlled on the outside of the chambers, and there were fewer distractions available to the animals. Additionally, different novel objects were selected to deter the animals from climbing on the objects and chewing the objects, which could have shifted the rewarding aspects of the objects away from the novelty of the object and also have affected recognition of the environmental cues.

2.2 Experiment 2

2.2.0 Methods

Subjects and housing

A total of 20 adult Lister-hooded rats, 12 males and 8 females, were tested in the second experiment. These animals were selected from in-house stock and were naïve to the CPP task. Although the number of males and females were not equal, the greatest number of available animals was selected to increase the power of the analysis. Housing was as previously described.

Apparatus

The rectangular apparatus was made from clear 6 mm scratch-resistant polycarbonate, with a pair of dark grey dividers separating it into right and left chambers, 48 cm x 48 cm x 45 cm (l x w x h), and a centre chamber, 22 cm x 48 cm x 45 cm (l x w x h). The outside of the apparatus was covered with patterned paper to provide distinct visual cues for each side; the left side with black triangles on a white background, and the right side with black squares on a white background, with approximately equal amounts of black and white on each side. The centre chamber, which was only

accessible on test day, had plain white background visible on the front and back, with the grey dividers on the sides. The left floor was metal mesh flooring taken from the previous apparatus; the right floor consisted of 3 mm rods, 13 mm on centre. The floor of the centre chamber was solid and made from the same dark grey polycarbonate as the divider walls. There was a gap under the floor with a pull-out tray (for ease of cleaning), that was spread with bedding during the trials. During conditioning trials, novel objects were plastic or glass containers filled with rocks, salt or beans, and a rock. During the preference test, the dividers, which had a semi-circular notch in the centre of one side large enough for the animals to pass through (13 cm wide x 13 cm tall), were adjusted to allow free access between all three chambers, which were now empty. Rather than remove the dividers completely, the animals would have a slightly obstructed view of each side of the apparatus, and thus perhaps more likely to enter each side to determine if an object was present or absent (**Figure 2**).

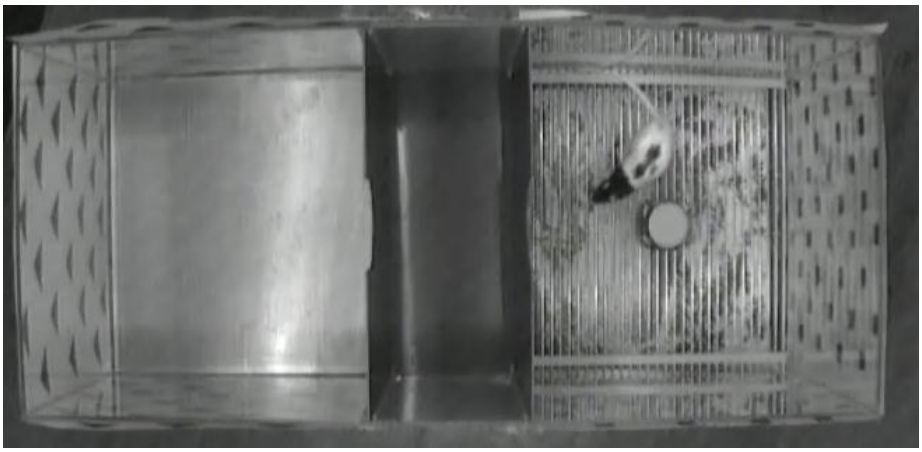

Figure 2. Conditioned place preference testing apparatus in use during a conditioning session with a novel object.

As new video capture software was used (see Behavioural Recording), the testing took place in a different, larger room than the first experiment. The apparatus

was located on the floor and was surrounded by a black curtain, while the video camera attached to the ceiling above relayed images to a computer. All tests were conducted in the same testing room under dim, white light (approximately 25 lux), and a white noise generator was used to mask external sounds.

Experimental design

Procedures were the same as in the previous experiment, with a slight difference in the preference test due to the new apparatus. On the sixth day, with the central dividers between the now empty chambers adjusted to allow free access between each side, the rat was placed in the central chamber and allowed to move between all three compartments for 10 minutes.

Behavioural recording

All CPP tests were digitally recorded directly onto the computer and analysed using EthoVision XT 5.0 software (Noldus Information Technology, Netherlands, 2008) and/or Observe software while watching the captured videos. EthoVision XT 5.0 is a behavioural tracking program which allows for video capture and analysis of behaviour, movement and animal activity as programmed by the experimenter. Animal detection is based on tracking movement of an animal on a contrasting colour background. When the contrast between the animal and the background is more pronounced, the tracking ability of the program is more accurate. Videos of sessions were stored and were available for review and further analysis if needed. Data captured included locomotion, object contact during conditioning, and time spent in each chamber during the testing session.

Statistical analyses

Statistical analyses were as previously described. However, for brevity, only performance during the preference test, not the conditioning trials is presented.

2.2.1 Results

Preference for novelty

A one sample *t*-test for all animals indicated no significant difference from zero in the preference coefficient (t_{19} = -.04, p = .967). Similarly, a paired sample *t*-test indicated no difference between time spent on the side previously paired with novel objects compared with the empty side (t_{19} = -.03, p = .978). Analysis of males and females separately showed no significant novelty preference for either males (t_{11} =.007, p = .995) or females (t_7 = -.13, p =.902). Analysis of the preference coefficient indicated a significant difference depending on which side had been previously paired with a novel object ($F_{1, 16}$ = 11.75, p =.003, η_p^2 = .42, β = .90), with all animals exhibiting a preference for the right, rod side of the apparatus, regardless of which side had been paired with a novel object during conditioning (**Figure 3**). This side preference was confirmed with a paired samples *t*-test (t_{19} = 3.40, p = .003, d = .76, β = .89; *M*s, *SD*s, in seconds: 128.99, 76.48 Left; 286.34, 135.99 Right). The interaction between sex and apparatus side was not significant ($F_{1, 16}$ = 1.15, p = .30), nor was there a sex difference ($F_{1, 16}$ = .007, p = .935). Total locomotion during the test trial did not show a significant correlation with the preference coefficient (r_{20} = .01, p = .966), and was not considered as a covariate in preference coefficient analysis.

During the preference test, there were 3 males that did not enter the left side of the apparatus. However, even if these subjects were excluded from the analyses, overall there was still not a significant preference for the side previously paired with a

novel object (t_{16} = -.78, p = .447), nor did the remaining males show a preference (t_8 = -.86, p = .415). The right side preference remained significant ($F_{1, 13}$ = 8.81, p =.011, η_p^2 = .40, β = .78), and the sex differences remained non-significant ($F_{1, 13}$ = .27, p =.612).

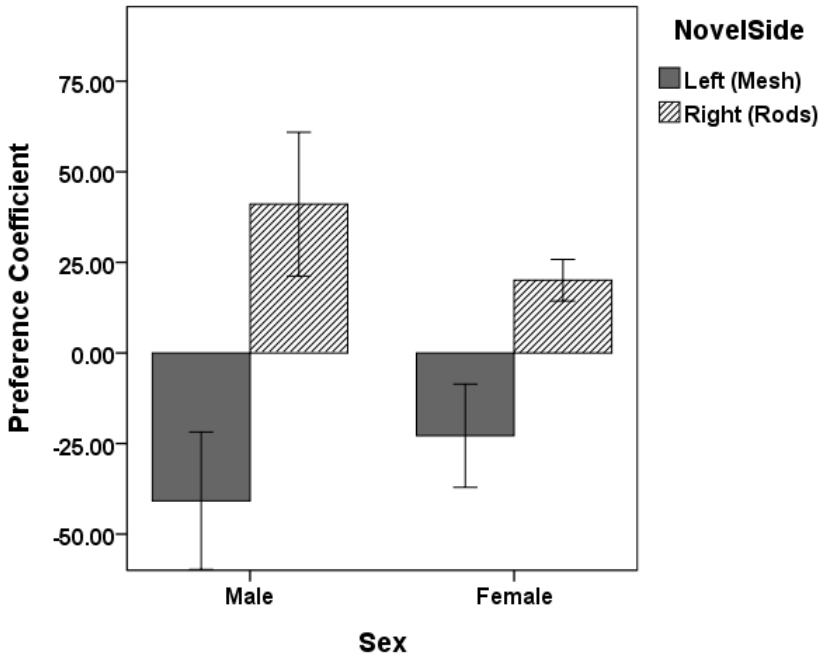

Figure 3. CPP 2 Mean preference coefficient during the preference test by sex and side previously paired with a novel object during conditioning trials. Error bars represent +/- 1 standard error of the mean.

Locomotion

There were no sex differences in locomotion during the preference test in either the side previously empty ($F_{1, 18}$ = .16, p = .697) or the side previously paired with a novel object ($F_{1, 18}$ = .07, p = .789). There was also no difference between males (*M*, 432.45; *SD*, 88.55, in seconds) and females (*M*, 389.65; *SD*, 50.71, in seconds) in total locomotion during the preference test ($F_{1, 18}$ = .15, p = .234). However, overall, locomotion was significantly greater on the right (rod floor) side of the apparatus (*M*, 286.34; *SD*, 135.99, in seconds) than the left (metal mesh floor) side (*M*, 128.99; *SD*,

76.48, in seconds), (t_{19} = 3.40, p = .003, d = .76, β = .90), although it did not differ based on whether a side was previously paired with a novel object (*M*, 206.86; *SD*, 134.73, in seconds) or was empty (*M*, 208.47; *SD*, 138.64, in seconds), (t_{19} = .03, p = .978). An ANOVA confirmed that this side difference in locomotion was not based on whether or not a side was previously paired with a novel object, since locomotion was higher on the right (rod floor) side of the apparatus both when it had been previously paired with a novel object ($F_{1, 18}$ = 12.49, p =.002, η_p^2 = .41, β = .92) and when it had been empty ($F_{1, 18}$ = 7.51, p =.013, η_p^2 = .29, β = .74).

There was no difference in the first side entered during the preference test based on the side previously paired with the novel object (χ^2 = .95, p = .329), nor was there a preference for either the right or left side as first entered (χ^2 = 3.20, p = .074), although the tendency was for the animal to enter the right side first (14 of the 20 test trials).

2.2.2 Summary

With the new testing apparatus, the test still did not elicit a conditioned preference for the side previously paired with a novel object. Instead, all animals showed a stronger preference for the right (rod floor) side of the chamber. Without the distractions of the previous apparatus, other differences could be affecting the preference other than the presence of a novel object. The metal mesh floor, as used in the first CPP test, seemed a likely candidate as the most striking difference between the two chambers. However, during the preference test, another design of the new apparatus could also have interfered with the display of preference. The first male animals with access to the centre chamber showed a need for the flooring of that section to be reinforced, as one of the males did not cross the apparatus during the test. However, even with the

floor temporarily reinforced for subsequent tests, two additional male animals exhibited the same behaviour pattern.

As the flooring seemed to be a problem, a third CPP test was designed with a new floor on the left side of the chamber. Instead of a metal mesh floor, a metal grid was used, with the idea of having both sides similar enough not to elicit a preference independent of pairing with a novel object. The floor of the central chamber was also reinforced.

2.3 Experiment 3

2.3.0 Methods

Subjects

A total of 18 adult Lister-hooded rats, 9 males and 9 females, were tested in the third experiment. Animals were from available stock and were naïve to the CPP task. Housing was as previously described.

Apparatus and experimental design

The left floor was changed on the third CPP test to a white wire grid, with approximately 1.5 cm squares. Under both sides, in both conditioning and test trials, standard bedding was scattered on removable trays. The floor of the centre chamber was reinforced with an additional piece of polycarbonate placed under the existing grey floor. Testing procedures remained the same as previously described.

Behavioural recording and statistical analyses

Both the behavioural recording and the statistical analyses were as previously described.

2.3.1 Results

Preference for novelty

A one sample *t*-test indicated no significant differences for all animals between times spent in the side paired with a novel object and the previously empty side as indicated by the novelty preference coefficient (t_{17} = 1.24, p = .233). Analysis of males and females separately showed no significant novelty preference for either sex (ts_8 < 1.09, ps > .309). An ANOVA of the preference coefficient indicated no difference depending on which side was paired with a novel object ($F_{1,14}$ = .32, p = .580) or sex ($F_{1,14}$ = .21, p = .655), nor was the interaction significant ($F_{1,14}$ = .04, p = .845; **Figure 4**). However, additional analysis indicated that animals spent a greater percentage of time in the centre chamber (*M*, 36.58; *SD*, 14.67, percent) relative to the side previously paired with a novel object (*M*, 26.99; *SD*, 11.96, percent), (t_{17} = 2.19, p = .043, d = .52, β = .54) with no difference between the centre and the empty sides (*M*, 36.43; *SD*, 19.23, percent), (t_{17} = .02, p = .984). There was no preference for either the right or left side of the apparatus (*M*s, *SD*s, in seconds: 198.63, 99.06 Left; 181.87, 101.03 Right), (t_{17} = .40, p = .697), and no preferences between either the right or left and the centre chamber (*M*, 219.50; *SD*, 88.01, in seconds), (ts_{17} < .99, ps > .337).

Analysis of the first 2 minutes of the preference test also indicated a non-significant preference coefficient, (t_{17} = 1.14, p = .271). However, further examination of the first 2 minutes of the preference test showed that 6 animals had not yet crossed the centre chamber to explore both sides of the apparatus. Additionally, 2 males at the end of the 10-minute trial had very little or no movement across the centre chamber.

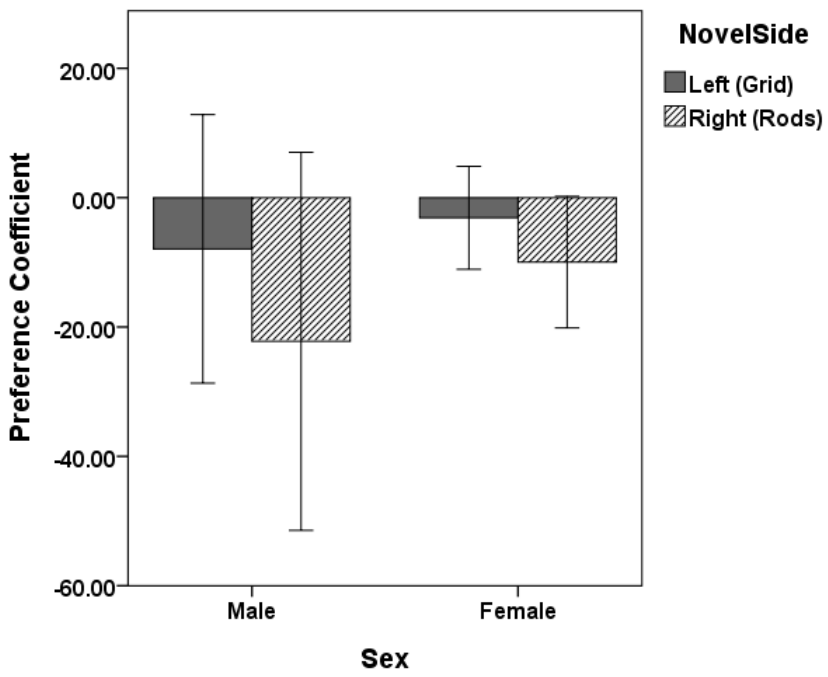

Figure 4. CPP 3 Mean preference coefficient during the preference test by sex and side previously paired with a novel object during conditioning trials. Error bars represent +/- 1 standard error of the mean.

Locomotion

There were no sex differences in locomotion during the preference test in either the side previously empty ($F_{1, 16}$ = 1.14, p = .302) or the side previously paired with a novel object ($F_{1, 16}$ = .16, p = .695). However, there was a tendency for males (*M*, 416.36; *SD*, 77.57, in seconds) to be higher than females (*M*, 344.64; *SD*, 86.90, in seconds) in total locomotion during the preference test ($F_{1, 16}$ = 3.41, p =.083, η_p^2 = .18, β = .41). There were no differences in locomotion between the right (rod floor) side of the apparatus (*M*, 181.87; *SD*, 101.03, in seconds) and the left (grid floor) side (*M*, 198.63; *SD*, 99.06, in seconds), (t_{17} = .40, p = .697). Locomotion also did not differ based on whether a side was previously paired with a novel object (*M*, 161.93; *SD*, 71.79, in seconds) or was empty (*M*, 218.57; *SD*, 115.40, in seconds), (t_{17} = 1.41, p = .178).

The first side entered on test day was not influenced by the side previously paired with the novel object (χ^2 = .3, p = .629), nor was there a preference for either the right or left side (χ^2 = .89, p = .346; 11 left first, 7 right first).

2.3.2 Summary

The third CPP test found no significant differences in the preference coefficient by sex or apparatus side. Both males and females did show a slight preference for the grid floor over the rods, although this was not significant and thus not able to explain the absence of preference. One of the possible influences could be the novelty of the centre chamber. Some of the animals exhibited hesitation to cross the centre chamber during the first preference test, which was thought to be a result of the floor. However, as 3 animals in CPP 2 exhibited the same behaviour, originally attributed to the problems with the floors, additional investigation was completed. When re-exposed to the apparatus 2 days after testing, these animals were willing to cross the central area. As the preference test was the first to expose the animals to the centre chamber, which must be crossed to explore the opposite side, the novelty of this chamber could either elicit avoidance behaviour, masking any conditioned preference, or elicit a preference as an attractor. The novel aspect of the centre chamber was also supported since the animals spent more time in the centre chamber relative to the side previously paired with a novel object. Therefore, perhaps familiarization with the entire testing apparatus before the conditioning trials could reduce any possible influences due to the novelty of the centre chamber.

Since no significant side preferences were found, (i.e., right, rod floor vs. left, grid floor) the problems associated with different floor surfaces seemed to have been resolved, and no additional changes were made to the testing apparatus. A

familiarization trial, exposing the animals to the entire apparatus before the onset conditioning, was added to the next test, in order to minimize the influence of the novelty of the centre chamber during the preference test.

2.4 Experiment 4

2.4.0 Methods

Subjects

A total of 10 adult animals, 5 males and 5 females, were tested in the last CPP experiment. Animals were from available stock and were naïve to the CPP task. This sample size was selected as sex was not to be included as a variable, but left open the option of including more subjects at a later stage. Housing was as previously described.

Apparatus and experimental design

On the first day, animals were allowed to freely roam between all three chambers of the apparatus for a period of 10 minutes. During the next three days, days 2 – 4, the animals were given conditioning procedures as previously described, with a novel object consistently present in one side of the apparatus, but not the other. The conditioning days were reduced to 3 (30 minutes total per side) in order to reduce the time to complete the CPP task, as future studies were planned to examine different age groups (see Discussion for more detail). Testing occurred on the fifth day, with animals again allowed access to all three chambers of the apparatus.

Statistical analyses

Data for males and females were analyzed together. In addition to the statistics previously described, side preferences were calculated for both the familiarization trial and the test trial, [(time left – time right)/(total time) * 100], with a negative value representing a left-side preference, and a positive value indicating a right-side preference. A preference change was then calculated as the difference between these preferences to see if an animal changed preference more toward the side paired with a novel object (+) or toward the empty side (-) of the apparatus.

2.4.1 Results

Preference for novelty

A one sample *t*-test indicated that overall the animals did show a preference for the side of the apparatus paired with a novel object during the conditioning trials (t_9 = 2.46, p = .036, d = .78, β = .59). An ANOVA indicated no difference in the preference coefficient due to whether the right or left side had previously been paired with novel objects ($F_{1,9}$ = 1.56, p = .247, **Figure 5**). Animals spent a greater percentage of time in the centre chamber (*M*, 40.09; *SD*, 9.39, percent) relative to the side that was empty during the conditioning trials (*M*, 24.47; *SD*, 5.57, percent), (t_9 = 4.43, p = .002, d = 1.40, β = .98) with no difference between the centre and the side previously paired with a novel object (*M*, 34.73; *SD*, 10.72, percent), (t_9 = .88, p = .404). There was no preference for either the right (rod floor) or left (grid floor) side of the apparatus during the preference test (t_{10} = 1.00, p = .344; *M*s, *SD*s, in seconds: 161.47, 46.25 Left; 193.68, 68.16 Right), although the animals spent more time in the centre chamber (*M*, 240.54; *SD*, 56.32, in seconds) than the left side (t_9 = 3.22, p =

.010, d = 1.02, β = .82), with no difference between the right side and the centre (t_9 = .88, p = .404).

Although there was no difference in preference coefficient depending on conditioning side, because there were differences in time spent in the centre chamber compared with the left (grid floor) side and compared with the side that was empty during the conditioning trials, the preference coefficient was examined separately by conditioning side. When the left (grid floor) side was paired with the novel object, the preference coefficient was not significant (t_4 = .76, p = .488). However, when the right (rod floor) side was paired with the novel object, the preference coefficient was significant (t_4 = 3.51, p = .025, d = 1.57, β = .89).

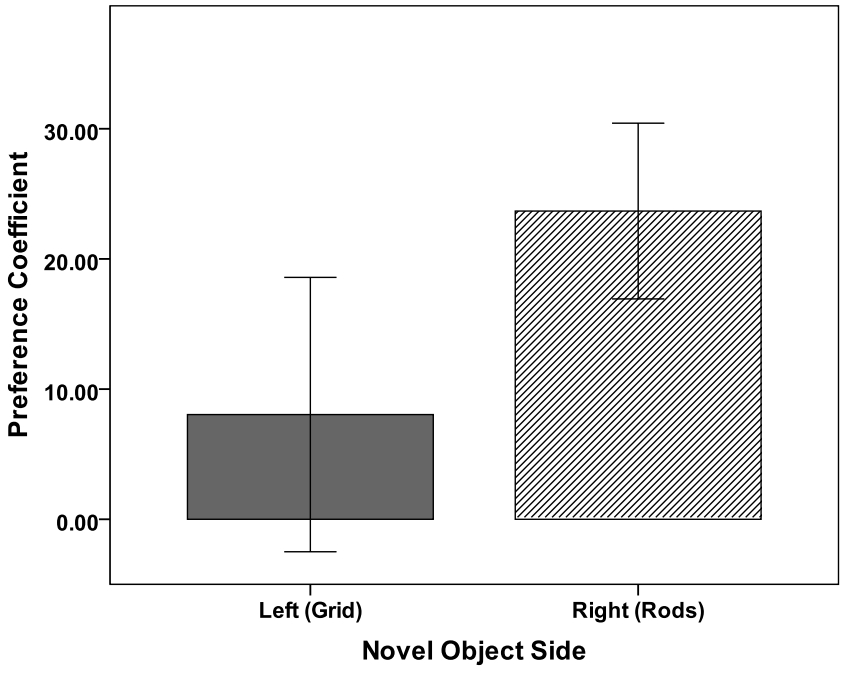

Figure 5. CPP 4 Mean preference coefficient during the preference test by side previously paired with a novel object during conditioning trials. Error bars represent +/- 1 standard error of the mean.

Preference change

During the familiarization trial, no side preference was displayed (t_9 = .13, p = .901). Analysis of the change in preference from the familiarization trial to the test trial indicated that preference did not shift significantly toward the side previously paired with a novel object (t_9 = .64, p = .538). The preference change also did not differ due the side of the apparatus previously paired with a novel object ($F_{1,8}$ = .56, p = .477; **Figure 6**).

In the familiarization session, although there was no difference between the percent of the total time spent on the right and left side of the apparatus (t_9 = .11, p = .917), the animals spent a significantly greater percentage of the total session time on the right side (rod floor; *M*, 36.18; *SD*, 8.20, percent) than in the centre chamber (*M*, 28.00; *SD*, 4.56, percent), (t_9 = 2.58, p = .030, d = .81, β = .63) with a similar trend toward a greater amount of time in the left side (grid floor; *M*, 35.63; *SD*, 8.89, percent) than the centre (t_9 = 2.16, p = .060, d = .68, β = .49). From the familiarization session to the preference test, there was no significant difference in the percentages of total time spent on the right side (rod floor) of the apparatus (*M*s, *SD*s, percent: 36.18, 8.20 familiarization; 32.51, 11.41 preference test), (t_9 = .94, p = .374). However, the animals spent a significantly lower percentage of the total time in the left side (grid floor) of the apparatus (*M*s, *SD*s, percent: 35.63, 8.69 familiarization; 27.11, 7.78 preference test) during the preference test compared to the familiarization session (t_9 = 3.00, p = .015, d = 1.62, β = .99), and a significantly higher percent of the total time in centre chamber (*M*s, *SD*s, percent: 28.00, 4.56 familiarization; 40.38, 9.45 preference test) during the preference test compared to the familiarization session (t_9 = 5.12, p = .001, d = .68, β = .49).

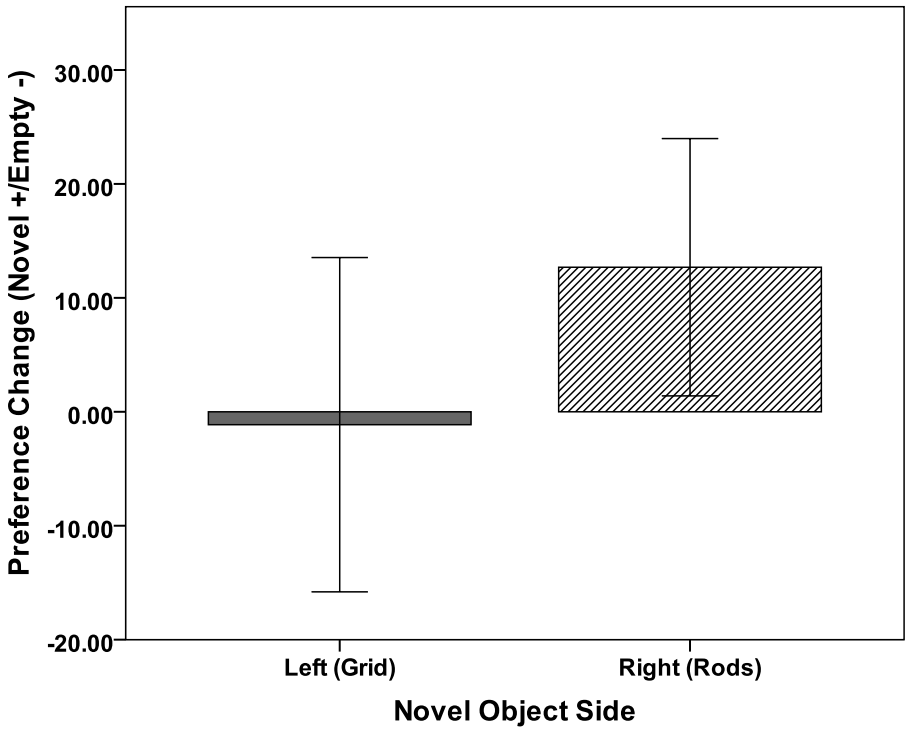

Figure 6. CPP 4 Mean preference change from familiarization trial to the preference test by side previously paired with a novel object during conditioning trials. Error bars represent +/- 1 standard error of the mean.

Locomotion

Total locomotion during the preference test did not differ depending upon whether the left side (grid floor; *M*, 397.92; *SD*, 12.04, in seconds) or right side (rod floor; *M*, 408.82; *SD*, 13.80, in seconds) was previously paired with a novel object ($F_{1, 8} = 1.77$, $p = .220$)

The first side entered during the preference test did not differ between right and left ($\chi^2 = 1.60$, $p = .206$; 7 left first, 3 right first), however it was influenced by the side previously paired with the novel object ($\chi^2 = 4.29$, $p = .038$), but not in the expected direction, given the preference for the rod floor. Of the 5 animals with the right (rod floor) side paired with novel objects, all 5 entered the left (grid floor) side

first; of the 5 animals with the left side paired with the novel objects, 3 entered the right side first.

2.4.2 Summary

The last CPP test included a habituation, or familiarization, trial, where animals were exposed to the full apparatus prior to the start of conditioning. This change in design also allowed within-subjects comparisons between the familiarization session and the preference test. As no preference for the right or left side of the apparatus was apparent during this familiarization trial, the previous problems due to the flooring seemed to have been addressed. After the period of conditioning, a preference for the side previously paired with the novel object was apparent, although only when the objects were paired with the rod floor. The apparent preference for the rod floor perhaps only emerged over repeated trials (i.e., by the end of testing). In the future, the conditioning data can be examined to assess locomotion in the two sides. However, when the change in preference from the familiarization session to the preference test was examined, similar to prior studies (Besheer et al., 1999; Bevins & Bardo, 1999; Bevins et al., 2002), the preference was no longer present. Therefore, although exposure to the novel objects appeared to elicit a conditioned preference for the side paired with the objects, the individual preferences of the animals to one side of the apparatus over the other may have diminished the strength of the preference.

Other factors could also have influenced the preference score. As this test used a small sample of animals and was aimed at examining the influence of a familiarization trial, individual differences in performance could have had a larger impact on findings. The choice of objects paired with the novel side could be another contributing factor: because the objects were selected to deter climbing and chewing,

the objects may not have sustained the interest of the animals in order to develop a preference during the conditioning trial. Perhaps an extended conditioning period, as used in previous CPP tests with novel objects, and/or the use of more attention sustaining novel objects, could strengthen the preference acquisition so it remains detectable using a within-subjects design.

When the percent of time spent in the centre of the apparatus during the preference test was compared to percentages of the time spent in the novel or empty sides, a higher proportion of the test time was spent in the centre chamber (≈ 40%) compared to the empty side. A higher percent of the total time was also spent in the centre during test day than during the familiarization session (≈28%), four days prior, with a corresponding decrease in time spent on the left side of the apparatus. The potential factors contributing to this finding will be discussed in the following section.

2.5 Discussion

As literature comparing male and female rat novelty-seeking behaviours is limited, the purpose of the preliminary experiments was not only to evaluate the effectiveness of the CPP task to determine novelty preference, but also to determine if there were sex differences in adult rats as previously suggested (Douglas et al., 2003). Additionally, these experiments were designed to refine procedures and equipment to elicit a preference for novelty in rats. Previous studies have used varying experimental designs as well as different statistical formulae of novelty preference, varies between researchers.

Several advantages of using CPP with drugs as a conditioning stimulus over other behavioural tests include the sensitivity of the test such that low doses can cause preference acquisition, that it measures both the rewarding and aversive properties of

drugs, and that surgical procedures are not required (Carr, 1989). However, several negative aspects of the test also need to be considered. One of the potential problems arises from individual differences in an animal's tendency to prefer one environment over another, so care must be taken during the design of the testing apparatus that one side should not be more aversive or attractive than the other. During our first two experiments, a stronger preference for one side over the other of the apparatus was apparent. As the animals only had access to one chamber at a time during conditioning, this preference was not noticeable until analysis of the test data. Modifications were made to the testing apparatus and, by the third experiment, side preferences were minimized.

An alternative way to address individual side preferences is by comparing changes in place preference away from a preferred side, determined during a familiarization trial administered prior to the start of conditioning to the test trial, then potentially having paired the stimulus with either the initially preferred or non-preferred side (Bardo & Bevins, 2000). However, Bardo and Bevins (2000) point out that these methods to alleviate side preferences can be problematic, as the effects of conditioning can be masked either by the animals reaching a plateau of preference, if paired with the preferred side, or from a reduction in aversion to the non-preferred side through repeated exposure rather than an increase in preference due to the conditioning stimuli. In our fourth experiment, although a preference for the side paired with the novel objects was found, this preference was not a significant increase over the preferences displayed by individuals in the familiarization session, even though, overall, right or left side preferences were not indicated in either session. Although the side paired with the novel object was randomized in our studies, as there was not a significant side preference overall indicated in the familiarization session,

the selected side was not based on the initial side preference of the individual animals. Therefore, it is possible that a preference plateau, as suggested by Bardo and Bevins (2000), was reached, which could have been minimized by pairing the novel objects to the non-preferred side of the apparatus.

As the novel object conditioning stimulus is not present during the preference test, another limitation of the CPP test arises from the change of 'object present' to 'object absent' on the conditioning side of the apparatus. During the final preference test, the previously paired side of the apparatus could now be seen as novel relative to the empty side during testing, which remained unchanged, and could interfere with assessment of the rewarding characteristics of the stimulus (Bardo & Bevins, 2000). Since rats are drawn to novelty (Berlyne, 1950) the introduction of an additional novel condition, the now empty conditioning side or access to the centre chamber, during the CPP preference test could act as an attractor and be enough to counter the effects of conditioning. In the third CPP experiment, the first access to the central chamber was during the test trial. Although the removal of the novel objects from the paired side would also have been novel, the preference for the central chamber over the side previously paired with a novel object seems to indicate that preference had shifted to that which was relatively more novel to the animal. Besheer and colleagues (1999), as well as Bardo and Bevins (2000), suggest that habituation trials where the animals are given free access to the entire testing chamber before the conditioning period would be enough to prevent a shift of preference based on the novel aspects of the apparatus instead of a shift due to conditioning with the novel objects.

Our fourth experiment included a familiarization session, and, while preference for the side paired with a novel object did not change between the familiarization session and the preference test, time on the always empty side

decreased and time in the centre chamber increased. The changes in times spent in the empty side and centre could suggest that although the rewarding aspects of the novel objects were sufficient to elicit a place preference for the side of the apparatus paired with a novel object, re-exposure to the centre of the apparatus was also novel, and was enough to affect the preference score. Studies of memory in adult rats suggest that memory for novelty object recognition can vary not only with inter-trial-intervals, but also with recency of exposure, and can be influenced by intervening events (Ennaceur, 2010). Thus, in the CPP preference test, the increase in exploration of the central chamber could be the result not only of prior exposure, where the familiarity of it would actually contribute to the increased exploration, but also due to the central chamber being more recently novel than either side. Bevins et al. (2002) have two days of habituation trials prior to conditioning, then use the average preferences of each individual subject to determine the non-preferred side with which side to pair the stimulus for that subject. The use of two, rather than one, habituation trial would also give additional exposure to the central chamber which could reduce the increase in centre exploration relative to the sides.

One additional limitation of novelty-induced CPP, not addressed by Bardo and Bevins (2000), is the amount of time required for habituation, conditioning and testing a sufficiently large sample. While time considerations are less important when testing adults, the eventual aim of this research was to examine age as well as sex differences in novelty preferences. Developmental changes of interest in rats occur from weanling stage, pnd 21, to early adolescence, pnd 28, through the onset of puberty at mid-adolescence, from pnd 33-44, and, finally into adulthood, beginning at pnd 60, and are linked with changes in gonadal hormones (Spear, 2000; Tirelli et al., 2003). While additional benefits of CPP are based on its sensitivity to low drug doses and

that place preference can be obtained after one period of conditioning in drug testing, eliciting a response to novelty is dependent on the period of habituation and conditioning (Bardo & Bevins, 2000).

In the one study found to compare CPP with novel objects in adolescent and adult animals, Douglas and colleagues (2003) tested the animals during the ranges of pnd 33 to 38 for adolescence, and pnd 65 to 70 for adults. However, Anderson and Teicher (2000) found D1 and D2 receptor density increased at the highest rate between pnd 25 and 40, then decreased through pnd 120, especially in male Sprague Dawley rats. While D2/D3 receptor antagonists were ineffective at blocking the acquisition of conditioned place preference with novel objects (Besheer, Jensen & Bevins, 1999), the effects were studied in adult animals and not adolescents. Therefore, the findings of Douglas and colleagues (2003) may not be as precise at measuring age differences in behavioural responses to novelty due to the age span of the animals over the time required to conduct the CPP test. As significant physiological changes occur relatively quickly in rats, the time needed to complete CPP task may reduce the sensitivity of the test to age differences.

Despite the difficulties in eliciting a novelty induced place preference in the CPP task, it is interesting to note that there were no sex differences in preference during any of the experiments. Although these findings are inconsistent with the prior study examining the performance of both males and females, where adult females, but not males, in the pair-housed condition exhibited a preference (Douglas et al., 2003), perhaps the testing apparatus and procedures used in this series of experiments were not yet sufficiently sensitive to detect a difference. However, as the sex differences during the conditioning sessions were not based on novelty, but influenced by the apparatus surfaces, they may have been partly based on body size differences which

could have influenced locomotion. The animals used in these tests were full-grown adults, utilized to minimize resources while determining the feasibility of the CPP task, compared with the early adults, pnd 65 – 70, tested by Douglas et al. (2003). Although a direct comparison cannot be made, it may be that sex differences in conditioned place preference with novel objects are apparent in young adulthood, but not at later stages of development.

One final factor that might explain the difference between our results and previous finding is the strain of rat. Our study examined the ability of novel objects to elicit a conditioned place preference in Listar-hooded (LH) rats, whereas the previous studies (Besheer et al., 1999; Bevins & Bardo, 1999; Bevins et al., 2002; Douglas et al., 2003), examined the effect in Sprague Dawley (SD) rats. McDermott and Kelly (2008) reported behavioural differences between the two strains, with LH rats exhibiting higher activity in a locomotor task and a higher percentage of open arm entries in an elevated plus maze test than SD rats. Strain differences in cognitive tasks such as the water maze, delayed match to position, and two-object discrimination task have also been reported (Andrews et al., 1995; Andrews, 1996). In the two-object discrimination task, the Long-Evans hooded rat, similarly behaved to the Listar-hooded, was better at discriminating a novel object than the SD (Andrews et al., 1995). Perhaps our LH rats were more active during the conditioning sessions, with a greater interest in the novel objects, than the SD rats and, thus, less attentive to the environmental cue differences, affecting the ability to form an association between apparatus side and novel objects.

Although the initial design of our CPP apparatus was based on apparatus designs from prior place preference studies, several modifications were required before a preference could be detected in this study. However, in the studies of Bevins

and Bardo (1999), for example, the testing apparatus used with novel objects had also been effective at eliciting a conditioned place preference with drugs. An additional study in our CPP apparatus could examine if drugs associated with sensation seeking, such as amphetamines or cocaine (Bardo, Donohew & Harrington, 1996), are able to elicit a conditioned place preference. Since both amphetamines and cocaine have been successful at producing a conditioned place preference in previous studies (Bardo & Bevins, 2000), the outcome of the same experiment in our apparatus could assist in determining the effectiveness of the apparatus design.

In summary, the study by Besheer et al. (1999) examined behavioural responses to novel objects in both the CPP task and a novel object recognition task, and found differences in the ability of novel objects to change behaviour over time (CPP) compared with the immediate preference for a novel stimulus (NOR). Douglas et al. (2003) also found a difference in novel object interaction during the conditioning sessions of the CPP task, that adolescents across in all conditions, male/female and group/isolate housed, which was not consistent with the differences found in the preference test. Both of these studies suggest that the CPP task is measuring a different dimension of novelty than the novel object recognition task. As drugs of abuse are able to elicit conditioned place preference with a single drug pairing (Bardo & Bevins, 2000; Tzschentke, 1998), the CPP task may be more sensitive to the rewarding aspects of novelty, and thus similarly reflective of risk taking behaviours. A test that is more sensitive to novelty seeking may be better suited to comparison with human measures of sensation seeking, such as the SSS-V (Zuckerman, 1994). Based on the limitations of the CPP task, the next set of tests to examine responses to novelty will be a different testing paradigm, the novel object recognition task.

Chapter 3: Adult sex differences in novel object recognition

3.0 Introduction

In mice and rats measures of novelty seeking are characterized by both approach and avoidance behaviours signified by the animal either approaching and exploring novel stimuli, or exhibiting what is thought to be a fear response to the stimuli by avoiding novel objects or environments (Montgomery, 1955; Montgomery & Monkman, 1955; Welker, 1957). Therefore, how animals respond to novelty can be seen as a balance between expressions of neophilia and neophobia (Barnett, 1958). Many different behavioural tests have been used to measure response to novelty in animals, including open fields, hole-board apparatus, arenas containing novel objects, and place preference tests (Brown & Nemes, 2008; Hughes, 1997; Renner, 1990). However, several of these tasks require 'forced' entry to a novel environment or measure interaction with a single, novel object rather than providing the animal a 'choice' of novel versus familiar objects or environments (Welker, 1957). In this chapter, animal performance is examined in a task that forces the animal to confront novelty and also provides subjects with the opportunity to choose between a novel and a familiar stimulus.

This series of novelty preference tests involves determining preference between two objects, one familiar and one novel, and is commonly referred to as the novel object recognition (NOR) task (Berlyne, 1950; Ennaceur & Delacour, 1988). In this task, an animal is first introduced to an empty testing apparatus in order to become familiar with the environment. In the second session, two objects, both novel to the animal and either identical to or distinct from each other, are placed into the arena and the animal is allowed to freely interact with the objects and the

environment. The previous exposure to the testing apparatus during the familiarization session should act to reduce novelty avoidance, and the animals should approach the novel stimuli (Sheldon, 1969). In the last session, one of the objects is replaced by a novel item, and the animal is given a 'choice' of interacting with either the novel or familiar object. When approach behaviours to the novel item versus the familiar item are compared, the animal is said to display a preference for novelty if a larger proportion of contact time is spent interacting with the novel object.

Early work consistently showed that animals tend to approach and explore novel objects more than familiar objects (Berlyne, 1950; Dember, 1956; Thompson, 1954). Berlyne (1950) was the first to use an object recognition task to examine rodent behaviour towards novel objects in rats. The subjects of this study were 12 adult male Wistar rats (n = 6 per group). After a 20 minute period of habituation to the testing apparatus, each subject in the experimental group were exposed to 3 identical objects for two sessions of 5 minutes each, with inter-trial intervals (ITIs) of 10 minutes, then one of the objects was replaced with a new, distinct object before the animal was reintroduced to the apparatus for a third 5 minute session. The control group was also exposed to three objects, two of which were identical and one different, in 2 sessions of 5 minutes each. Both the objects presented as novel or familiar (wooden cube or cardboard ring) and placements of the distinct object (left, middle or right position) were counterbalanced. Animals in the experimental group spent more time interacting with the novel object than the familiar objects in the last session, whereas the control animals did not interact more with the distinct object during any trial, including the first trial, when they were first exposed to a similar combination of objects as the experimental group. The increased exploration of the new object by the experimental group in the third trial was attributed to the novel

aspect of the new object compared to the familiarity of the previously encountered objects, rather than to the physical properties of the object.

Although Berlyne (1950) developed this test to examine exploratory behaviour, Ennaceur and Delacour (1988) promoted this test as a working memory task in rodents. The NOR task is similar to delayed matching or non-matching to sample tests that are commonly used in primates, thus allowing for interspecies comparisons. In a series of studies, Ennaceur and Delacour (1988) examined novel object exploration in adult male Wistar rats (n = 10 to 23 per group) using two different testing designs and four different ITIs – 1 minute, 1 hour, 4 hours and 24 hours. In all the tests, animals were exposed to the apparatus on the day prior to the start of testing for 2 minutes as a familiarization session. In the first testing design, the animals were exposed to a single object either for a period of 5 minutes or until they had contacted the object for a minimum of 20 seconds, then removed from the apparatus for the various ITIs. Subjects were finally reintroduced to the apparatus, which now contained a second copy of the object in a new position in addition to the first object, for a 3-minute test session. When the first session was 5 minutes, animals in each of the ITI groups spent more time interacting with the new object compared to the familiar object, although this difference was significantly smaller in the 24 hour interval group. However, when exposure to the object in the first trial was limited to 20 seconds, only the 1-minute ITI group displayed a preference for the novel object. These findings suggest that a preference for novel objects is dependent upon the amount of exposure to familiar objects as well as to the duration of the ITI.

In the second design, after a familiarization session, the animals were exposed to two identical objects in the first trial, then after ITIs of 1 minute, 1 hour or 24 hours, the animals were reintroduced to the apparatus for a second trial with one of

the familiar objects now replaced by a completely different object. Unlike Berlyne (1950), Ennaceur and Delacour (1988) did not have a control condition to assess animal response to two distinct objects in a first trial, when both would be novel to the subject. Although the objects used by Ennaceur and Delacour were counterbalanced as either novel or familiar, the only exposure to two distinct objects was when one object was novel and one object was familiar in the testing session. However, in the two-object design, interaction with the novel object was greater than the interaction with the familiar object after the 1 minute and the 1 hour ITIs, but not after 24 hours. Ennaceur & Delacour (1988) demonstrated that rodents spent more time exploring novel objects over familiar objects, which was influenced by the amount of exposure to the familiar objects as well as the interval between trials. Therefore, to examine memory, inter-trial intervals (ITIs) of varying lengths are used, and a decrease in novelty preference with increasing interval length is assumed to result from reduced recognition of the familiar object (Ennaceur, 2010).

The NOR task is also used to examine spatial memory, by moving familiar objects into novel locations, as well as contextual memory, by introducing familiar objects in a novel environment. A current area of research utilizes a variation of the recognition task with object, spatial and contextual components tested together in order to model episodic memory in rodents (Eacott & Norman, 2004; Langston & Wood, 2010) Lesions to the hippocampus, as well as to various limbic areas with connections to the hippocampus, generally have little effect on preference for the novel object in the NOR task (Ainge et al., 2006). Deficits are reported in spatial and contextual memory performance in animals with hippocampal or fornix lesions, suggesting that object recognition alone utilizes different neural areas than memory (Ainge et al., 2006; Eacott & Gaffan, 2005; Ennaceur, Neave & Aggleton, 1997;

Mumby, 2001; Mumby et al., 2002). Lesions to the parahippocampal region, specifically the perirhinal cortex, do cause deficits in object recognition when the objects are very similar (Brown & Aggleton, 2001; Mumby & Pinel, 1994). However, when the novel stimulus is more visually distinct from the familiar, as in Ennaceur and Delacours' second design (1988), animals with perirhinal lesions continue to show a preference for the novel object in the object recognition task (Bartko et al., 2007; Norman & Eacott, 2004). Therefore, while memory may be a component of novelty preference as measured by behaviour in the NOR task, responses to novelty also may be mediated by other neural actions (Hughes, 2007). Keeping the inter-trial interval short and ensuring that the novel object is visually distinct from the familiar objects should minimize the probability that any differences in novelty preference detected between groups are due to differences in memory.

Relatively few studies have examined sex differences in novelty preference in adult rodents. Most studies use male rodents and mainly examine spatial and non-spatial memory processes. Among those studies that have examined sex differences in NOR performance, the results are contradictory. A study by Ghi and colleagues (1999), using a two-arm maze in a different testing design from the standard NOR test, examined sex differences in memory performance on an object recognition task. Male and female Wistar rats (n = 8 – 18 per group), aged 40 days when sorted into testing groups, were handled and placed in an unfamiliar cage for 2 minutes per day for one week prior to testing in a two-arm maze (exact age at testing not indicated). The animals were first exposed to the maze, which had an identical object at each end of the two arms, for 12 minutes, before being removed for an ITI of 30, 60, 90 or 120 minutes. With one of the two objects replaced by a novel object, the animals were returned to the maze for an additional 8 minutes. Both sexes spent approximately

twice as much time exploring the novel object than the familiar object at ITIs of 30 and 60 minutes, and no sex difference was indicated. However, there was a sex difference at 90 minutes, with females continuing to show a novelty preference, but not males, while neither sex exhibited a preference at 120 minutes (Ghi, Orsetti, Gamalero & Ferretti, 1999).

Another study by Sutcliffe and colleagues (2007) examined sex differences and the influence of the oestrus cycle on working and spatial memory using a standard NOR task. Adult male and female Lister-hooded rats (n = 6 per group) were exposed to the empty testing apparatus for 3 days prior to the start of testing for a period of 30 minutes per day, as well as an additional 3 minutes just prior to the start of the test. During the first test trial, the animals were able to explore two identical objects for a period of 3 minutes. After a specified interval (3 or 30 minutes; 1, 2, 3, 4, 5, 24 or 48 hours), the animals were again exposed to objects for a period of 3 minutes, but in this second session one was a duplicate of the first object and the other a novel object. A discrimination index was calculated from object interaction during the second session by subtracting the time exploring the familiar object from the time exploring the novel object then dividing this difference by the total exploration time. If the index was positive, the animals spent more time exploring the novel object. Sutcliffe and colleagues (2007) first found no difference based on oestrus cycle in females for novel object preference, which was assessed at the 1 hour ITI only. They also found no difference between males and females in object exploration during the first test trial. However, although both male and females exhibited a preference for the novel object in both the 3 and 30 minute ITI conditions, only females continued to show novelty preference for intervals up to 3 hours, indicated by sex differences in preferences at intervals of 2 and 3 hours. Neither sex showed a preference for the

novel object at the intervals of 4, 5, 24 or 48 hours (Sutcliffe, Marshall & Neill, 2007). Although Sutcliffe et al. (2007) and Ghi et al. (1999) used different testing designs and different aged animals so that their results can not be directly compared, both found female rats showed a preference for the novel object at longer intervals than males. With shorter ITIs, both males and females exhibited a preference for the novel object over the familiar. Both sets of authors suggest the sex differences with longer ITIs are possibly influenced by hormonal and neurotransmitter system interactions.

In contrast to the results of Ghi et al. (1999) and Sutcliffe et al. (2007), three other studies have reported that males outperformed females on the NOR task or found no difference between male and female performance. A study on the effects of neo-natal handling in Sprague Dawley rats found male control animals, those not handled between pnd 1 and 21, outperformed female controls after a 3 hour ITI (Kosten, Lee & Kim, 2007). Kosten and colleagues (2007) tested adult rats (n = 6 per group) in an object recognition task. Animals were habituated to the testing apparatus for 5 minutes per day over 5 days, plus one additional minute immediately prior to the first testing trial, where the rats were exposed to 2 identical objects until a period of 30 seconds total object exploration had occurred. After an ITI of 3 hours, one of the objects was replaced by an entirely new object, and the animals were reintroduced to the testing apparatus for a second trial until 30 seconds of total object exploration had been reached. Over both sessions, male rats took a longer period of time to reach the 30 seconds of total object exploration than females. After the 3 hour ITI, control males spent more time exploring the novel object than the familiar object during the 30-seconds of contact, whereas control females did not differ in exploration between the familiar and the novel object.

Frick and Gresack (2003) used a series of seven 5-minute sessions with short 3-minute ITIs to test sex differences in both spatial novelty (session 6) and object novelty (session 7) in adult C57BL/6 mice (n = 23 – 25 per group), and found that males displayed a preference for the novel object in session 7, but that females did not. However, a similarly designed seven session study by Ricceri and colleagues (Ricceri, Colozza & Calamandrei, 2000), which will be discussed in more detail in the next chapter, found no sex difference in the preference for the novel object exhibited by male and female adult CD-1 mice (n = 8 – 10 per group) after short ITIs of 2 minutes. Kosten and colleagues (2007) attributed discrepancies in findings between studies to differences in handling, strains, ages and testing protocols. Frick and Gresack (2003) agreed that strain differences could contribute to different findings, noting that CD-1 and C57BL/6 strains show different responses to oestrogens. Based on the different findings across studies, additional investigation of sex differences in the novel object recognition task could add to the available literature.

The aims of this study were to investigate whether male and female rats behave differently on the NOR task and whether the task is a reliable measure of novelty preference. To minimize and reduce the influence of memory differences, the ITI was kept to 2 minutes. As in previous studies, a familiarization session was used to reduce the potential aversive qualities of exposure to a novel testing apparatus and increase the chance that animals would exhibit approach behaviours to the objects presented during the test trials. Besides collecting data on interaction with the objects, other measures, such as locomotion either in both the test apparatus and in automated locomotor boxes, were examined as potential contributors to sex differences in novelty preference. Both males and females rats were predicted to exhibit a preference for novelty with a short inter-trial-interval.

3.1 Experiment 1

3.1.0 Methods

Subjects and housing

A total of 24 adult Listar-hooded rats (12 males and 12 females) were tested on both unconditioned novel object preference and locomotor activity. Rats were housed in same-sex pairs in cages (measuring 25cm x 45cm x 15cm) with soy-free rodent pellets and water provided *ad libitum*. Housing rooms were controlled for temperature (20 ± 1ºC) and humidity (55 ± 5%), and maintained on a 12-hour light: dark cycle (lights on 7am).

All appropriate guidelines and requirements were adhered to, as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85-23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986.

Apparatus

The novel object recognition (NOR) testing apparatus was a wooden, grey-painted square chamber, 67cm x 67cm x 45cm (l x w x h), with a solid floor constructed of the same material. The chamber was raised 28cm above the ground on a metal stand. In the first test, familiar objects were identical combinations of large LEGO® Duplo blocks, and novel objects were a small clay brick and an arrangement of clear plastic blocks.

The testing took place under dim, white light (approximately 25 lux), while the observer was seated approximately 90 cm away from the apparatus. All tests were conducted between 09:00-14:00 hours in the same testing room.

The apparatus used for measuring locomotor activity was a set of clear polycarbonate chambers (3 shelves stacked vertically, 2 boxes per shelf), each measuring 46 cm x 24.5 cm x 22cm (l x w x h), with a lid and a set of photo-beams (LEDREARING model, Hamilton-Kinder, L.L.C.). These chambers were used under red-light condition.

Experimental design

Animals were tested for locomotor activity during a 30-minute session. Locomotor testing was conducted the day prior to NOR testing for half the animals, and the day after NOR testing for the other half, to control for test order effects. As the locomotor boxes were transparent, of the six locomotor boxes available for use, only three were used in each 30-minute session, one per shelf, to minimize any potential influence of visual contact with a neighbouring animal. To minimize disruption, no observer was present during the locomotor testing sessions. On the day prior to the NOR test, each subject was given a 30-minute familiarization session where the animal was allowed to freely explore the apparatus. During the first session on test day, two identical objects (multi-coloured LEGO® Duplo blocks tower) were placed against the wall, approximately 12 cm apart, in adjacent quadrants of the apparatus, and the animal was placed into an opposite, empty quadrant, facing away from the objects (Trial 1; T1; **Figure 7**). After 10 minutes, the animal was removed to an empty carrier container measuring 42cm x 26cm x 13cm (l x w x h) for a period of 2 minutes while the apparatus and objects were cleaned with a 70% ethanol solution and allowed to air dry, after which one of the objects was replaced by the novel object. The object that remained was considered the familiar object. The animal was then reintroduced to the apparatus as in the first session and allowed to interact with the objects for an

additional 10 minutes (Trial 2; T2). After testing, the subject was returned to the home cage and the apparatus and objects were cleaned with a 70% ethanol solution and allowed to air dry before a new subject was tested.

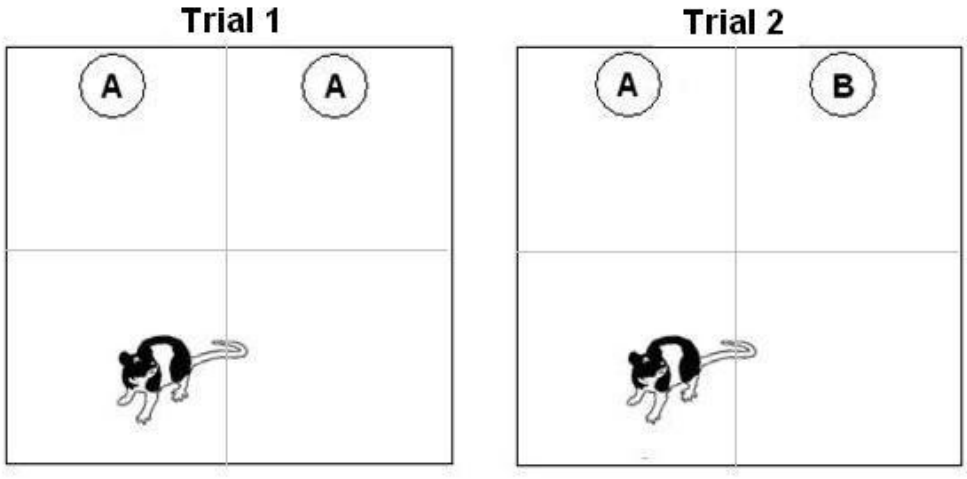

Figure 7. A diagram of object position and animal placement during Trial 1 and Trial 2. Grey lines denote quadrant boundaries but were not present in the apparatus.

Behavioural recording

Data during the first and second trials were captured by the seated observer by means of real-time manual input using Observe software, previously developed in-house, onto a laptop computer. Various keystrokes were toggled to start or stop recording the duration of an activity on each side (e.g. locomotion, object contact), with the elapsed time before each key was depressed also captured (e.g. latency to contact). During NOR Trials 1 and 2, time spent in contact with the objects, time spent in each quadrant, and number of quadrant crossings were recorded. Object interaction was recorded separately for each object and was defined as time spent when the nose was in contact with the object, which excluded behaviours such as backing into the object, tail only contact, or time resting next to the object without direct interaction. An animal was considered to have entered a quadrant when all four paws were located in that quadrant.

During the locomotor test, basic movements were detected by photobeam disruption and automatically collected by MotorMoniter software (version 4.14, Hamilton-Kinder, L.L.C).

Behavioural measures

Time spent with the novel object in Trial 2 was converted to a percentage of the total time spent with both the familiar and novel objects. This measure, referred to as *preference for novelty*, was calculated as the proportion of time spent interacting with the novel versus the familiar object in Trial 2, converted to a percentage [(Time with novel – Time with familiar)/(Time with novel + Time with familiar) * 100]. A positive value indicates a preference for the novel object, while a negative value indicates a preference for the familiar object, and a score of zero indicates equal preference for the two objects.

A second measure, referred to as *preference change*, takes into account any initial left/right side-bias shown by the subject. To take into account any such bias and also any individual object preference bias, a side preference was first calculated for both Trials 1 and 2 [(Time with right object – Time with left object)/(Total time with both objects) * 100], with a negative value representing a left-side preference, and a positive value indicating a right-side preference. *Preference change* was then calculated as the change in object contact times from Trial 1 (T1) to Trial 2 (T2), [(T2Right – T2Left)/(T2Right + T2Left)]*100 – [(T1Right – T1Left)/(T1Right + T1Left)] *100. Once the data had been tabulated, the preference change value was changed to positive (+) if contact changed toward the novel object, or to negative (–) if more toward the familiar object. For example, in Trial 1, if an animal spent 80% of the total contact time with the left object, a side preference score would be calculated

as -60, indicating that 60% more of the total time was spent in contact with the left object than the right object. Then, in Trial 2, if the same animal now spent 60% of the total contact time with the left object after the right object was replaced with a novel object, the side preference score would be -20, or 20% more time was spent with the left object than the right object. Although the difference between Trial 1 and Trial 2 side preference scores was -40, the preference change was in the direction of the novel object, to the right, so the preference change measure would be a score of + 40. If no bias was present in Trial 1, then the novelty preference score and the preference change score would be the same. In this example, the novelty preference score would be -20.

Statistical analyses

One-sample *t*-tests were carried out to examine if animals showed a significant preference for the novel object compared to chance, as indicated by a score significantly greater than zero. Analysis of variance (ANOVA) was used to examine whether the novelty preference (the dependent variable) differed by sex (the independent variable) as well as if preferences differed between each side of the apparatus. Although the objects used in Trial 1 and Trial 2 were randomized as novel or familiar between animals, preferences and object contact were also examined by object in order to look at any potential biases. Locomotor activity (from both the locomotor box session and Trials 1 and 2), object interaction, and novelty preference measures were compared using Pearson correlations. Locomotor activity was measured by the number of photobeam disruptions per minute in the locomotor box, with the total number of disruptions during the 30-minute trial considered in the correlations, and by the total number of quadrant crossings during the NOR test. If a

significant correlation was found between the novelty preference and another behavioural measure, an analysis of covariance (ANCOVA) was carried out to see if any significant differences remained. A repeated measures ANOVA was also used to analyse locomotor and object contact measures for sex differences, with Greenhouse-Geisser correction if needed.

A significant α of .05 was used for all comparisons and Bonferroni pairwise or post hoc comparisons were used to investigate sex differences. Non-parametric data were analysed with Chi-Square tests.. All data were analysed using SPSS version 17.0 for Windows (2009). Effect size (partial-eta squared, η_p^2) and power (β) values for ANOVAs were calculated by SPSS, and Cohen's *d* and power for *t*-tests were calculated with G*Power Version 3.0.8.

3.1.1 Results

Locomotion

Analysis of locomotor box activity indicated a significant sex difference, with females more active during the 30-minute test than males, ($F_{1, 22}$ = 24.97, p < .001, η_p^2 = .53, β = 1.00; **Table 1**). There was a significant decrease in locomotion for both sexes throughout the session ($F_{29, 638}$ = 41.67, p < .001, η_p^2 = .65, β = 1.00), with the first minute higher than any other (ps < .001; **Figure 8)**. The sex by minute interaction was not significant ($F_{29, 638}$ = 1.24, p = .183).

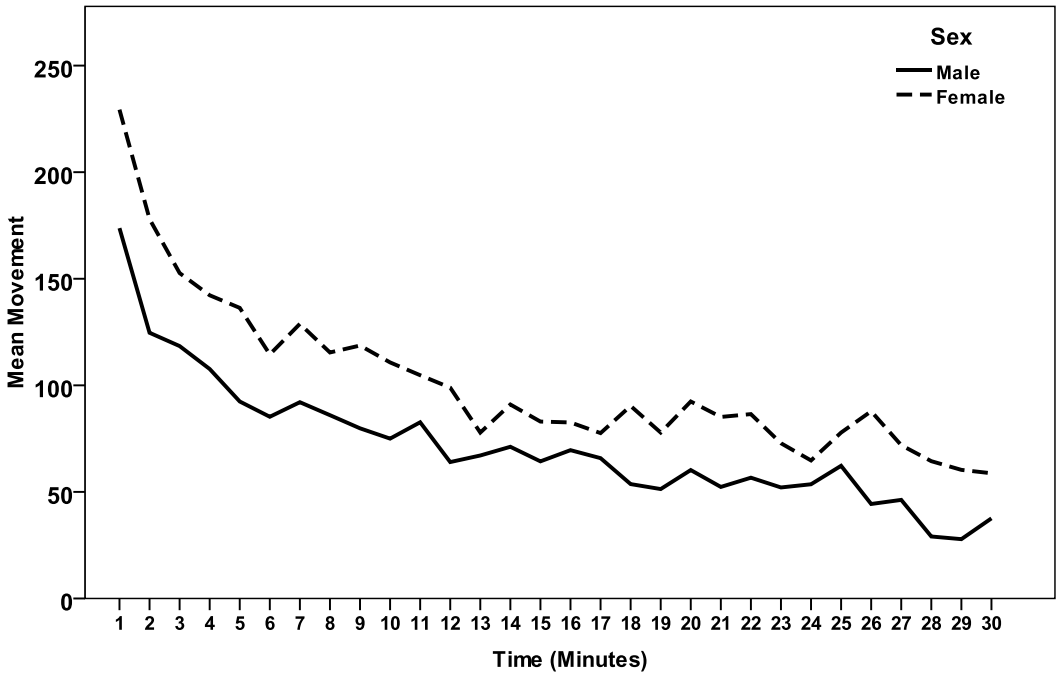

Figure 8. Number of detected movements per minute of males and females in a 30-minute locomotor test.

In the NOR test, although females had slightly higher locomotion than males during both trials, as measured by the number of quadrant crossings in the apparatus during the trials, sex differences were only a trend ($F_{1, 22}$ = 3.03, p = .096, η_p^2 = .12, β = .38). Movement decreased from Trial 1 to Trial 2 ($F_{1, 22}$ = 30.21, p < .001, η_p^2 = .58, β = 1.00), with a non-significant interaction between sex and trial ($F_{1, 22}$ = .08, p = .778; **Table 1**).

Total amount of contact with objects

In Trials 1 and 2, total object contact did not differ by sex ($F_{1, 22}$ = .08, p = .779) or between trials ($F_{1, 22}$ = .24, p = .629), nor was the trial by sex interaction significant ($F_{1, 22}$ = 1.03, p = .321; **Table 1**).

There was a significant trial by object interaction ($F_{1, 22}$ = 39.11, p < .001, η_p^2 = .64, β = 1.00), with no difference in total object contact in Trial 1 (p = .762), but a difference in Trial 2: there was higher total contact with both objects when plastic blocks were novel objects than with both objects when the clay brick was the novel object (p < .001; **Figure 9**).

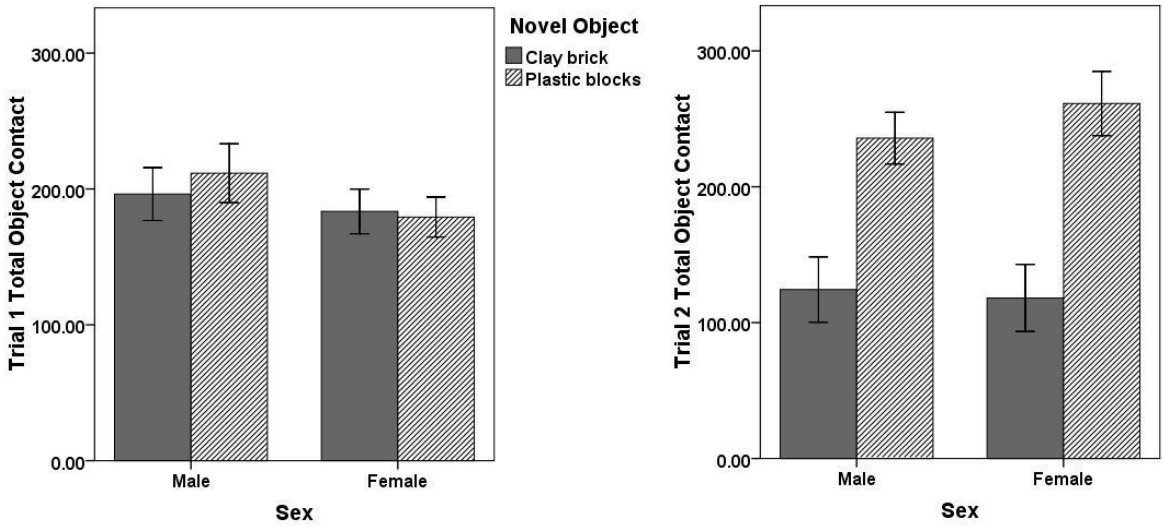

Figure 9. NOR 1 mean total object contact during Trial 1 (Left) and Trial 2 (Right), in seconds, by sex and novel object. Both objects in Trial 1 were identical (multi-coloured LEGO® Duplo blocks tower). Error bars represent +/- 1 standard error of the mean.

Preference for novelty

A one-sample *t*-test indicated that when all subjects were considered together, the animals did show a significant preference for the novel object compared to the familiar object in Trial 2 (t_{23} = 3.78, p = .001, d = .77, β = .95), spending approximately 64% of the time with the novel object. Analysis of male and female rats separately indicated that females displayed a significant preference for the novel object (t_{11} = 4.14, p = .002, d = 1.19, β = .96), but males did not (t_{11} = 1.43, p =.180). An ANOVA indicated there were significant differences in novelty preference by sex

($F_{1, 22}$ = 5.47, p = .029, η^2 = .20, β = .61), with females greater than males (**Figure 10**; **Table 1)**.

A Pearson correlation indicated significant positive relationships between the total locomotor activity in the locomotor box and novelty preference (r_{24} = .43, p = .037), and between total object contact in Trial 2 and novelty preference (r_{24} = .57, p = .004). The correlation between locomotion in Trial 2, as indicated by the number of quadrant crossings, and novelty preference also approached significance (r_{24} = .40, p = .056). Higher locomotion and object contact was associated with greater novelty preference. Therefore, with both locomotor measures and object contact in T2 as covariates, an ANCOVA indicated a significant influence of total object contact in Trial 2 ($F_{1, 19}$ = 11.53, p = .003, η_p^2 = .38, β = .90), such that the sex difference in novelty preference was no longer significant ($F_{1, 19}$ = 2.30, p = .146; R^2_{adj} = .455).

An ANOVA showed a significant difference in novelty preference by novel object ($F_{1, 22}$ = 6.43, p = .019, η_p^2 = .23, β = .68), with higher novelty preference found when the blocks were the novel object compared to when the clay brick was the novel object (**Figure 10)**. However, when total object contact in Trial 2 was considered in an ANCOVA, the difference in novelty preference due to novel object was no longer significant ($F_{1, 21}$ = .09, p = .765), and the influence of contact approached significance ($F_{1, 21}$ = 3.19, p = .088, η_p^2 = .13, β = .40).

Since there were differences in novelty preference due to both sex and novel object, an additional ANOVA and ANCOVA were conducted to examine the interaction of both variables along with the influence of object contact and locomotion. The ANOVA replicated both the significant sex difference ($F_{1, 20}$ = 7.43, p = .013, η^2 = .27, β = .74) and the difference due to the novel object used ($F_{1, 20}$ = 8.44, p = .009, η^2 = .30, β = .79), but the interaction was non-significant ($F_{1, 20}$ = 1.45,

p = .242). In the ANCOVA, although neither of the locomotor measures (Fs$_{1, 20} \leq$ 1.75, ps $\geq$ .203) nor total object contact in T2 ($F_{1, 20}$ = 2.31, p = .147) showed a significant contribution, both the sex ($F_{1, 20}$ = 2.17, p = .159) and novel object ($F_{1, 20}$ = .42, p = .523) differences were no longer significant, and the interaction remained non-significant ($F_{1, 20}$ = .81, p = .380; R^2_{adj} = .428).

Preference change

In Trial 1, a significant side-bias was indicated (t_{23} = 2.37, p = .027, d = 0.48, β = .62), with the animals spending more time in contact with the right object than the left, even though the objects were identical (*M*s, *SD*s, in seconds: 106.20, 35.32, right; 86.47, 23.20, left). A side-bias was not significant in Trial 2 (t_{23} = 1.12, p = .275), although more contact was made with the object on the left (*M*s, *SD*s, in seconds: 104.92, 73.03, right; 79.99, 64.43, left). Analysis of the preference change measure, which adjusts the preference score for side-biases, indicated a trend for a significant difference between males and females ($F_{1, 20}$ = 3.47, p = .077, η^2 = .15, β = .43; **Table 1**), with females higher than males, and a significant difference by novel object ($F_{1, 20}$ = 4.22, p = .053, η^2 = .17, β = .50), with the preference change higher when the plastic blocks were the novel object than with the clay brick **(Figure 10)**. The sex by object interaction was not significant ($F_{1, 20}$ = .02, p = .889).

A significant correlation was found between preference change and total object contact in Trial 2 (r_{24} = .53, p = .008), but not between preference change and either of the locomotor measures, locomotor box activity (r_{24} = .32, p = .130) or total quadrant crossings in Trial 2 (r_{24} = .31, p = .138). With total object contact in T2 as a covariate, an ANCOVA indicated there was some influence of total object contact on preference change ($F_{1, 19}$ = 3.34, p = .083, η_p^2 = .15, β = .41), resulting in a reduced

difference between males and females ($F_{1, 19} = 3.20$, $p = .090$, $\eta_p^2 = .14$, $\beta = .40$), and an elimination of the difference between novel objects ($F_{1, 19} = .004$, $p = .953$). The sex by object interaction remained non-significant ($F_{1, 19} = .02$, $p = .898$).

When the novelty preference and preference change measures were compared, a significant correlation was found between the two measures ($r_{24} = .87$, $p < .001$), with neither measure higher or lower than the other ($t_{23} = 1.43$, $p = .167$).

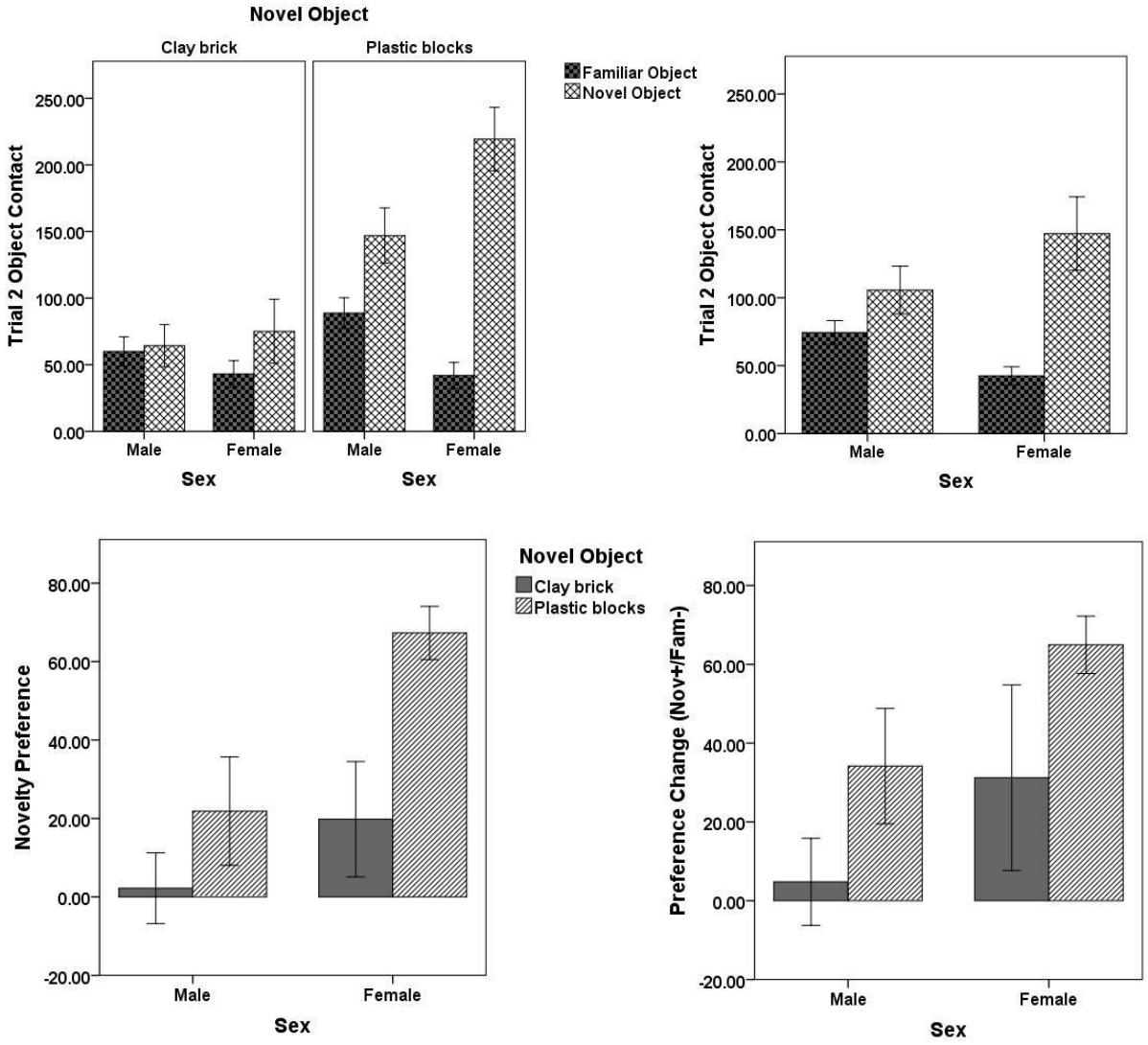

Figure 10. NOR 1. **Top**: Mean contact time, in seconds, with Familiar and Novel objects in Trial 2 by object and sex (Left) and overall by sex (Right). **Bottom**: Mean novelty preference (Left) and mean preference change (Right) during Trial 2 by sex and novel object. Error bars represent +/- 1 standard error of the mean.

Table 1

NOR 1. *Means, in seconds (except where noted), and standard error of mean of behavioural measures and preference scores by sex (n = 12 per group).*

	Males	Females	Totals
Locomotion			
Locomotor Box	2147.00	3033.33	2590.17
	(78.60)	(159.01)	(126.74)
Trial 1	55.58	64.25	59.92
(Quadrant crossings)	(4.10)	(3.25)	(2.71)
Trial 2	44.92	52.42	48.67
(Quadrant crossings)	(3.29)	(3.66)	(2.53)
Total Object Contact			
Trial 1	203.95	181.39	192.67
	(14.08)	(10.53)	(8.91)
Trial 2	180.09	189.74	184.91
	(22.29)	(27.03)	(17.16)
Trial 2 Novel Object Contact	105.68	147.22	126.45
	(17.61)	(27.07)	(16.38)
Trial 2 Familiar Object Contact	74.41	42.52	58.46
	(8.74)	(6.69)	(6.32)
Novelty Preference	12.05	43.56	27.80
	(8.41)	(10.53)	(7.36)
Preference Change	19.48	48.09	33.78
	(9.80)	(12.81)	(8.43)

Note. Numbers in parentheses are standard error of mean.

3.1.2 Summary

The first NOR was designed to replicate the early work of Ennaceur & Delacour (1988), with two identical objects used during Trial 1, and to assess the ability of the task to elicit a novelty preference. Although a preference for the novel objects in Trial 2 was found, there were also differences due to the objects. During Trial 2, the

animals spent more time with the plastic blocks than with the clay brick, which could have been due to the different properties of the objects. It was observed that the smaller females were able to climb on the plastic blocks, which were taller and more flat than the brick and, as the objects were against the outside of the apparatus, could have been contacted as a means of escape. Although females did interact more with the novel objects in Trial 2 than males, as indicated by higher novelty preference, sex differences were not present in total object contact. When total object contact was considered with novelty preference, the sex difference was no longer significant, and could be more a result of the increased attempts to leave the apparatus. This behaviour could indicate that the biased preference of the females was not due to the item's novelty but due to the object used. Therefore, for the next set of experiments a new set of objects were selected that deterred climbing and were of a similar size to each other.

In this experiment, as with Ennaceur and Delacour (1988), the animals were exposed to two identical objects in the first trial. When testing for memory performance over varied inter-trial-intervals, this could strengthen the recognition that the new object in the test trial is indeed novel. However, due to differences in object preference, inconsistent preferences for the novel object in Trial 2 could occur. In the current study, the novelty preference score was higher when the plastic blocks were the novel object in Trial 2, after having been exposed only to the Duplo in Trial 1, than when the clay brick was the novel object after the familiar objects. The relative complexity of the blocks, as the preferred object, compared to the Duplo was higher than the brick to the Duplo, and so the blocks were potentially better at eliciting approach behaviours (Berlyne, 1950). If the objects used in Trial 1 were counterbalanced such that the two identical objects were either Duplo, clay bricks or

blocks, the preferences based on the characteristics of the objects could have been reduced. Therefore, in order to minimize the influence of individual preferences of the animals to specific objects, the experimental design was changed to utilize two distinct objects in Trial 1, one of which was replaced with a novel object in Trial 2. The objects used and replaced were counterbalanced between animals, and whether the left-hand or right-hand object was replaced in Trial 2 was also counterbalanced. The inter-trial-interval was kept at 2-minutes to reduce the influence of differences in memory.

As expected, the preference change measure was able to eliminate the object preference biases when total object contact was considered as a covariate. A significant preference change measure indicates that relative to the first trial, the change in object contact was more toward the novel object. Because this measure was effective at eliminating bias when present, it will continue to be analyzed when object and/or side biases are indicated as well as to examine if object preference changes occur and if they are toward the novel object.

3.2 Experiment 2

3.2.0 Methods

Subjects and housing

In experiment 2, 20 adult rats, 10 males (*M*, 471.88; *SD*, 30.75, in grams) and 10 females (*M*, 213.01; *SD*, 11.24, in grams), were tested for unconditioned novel object preference. Housing was as previously described.

Apparatus

In this series of tests, the objects were changed to a yellow hedgehog rubber toy, an orange plastic toy watering can, a green frog chew toy and a blue hard rubber chew toy similar to a KONG®. All these objects were of similar size and chosen to deter climbing on the objects. The apparatus was as previously described: a wooden, light grey-painted square chamber, measuring 67cm x 67cm x 45cm (l x w x h), with a solid floor constructed of the same material.

Experimental Design

The novel object recognition test was conducted as previously described, and included a 30-minute familiarization session on the day prior to testing, and two 10-minute sessions on test day. However, in these tests, the experimental design was changed to utilize two distinct objects in Trial 1, one of which was replaced with a novel object in Trial 2. The objects used in each trial were counterbalanced across subjects and between age groups, and whether the left-hand or right-hand object was replaced in Trial 2 was also counterbalanced (**Figure 11**). The inter-trial-interval was kept at 2-minutes to reduce the influence of differences in memory.

The test trials were no longer observed directly, and a video camera attached to the ceiling relayed images to a computer. The apparatus was surrounded by a black curtain to reduce visual distractions and a white noise generator was used to mask external sounds.

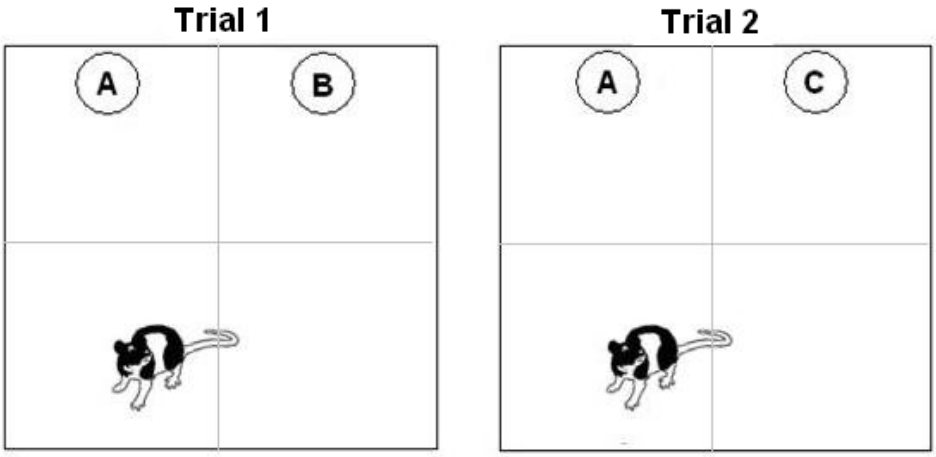

Figure 11. A diagram of object position and animal placement in the novel object recognition task during Trial 1 and Trial 2 for experiments 2 and 3. Grey lines denote quadrant boundaries but are not present in the apparatus.

Behavioural recording

In the second and subsequent NOR tests, all sessions, including the familiarization session, were recorded and analysed by EthosVision XT 5.0 software (Noldus Information Technology, Netherlands, 2008) and/or the Observe software while viewing the captured videos. Behavioural measures were as previously described, and included time spent in contact with each object, time spent in each quadrant, and number of quadrant crossings.

3.2.1 Results

Locomotion

Locomotion, as measured by the number of quadrant crossings in the apparatus during the trials, significantly decreased from Trial 1 to Trial 2 ($F_{1, 18} = 47.56$, $p < .001$, $\eta_p^2 = .73$, $\beta = 1.00$), with no difference between males and females, either overall ($F_{1, 18} = 2.60$, $p = .125$) or in the interaction of sex with trial ($F_{1, 18} = 1.34$, $p = .262$; **Table 2**).

Total amount of contact with objects

Total object contact decreased significantly from Trial 1 to Trial 2 ($F_{1, 18}$ = 9.99, p = .005, η_p^2 = .36, β = .85; **Figure 12**). There was a main effect of sex, with females spending more time in contact with the objects than males ($F_{1, 18}$ = 6.72, p = .018, η_p^2 = .27, β = .69). Although the trial by sex interaction was non-significant ($F_{1, 18}$ = 1.59, p = .223), females spent more time in contact with the objects than males in Trial 1 (p = .003) but there was no sex difference in Trial 2 (p = .402). Females had a decrease in contact between trials (p = .006), whereas males had no difference between trials (p = .196; **Table 2**).

In Trial 2, total object contact did not differ between novel objects ($F_{1, 12}$ = 1.38, p = .297). The sex by novel object interaction was non-significant ($F_{1, 12}$ = 1.38, p = .297), indicating no difference between males and females in response to the properties of the object.

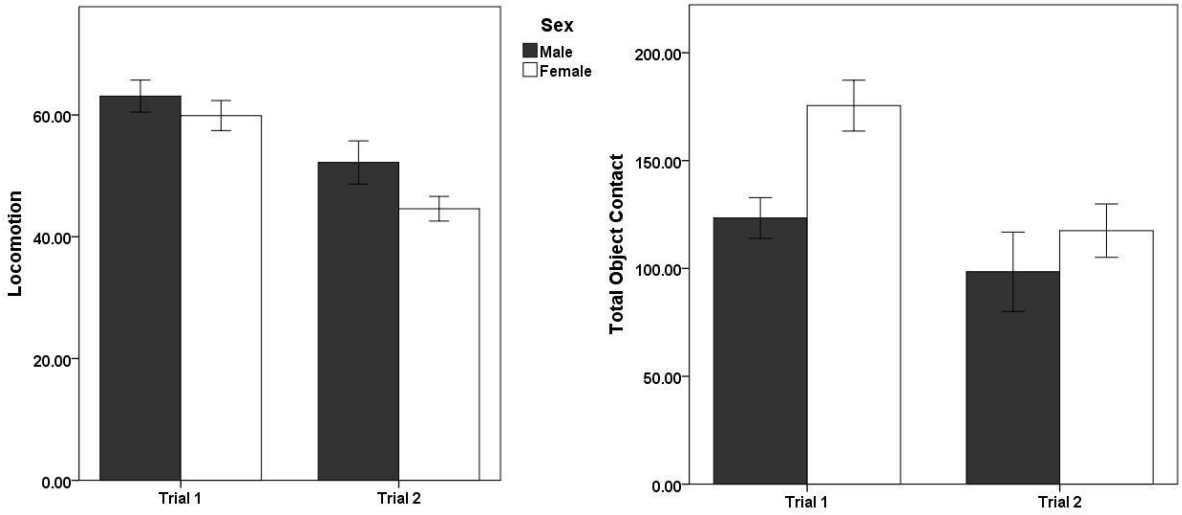

Figure 12. NOR 2 mean total locomotion (quadrant crossings) (Left), and total object contact, in seconds (Right), during Trial 1 and Trial 2 by sex. Error bars represent +/- 1 standard error of the mean.

Preference for novelty

A one-sample *t*-test indicated that animals did show a significant preference for the novel object compared to the familiar object ($t_{19} = 6.26$, $p < .001$, $d = 1.40$, $\beta = 1.00$), spending approximately 73% of the total contact time with the novel object during Trial 2. Both males ($t_9 = 3.58$, $p = .051$, $d = 1.13$, $\beta = 1.00$) and females ($t_9 = 5.35$, $p < .001$, $d = 1.69$, $\beta = 1.00$), when analysed separately, displayed a significant preference for the novel object in Trial 2. An ANOVA indicated no significant difference in novelty preference by sex ($F_{1, 12} = 1.37$, $p = .265$; **Table 2**). However, there was a significant difference by novel object ($F_{3, 12} = 9.28$, $p = .002$, $\eta^2 = .70$, $\beta = .97$), with higher novelty preference found when the plastic watering can or the frog were the novel objects than when either the blue KONG ($ps \leq .040$) or the yellow hedgehog was the novel object ($ps \leq .013$). There were no significant differences between the other objects ($ps = 1.00$; **Figure 13**). The sex by novel object interaction was non-significant ($F_{3, 12} = .79$, $p = .523$).

The other behavioural measures, total object contact or quadrant crossings in either Trial 1 or Trial 2, did not show a significant correlation with novelty preference ($rs_{20} \leq .36$, $ps \geq .122$) and were not considered as covariates for further analysis.

Preference change

Side biases in object contact were not found in either Trial 1 ($t_{19} = .22$, $p = .829$; *M*s, *SD*s, in seconds: 76.19, 38.70, right; 73.24, 34.84, left) or Trial 2 ($t_{19} = 1.45$, $p = .163$; *M*s, *SD*s, in seconds: 39.95, 27.62, right; 62.67, 48.59, left). Therefore, the preference change analysis findings were similar to those of novelty preference. There was still no significant difference between males and females ($F_{1, 12} = .0005$, $p = .983$; **Table**

2). The difference between novel objects remained ($F_{3, 12}$ = 5.03, p = .017, η^2 = .56, β = .80), with higher novelty preference found when either the plastic watering can (p = .023) or the green frog (p = .023) was the novel object than when the blue KONG was the novel object, but not between any of the other objects (ps ≥ .638; **Figure 13**). The sex by novel object interaction remained non-significant ($F_{3, 12}$ = .151, p = .927).

There was a significant correlation between the preference change measure and the novelty preference measure (r_{20} = .53, p = .015), with preference change percentages significantly higher than those of novelty preference (t_{19} = 2.39, p = .027, d = .24, β = .22; **Figure 14**).

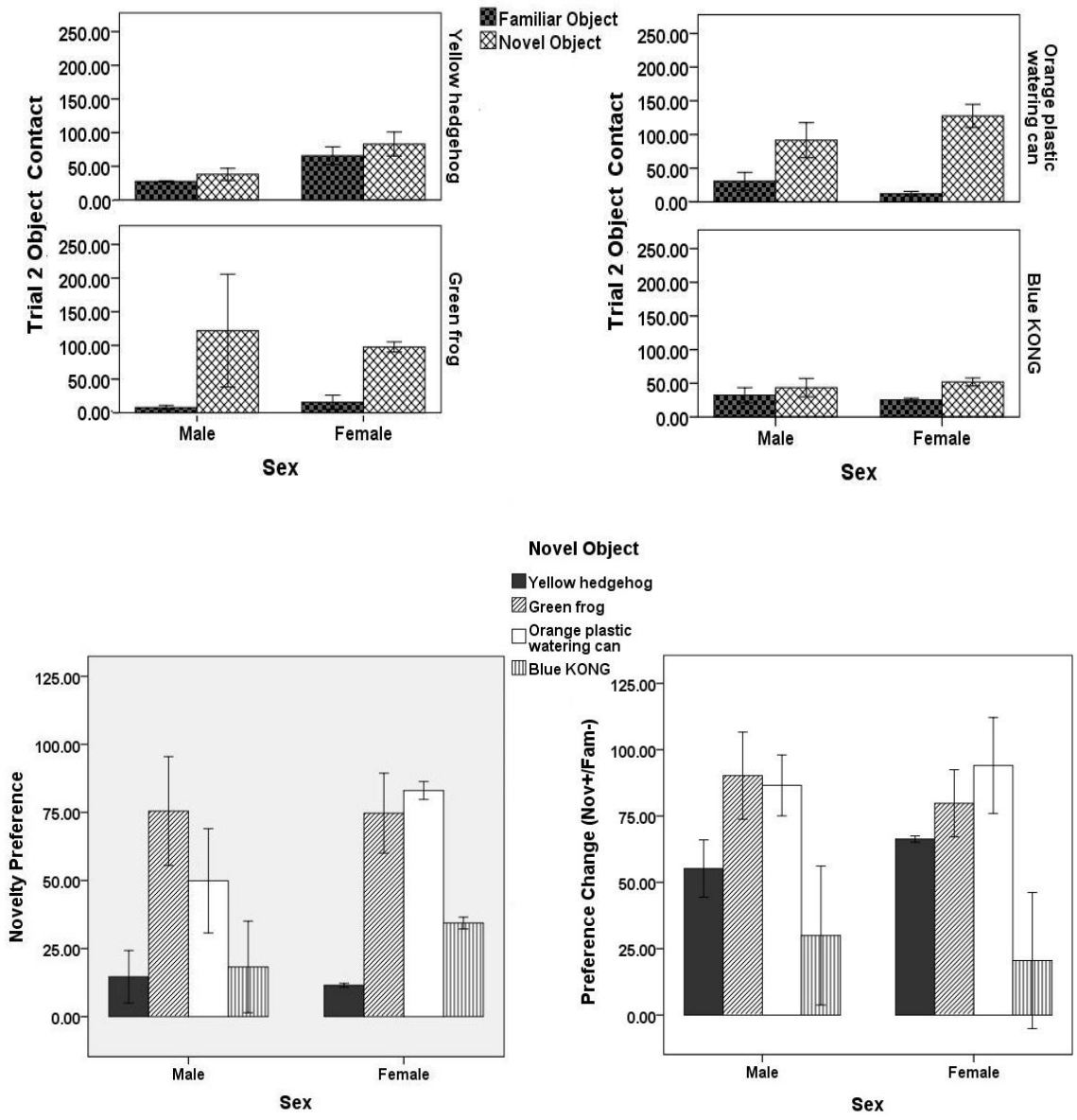

Figure 13. NOR 2. **Top 4 graphs**: Mean contact time, in seconds, with Familiar and Novel objects in Trial 2 by object and sex. **Bottom**: Mean novelty preference (Left) and mean preference change (Right) during Trial 2 by sex and novel object. Error bars represent +/- 1 standard error of the mean.

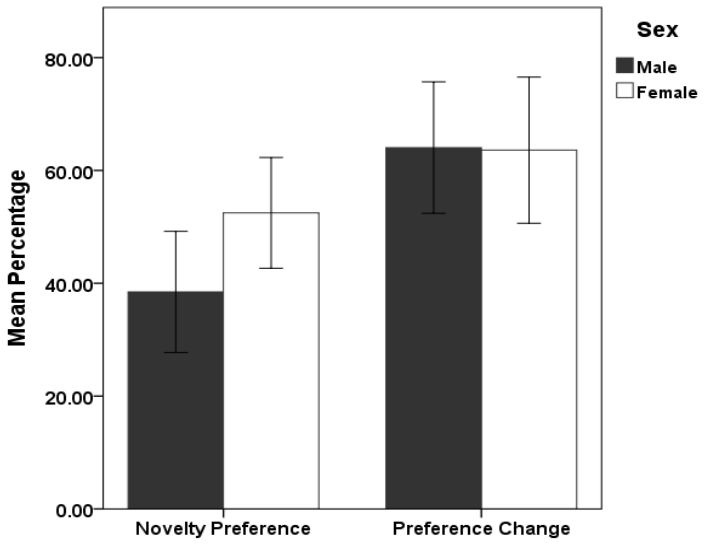

Figure 14. NOR 2 mean preference measures during Trial 2 by sex. Error bars represent +/- 1 standard error of the mean.

Table 2

NOR 2. *Means, in seconds (except where noted), and standard error of mean of behavioural measures and preference scores by sex (n = 10 per group).*

	Males	Females	Totals
Locomotion			
Trial 1	63.10	59.90	61.50
(Quadrant crossings)	(2.64)	(2.46)	(1.79)
Trial 2	52.20	44.60	48.40
(Quadrant crossings)	(3.55)	(2.02)	(2.17)
Total Object Contact			
Trial 1	123.38	175.50	149.44
	(29.98)	(11.80)	(9.49)
Trial 2	98.47	117.52	107.99
	(18.42)	(12.35)	(11.01)
Trial 2 Novel Object Contact	72.52	90.00	81.26
	(18.34)	(11.33)	(10.68)
Trial 2 Familiar Object Contact	25.94	27.52	26.73
	(5.45)	(7.15)	(4.38)

Novelty Preference	38.47 (10.74)	52.48 (9.81)	45.47 (7.26)
Preference Change	64.05 (11.65)	63.60 (12.96)	63.82 (8.48)

Note. Numbers in parentheses are standard error of mean.

3.2.2 Summary

The design of this novel object recognition task was sufficient to elicit a preference for novel objects in rats. Although there were some behavioural differences due to the objects used, with lower novelty preference when the blue KONG was present, a sex difference was found only in total object contact in Trial 1. No sex differences were found in Trial 2. These findings are consistent with previous studies that did not find adult sex differences in rodents at short inter-trial-intervals (Ghi, Orsetti, Gamalero & Ferretti, 1999; Ricceri, Colozza & Calamandrei, 2000; Sutcliffe, Marshall & Neill, 2007).

One of the methodological issues associated with NOR testing is inconsistent behavioural response across objects due to the properties of the objects. Differences can arise from the inability to discriminate between objects, if they are too similar, or the increased attraction to one object over the other due to stimulus complexity (Dember, Earl & Paradise, 1957; Ennaceur, 2010). Since individual objects continued to affect behaviours (i.e., the blue KONG eliciting less preference) interaction with a variety of objects was examined before additional NOR tests were conducted. In a 10-minute session, animals (n = 8 males, 12 females) were exposed to 4 different objects (from 12 objects in total), one placed against the wall in each quadrant of the testing apparatus, while object contact, latency to approach and frequency of contact were measured. Objects excluded from future tests were those that elicited either the least (i.e. blue KONG, green frog, clay brick) or the most (i.e. jar filled with beans; jar

with salt) amount of contact. The objects selected for presentation in subsequent NOR tests were those with similar levels of contact (e.g., blue plastic bottle, orange watering can). Although individual differences for certain objects may remain, counterbalancing objects as either novel or familiar between animals should minimize novelty preference differences due to object interaction.

Since additional studies would examine NOR performance in adolescents, as detailed in the next chapter, the experimental design was revised to minimize the time needed to administer the test. In the seven-session design used to test both spatial and object novelty (Frick & Gresack, 2003; Ricceri et al., 2000), and in the early work of Berlyne (1950), habituation to the testing apparatus was done immediately preceding the test trials, rather than the day(s) before testing (Ennaceur & Delacour, 1988; Ghi et al., 1999). Additionally, the length of the testing trials varied from a minimum total object contact time of 20-30 seconds (Ennaceur and Delacour, 1988; Kosten, Lee & Kim, 2007) to trials of a set time of 3-5 minutes (Berlyne, 1950; Sutcliffe et al., 2007). Despite the differences in the designs of the studies, in each the animals continued to display a preference for the novel object with short ITIs. Therefore, the design was changed to include a 10-minute familiarization session (rather than 30-minute) on the same day as testing to habituate the animals to the testing apparatus, followed by two 5-minute testing trials (rather than 10-minute) with the objects. As the ITI was kept at 2-minutes, the total time required for each testing session, and thus the time the animal was kept out of its home cage, was reduced to less than 30-minutes per animal.

Object placement within the apparatus was another factor that could lead to differences in object interaction. As in prior studies, objects in T1 and T2 were placed against the wall of the apparatus such that animals were able to interact with

the objects from the front or sides, but not able to get behind the objects (Ennaceur & Delacour, 1988; Kosten et al., 2007). However, when tested in an open field, animals spend less time in the centre of the apparatus than in the periphery, also known as thigmotaxis (Barnett, 1958; Lynn & Brown, 2009; Prut & Belzung, 2003). When objects are located on the wall of the apparatus, the animals have to locomote toward the open centre to contact the adjacent object, introducing the possibility that anxiety could affect object exploration. To reduce the influence of anxiety, the objects were now placed approximately 8 cm from the wall of the apparatus in each of the object trials to allow subjects to move behind them, although the objects remained the same distance relative to each other as in prior tests.

The final experiment with adult rats used the new experimental design, and, as a repeated measure, added two additional trials with novel objects. Along with counterbalanced objects, the repeated design can reinforce that the animals are attracted to the novel aspect of the objects rather than the physical characteristics of the objects. If the animals repeatedly show a preference for the novel object in each trial, this could demonstrate the distinction between object preference and novelty preference (Ennaceur, 2010).

3.3 Experiment 3

3.3.0 Methods

Subjects and housing

The subjects were 15 male and 15 female adult Lister-hooded rats bred in-house from stock supplied by Harlan, U.K., ranging in age from 98 to 102 postnatal days at the time of testing. Housing was as previously described. Previous testing on some

animals (*n* = 7 females) was behavioural, and a minimum of 20 days had elapsed after completion of prior tests.

Apparatus

Because of the number of trials, a total of fourteen objects were used during this experiment (e.g., yellow hedgehog rubber toy, clear glass jar filled with rocks, blue plastic bottle filled with sand, orange plastic toy watering can, multi-coloured LEGO® Duplo blocks tower) and were chosen to deter climbing and chewing. The testing apparatus, time of testing and room conditions were as previously described.

Experimental design

As with previous testing, at the beginning of the testing session, an animal was brought to the testing room in a carrying box measuring 42cm x 26cm x 13cm (l x w x h). However, for this testing session, the subject was first placed into the empty apparatus and given a 10-minute familiarization session. The animal was then removed to the carrying box for a period of 2 minutes while the apparatus was cleaned with a 70% ethanol solution and allowed to air dry. During the second session, Trial 1 (T1), two objects were placed in adjacent quadrants, 15 cm apart and 8 cm from the wall (**Figure 15**), after which the animal was placed into an empty quadrant, facing away from the objects. After 5 minutes, the animal was again removed to an empty carrier container for an inter-trial interval of 2 minutes while one of the objects was replaced by the novel object and both the apparatus and the remaining object were cleaned with the alcohol solution. The object that remained was considered the familiar object. The animal was then reintroduced to the apparatus as in the second session, Trial 2 (T2), and allowed to interact with the objects for an

additional 5 minutes. This process was repeated for two additional trials, Trial 3 (T3) and Trial 4 (T4), where a new novel object was introduced, and the previous novel object was now considered the familiar object. At the end of all trials, both objects and the apparatus were cleaned with the alcohol solution in preparation for the next Trial or subject. All the objects were used in either the familiar or novel condition and were counterbalanced between individuals, trials, apparatus side and novelty status.

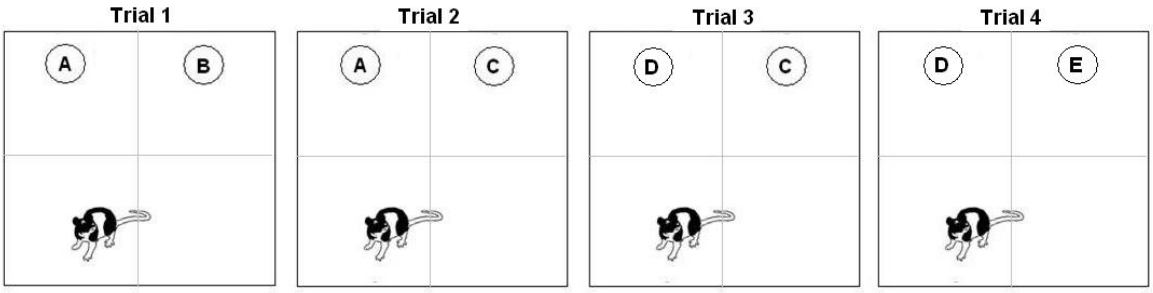

Figure 15. A diagram of object positions and animal placement during the four sessions with objects, Trial 1 (T1), Trial 2 (T2), Trial 3 (T3) and Trial 4 (T4). If more object contact was made with object C than A in T2, object D than C in T3 and object E than D in T3, then a preference for novelty was indicated.

Behavioural recording

As in prior testing, all sessions were digitally recorded direct to a computer and were analysed using EthoVision XT 5.0 software (Noldus Information Technology, Netherlands, 2008).

During all sessions, locomotor activities measured included the duration of movement, total distance moved and time spent in each quadrant. Behavioural measures were as previously described, and included time spent in contact with the objects and latency of approach to each object.

Statistical analyses

All statistics were analysed as previously indicated, including the calculation of the *preference for novelty* and *preference change* measures. Repeated measures Analysis of Variance (ANOVA) was used to examine sex differences in the preference measures, object interaction and locomotion over the three novel object trials. As additional criteria, an animal must have met a minimum of 5 seconds total object contact in Trial 1 and at least 1 second total contact with the objects in each of the novel object trials in order to be included in the data analyses. No animals were removed based on these criteria.

3.3.1 Results

Locomotion

Locomotion was found to differ significantly between object trials in distance moved ($F_{3, 84} = 52.00$, $p < .001$, $\eta_p^2 = .65$, $\beta = 1.00$) and duration of movement ($F_{3, 84} = 43.39$, $p < .001$, $\eta_p^2 = .61$, $\beta = 1.00$), with a trend between trials in total transitions ($F_{2.31, 64.68} = 2.68$, $p = .068$, $\eta_p^2 = .09$, $\beta = .55$). Generally, locomotion decreased from T1 to T2, when novel objects were introduced (ps < .001), then either decreased only slightly or remained steady through T4 (ps ≥ .097; **Figure 16**). Sex by trial interactions were not significant in any of the locomotor measures: distance moved ($F_{3, 84} = .55$, $p = .653$); total transitions ($F_{2.31, 64.68} = .21$, $p = .838$); or duration of movement ($F_{3, 84} = 1.02$, $p = .387$; **Table 3**). Main effects of sex were also not significant in either distance moved ($F_{1, 28} = 3.18$, $p = .085$) or total transitions ($F_{1, 28} = .05$, $p = .828$); however, there was a trend for a main effect of sex in duration of movement ($F_{1, 28} = 3.18$, $p = .085$, $\eta_p^2 =$

.10, β = .41), with males (*M*, 179.78; *SD*, 5.96, in seconds) spending more time moving over the four trials than females (*M*, 164.76; *SD*, 5.96, in seconds).

Total amount of contact with objects

Examining the four object trials, T1 to T4, there were no differences in total object contact between the trials ($F_{3, 84}$ = .69, p = .558). The sex difference was non-significant ($F_{1, 28}$ = .33, p = .572), as was the sex and trial interaction ($F_{3, 84}$ = .27, p = .849; **Figure 16**; **Table 3**).

Although means indicated the left object was contacted first in each of the object trials (latency data not shown), the tendency was significant only in T2 (T1: t_{29} = 1.60, p = .120; T2: t_{29} = 2.17, p = .038, d = .40, β = .56; T3: t_{29} = 1.35, p = .188; T4: t_{29} = 1.82, p = .080). There were no significant differences between the duration of contact with the left object compared to the right object in any of the object trials, T1 – T4 ($ts_{29} \leq 1.01$, $ps \geq .321$).

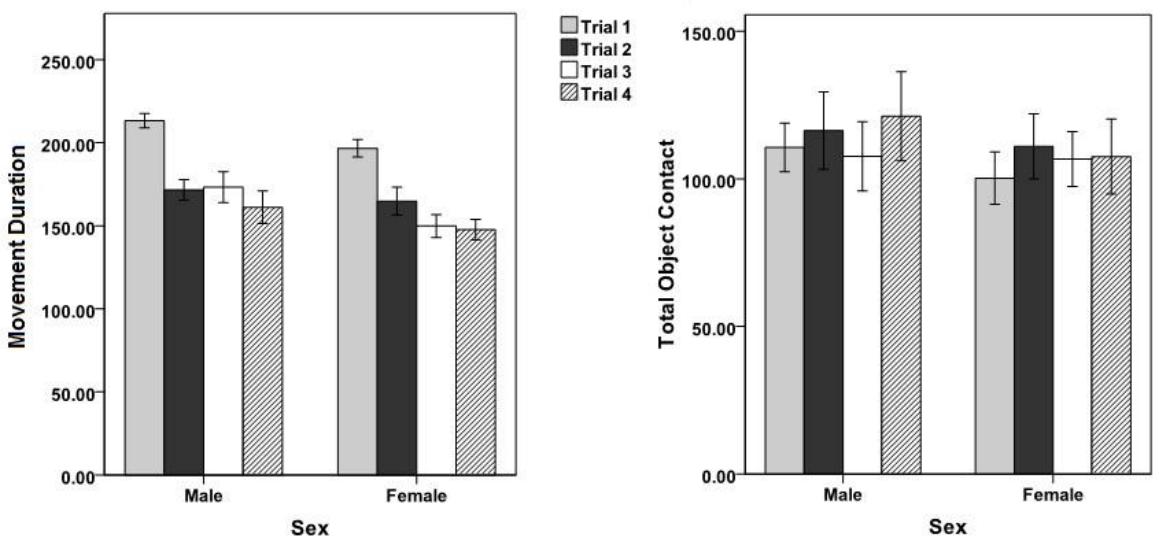

Figure 16. Mean duration of movement, in seconds (Left) and mean total object contact (Right), in seconds, during Trial 1, Trial 2, Trial 3 and Trial 4 by sex. Error bars represent +/- 1 standard error of the mean.

Preference for novelty

One-sample *t*-tests indicated that animals did show a significant preference for the novel object in each of the trials with novel objects, T2 – T4 (T2: $t_{29} = 2.74$, $p = .011$, $d = .50$, $\beta = .75$; T3: $t_{29} = 4.06$, $p < .001$, $d = .74$, $\beta = .98$; T4: $t_{29} = 3.45$, $p = .002$, $d = .63$, $\beta = .92$), with animals spending greater than 61.25% of their time with the novel object in each trial. When each sex was analyzed separately, males displayed a significant preference for the novel object in T3 ($t_{14} = 5.08$, $p < .001$, $d = 1.31$, $\beta = 1.00$), but not in either T2 ($t_{14} = 1.02$, $p = .326$) or T4 ($t_{14} = 1.58$, $p = .135$). Females, however, showed a significant preference for the novel object in both T2 ($t_{14} = 3.30$, $p = .005$, $d = .85$, $\beta = .87$) and T4 ($t_{14} = 3.38$, $p = .004$, $d = .87$, $\beta = .88$), but not in T3 ($t_{14} = 1.62$, $p = .128$).

A repeated measures ANOVA indicated no significant differences in novelty preference between the novel object trials, T2 – T4 ($F_{2, 56} = .67$, $p = .514$), and a non-significant trial by sex interaction ($F_{2, 56} = 1.88$, $p = .162$). Over all trials, the main effect of sex was also non-significant ($F_{1, 28} = .21$, $p = .648$). As variances were not equal in T3, as indicated by a significant Levene's test ($F_{1, 28} = 5.53$, $p = .026$), Mann-Whitney tests confirmed a lack of significant sex differences in each of the three novel object trials (*U*s, ≥ 85.00, *p*s $\geq .267$; **Figure 17**; **Table 3**).

The variances of novelty preference by novel object were not homogeneous, and so were analyzed with the Kruskal-Wallis non-parametric test. Analysis indicated no significant differences in preference for the novel object by the object presented in T2 ($\chi^2_{12} = 16.81$, $p = .157$), T3 ($\chi^2_{10} = 16.33$, $p = .091$) or T4 ($\chi^2_{10} = 9.99$, $p = .442$).

Pearson correlation revealed no relationships between the locomotion measure of movement duration, total object contact and novelty preference in any of the novel

object trials (T2: $-.16 \leq rs_{30} \leq -.14$, $ps \geq .410$; T3: $.01 \leq rs_{30} \leq .24$, $ps \geq .211$; T4: $.10 \leq rs_{30} \leq .15$, $ps \geq .442$).

As some of the animals had previously been tested in a similar behavioural test ($n = 7$), analysis confirmed no differences due to prior testing on novelty preference, either by trial ($F_{2, 56} = .33$, $p = .723$) or overall ($F_{1, 28} = .30$, $p = .586$).

Preference change

Side biases between the left and right sides of the apparatus were not apparent in any of the object trials, T1 – T4 ($ts_{29} \leq .73$, $ps \geq .473$), so preference change should be similar to novelty preference. However, a one-sample *t*-test indicated animals did not show a significant preference change toward the novel object from T1 to T2, although the trend was in the direction of the novel object ($t_{29} = 1.71$, $p = .097$), but did show a significant preference change toward the novel object from T2 to T3 ($t_{29} = 6.55$, $p < .001$, $d = 1.20$, $\beta = 1.00$) and from T3 to T4 ($t_{29} = 7.03$, $p < .001$, $d = 1.28$, $\beta = 1.00$). Males did not show a significant change in preference toward the novel object from T1 to T2 ($t_{14} = .44$, $p = .664$), whereas females showed a non-significant trend in preference change toward the novel object between the same trials ($t_{14} = 1.86$, $p = .083$). Both males and females had significant preference changes toward the novel object from T2 to T3 ($ts_{14} \geq 4.27$, $ps \leq .001$, $ds \geq 1.10$, $\beta s \geq .98$) and from T3 to T4 ($ts_{14} \geq 4.90$, $ps \leq .001$, $ds \geq 1.01$, $\beta s \geq .96$).

An ANOVA indicated a significant difference in preference change between trials ($F_{1.60, 44.73} = 8.24$, $p = .002$, $\eta^2 = .23$, $\beta = .91$), with a higher change in preference toward the novel object in both T3 and T4 than in T2 ($ps \leq .020$), and no difference between T3 and T4 ($p = 1.00$). The difference between males and females was non-significant ($F_{1, 28} = .02$, $p = .884$) as was the trial by sex interaction ($F_{1.60, 44.73} = .92$, p

= .385; **Figure 17**; **Table 3**). Preference change did not differ by the novel object presented in any of the trials (χ^2s$_{10\text{-}12} \leq 16.98$, ps $\geq .150$).

Examining correlations between the object contact and locomotion measures and preference change in all the trials, T1-T4, the only significant correlation was between total object contact in T1 and the preference change between T1 and T2 ($r_{30} = -.60$, $p < .001$), with lower total object contact in T1 associated with higher preference change. Thus, when T1 total object contact was examined as a covariate with preference change across the trials, there was a significant influence of T1 object contact ($F_{2, 54} = 7.66$, $p = .001$, $\eta^2 = .22$, $\beta = .94$), although the difference between trials remained significant ($F_{2, 54} = 3.67$, $p = .032$, $\eta^2 = .12$, $\beta = .65$). As variation between the trials was reduced by the inclusion of T1 total object contact, the higher change in preference toward the novel object in both T3 and T4 than in T2 was more defined (ps $\leq .006$).

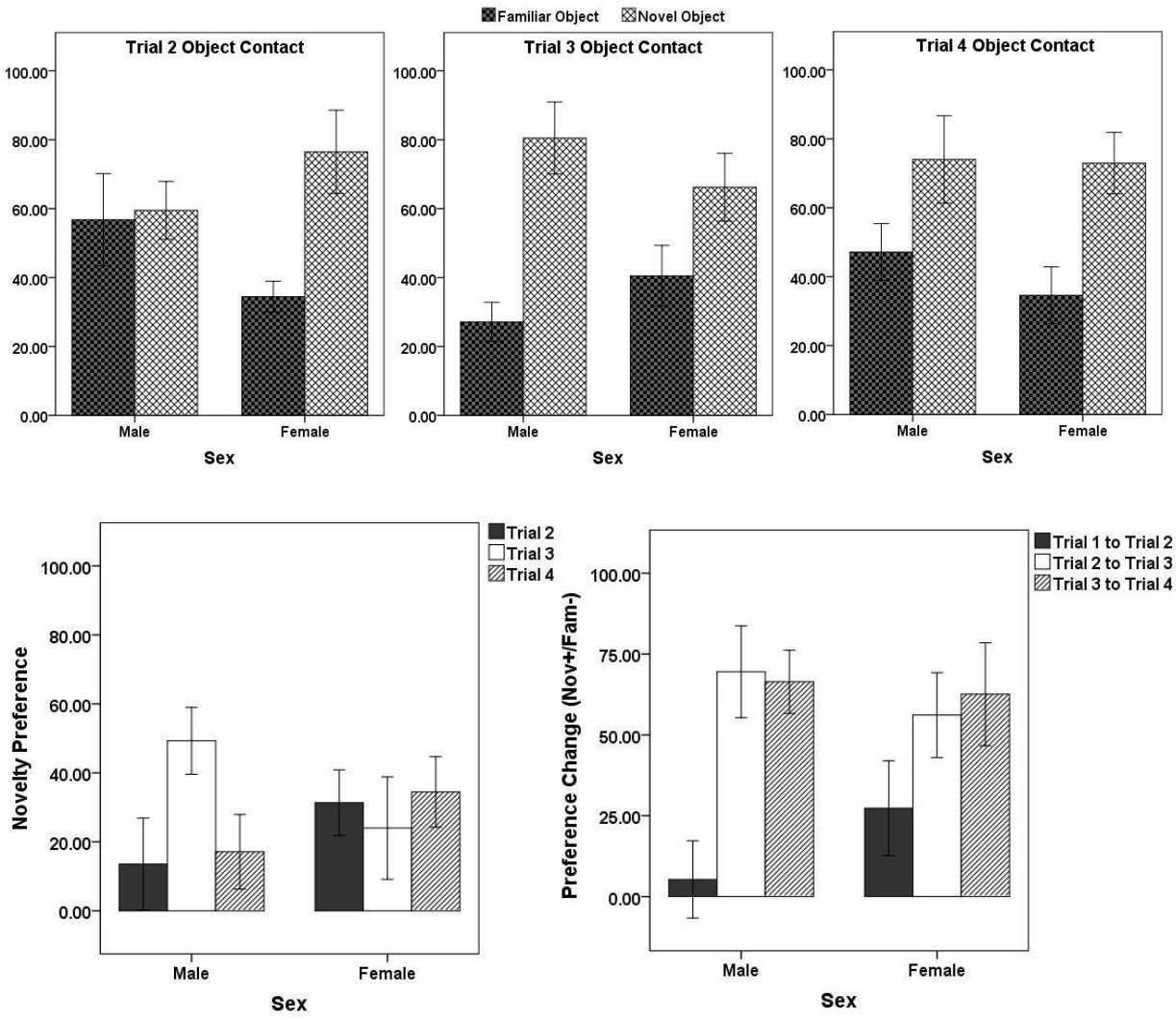

Figure 17. **Top 3 graphs**: Mean contact time, in seconds, with Familiar and Novel objects in Trial 2, Trial 3 and Trial 4 by sex. **Bottom**: Mean novelty preference (Left) and mean preference change from the previous trial (Right) during Trial 2, Trial 3 and Trial 4 by sex. Error bars represent +/- 1 standard error of the mean.

In each of the novel object trials, T2-T4, the preference change measures and the novelty preference measures were significantly correlated (T2: $r_{30} = .67$, $p < .001$; T3: $r_{30} = .56$, $p = .001$; T4: $r_{30} = .44$, $p = .015$). In Trial 2, the difference between novelty preference and preference change was non-significant (T2: $t_{29} = .85$, $p = .403$), but in both T3 and T4, preference change was significantly higher than novelty

preference (T3: $t_{29} = 3.01$, $p = .005$, $d = .55$, $\beta = .83$; T4: $t_{29} = 4.33$, $p < .001$, $d = .79$, $\beta = .99$; **Figure 18**)

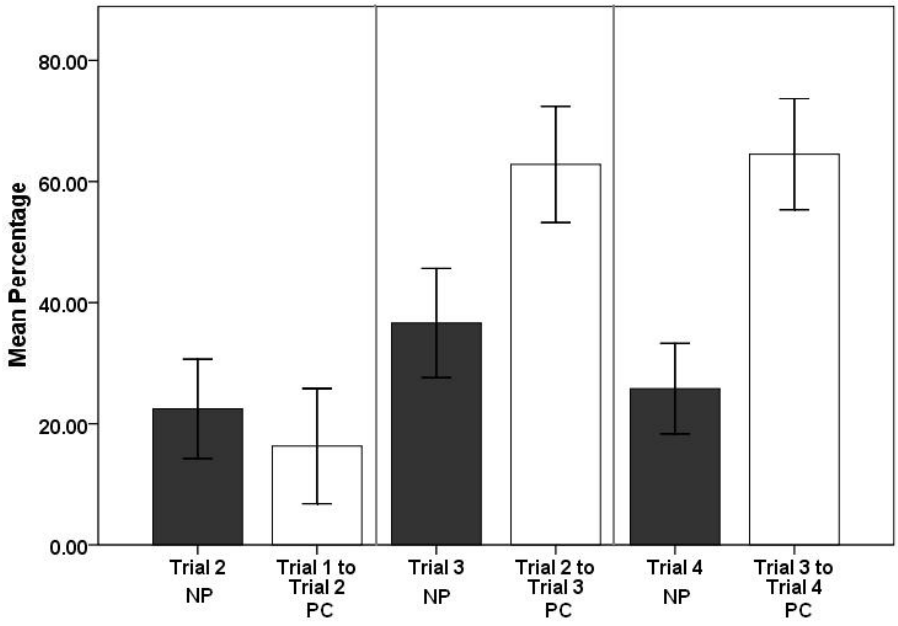

Figure 18. Mean preference measures, novelty preference (NP) and preference change (PC), during Trial 2, Trial 3 and Trial 4. Error bars represent +/- 1 standard error of the mean.

Table 3

NOR 3. *Means, in seconds, and standard error of mean of behavioural measures and preference scores by sex (n = 15 per group).*

	Males	Females	Totals
Movement Duration			
Trial 1	213.20	196.66	204.93
	(4.33)	(5.33)	(3.71)
Trial 2	171.53	164.82	168.17
	(6.18)	(8.37)	(5.15)
Trial 3	173.23	149.88	161.56
	(9.31)	(6.87)	(6.08)
Trial 4	161.15	147.67	154.41
	(9.77)	(6.26)	(5.84)

Total Object Contact			
Trial 1	110.63	100.24	105.43
	(8.38)	(8.89)	(6.05)
Trial 2	116.32	110.97	113.64
	(13.11)	(11.04)	(8.43)
Trial 3	107.64	106.70	107.17
	(11.74)	(9.33)	(7.37)
Trial 4	121.16	107.54	114.35
	(15.09)	(12.70)	(9.77)
Trial 2 Novel Object Contact	59.52	76.51	68.02
	(8.40)	(12.08)	(7.40)
Trial 2 Familiar Object Contact	56.80	34.45	45.63
	(13.39)	(4.45)	(7.24)
Trial 3 Novel Object Contact	80.51	66.21	73.36
	(10.43)	(9.85)	(7.17)
Trial 3 Familiar Object Contact	27.14	40.50	33.82
	(5.68)	(8.82)	(5.30)
Trial 4 Novel Object Contact	74.00	72.94	73.47
	(12.67)	(8.92)	(7.61)
Trial 4 Familiar Object Contact	47.16	34.59	40.88
	(8.25)	(8.26)	(5.85)
Novelty Preference			
Trial 2	13.58	31.35	22.46
	(13.33)	(9.51)	(8.21)
Trial 3	49.30	23.98	36.64
	(9.71)	(14.84)	(9.03)
Trial 4	17.12	34.48	25.80
	(10.81)	(10.20)	(7.48)
Preference Change			
Trial 2	5.30	27.32	16.31
	(11.95)	(14.66)	(9.51)
Trial 3	69.52	56.12	62.82
	(14.20)	(13.14)	(9.58)
Trial 4	66.42	62.58	64.50
	(9.77)	(15.91)	(9.18)

Note. Numbers in parentheses are standard error of mean.

3.3.2 Summary

The findings with the repeated NOR test confirmed the lack of a sex difference in adults in the preference for novelty over 3 trials in which novel objects were introduced, in agreement with other studies examining sex differences in NOR (Ghi, Orsetti, Gamalero & Ferretti, 1999; Ricceri, Colozza & Calamandrei, 2000; Sutcliffe, Marshall & Neill, 2007). Although total object contact did not change between trials, the change in preference from the familiar to the novel object did increase in the repeated preference trials, T3 and T4, compared with T2. In this experiment, the location of the novel object alternated between trials, either right-left-right or left-right-left, which could affect the novelty change measure because of spontaneous alternation (Dember & Fowler, 1958; Dennis, 1939). Dember and Fowler (1958) describe spontaneous alternation as a behaviour pattern over successive trials, e.g., if a rat selected the right arm of a T-maze in a first trial, then the animal would be more likely to select the left arm of the same maze in a second trial. This effect is more pronounced with short inter-trial-intervals of 2-minutes or less, and in forced, rather than free-choice, situations (Dember & Fowler, 1959). If the increase in preference change from T2 to T3 in this experiment was due to spontaneous alternation, then the preference change might have predicted a similar increase between T3 and T4, which did not occur.

Additionally, although the location of the novel object alternated between trials, it was counterbalanced, left-right-left in T2-T4 for 14 animals, and right-left-right in T2-T4 for 16 animals, with the animals 'free' to choose either object. If spontaneous alternation did occur, there should not have been a difference in contact latency between the right and left object, yet the animals showed a tendency to contact the left object first in each of the object trials (T1-T4). The placement of the

animal within the apparatus at the start of each trial may have contributed to this effect, thereby eliminating or reducing the effect of spontaneous alternation.

Studies examining spontaneous alternation typically look at the first choice of the animal, and not the continued behaviour over a set duration of time after the choice has been made (Dember & Fowler, 1958). Examining total object interaction over a 5-minute trial, rather than for a minimum amount of contact, should also reduce the influence of spontaneous alternation. Indeed, in each of the object trials, T1-T4, the duration of object contact over the entire 5-minute trial did not differ based on the location of the object within the testing apparatus, similar to findings of prior studies examining exploratory behaviours and spontaneous alternation (Montgomery, 1951). Although other researchers used either shorter trials (Sutcliffe, Marshall & Neill, 2007) or a minimum amount of contact to determine trial length in the NOR task (Kosten, Lee & Kim, 2007), the preference for the novel object over the familiar remained robust over the 5-minute trial used in this study, with no apparent alternation indicated. Therefore, the 5-minute trial length was considered suitable to elicit the novel-preference response for future NOR tests.

In the novel object trials, T2-T4, the only significant differences apparent in object contact were due to the relative novelty or familiarity of the objects. However, although neither side biases nor differences due to the novel objects were found in the analyses, the difference between the novelty preference measure and the preference change measure in T3 and T4 could be due to the accumulation of variations in the individual responses to specific objects. The recommendation for future tests, therefore, would be to keep the total number of objects used to the minimum number needed to complete the trials and thereby reduce added variation.

3.4 Discussion

In this series of experiments, the novel object recognition task was assessed for its reliability at eliciting a preference for novel objects over familiar objects in adult rats, while also evaluating sex differences in novelty preference behaviour. While the NOR task has been used in prior studies of both novelty preference and memory, the design of the task varies across studies as to the quantity and type of objects used in each of the trials, the inter-trial-interval lengths, the duration of both the trials and familiarization sessions, as well as in the number of trials needed to determine if an object preference is present (e.g. Ennaceur & Delacour, 1988; Ghi et al., 1999; Sutcliffe et al., 2007).

In the current study, a preference for the novel object over the familiar object was observed in adult rats in each of the NOR experiments, with any sex differences, if present, mediated by differences in total object contact. No sex differences in the NOR task were found in adult rats when a 2-minute ITI was used, and thus, our absence of sex differences in novelty preference is similar to the findings of prior researchers (Ghi et al., 1999; Ricceri, Colozza & Calamandrei, 2000; Sutcliffe, Marshall & Neill, 2007). The preference for the novel object over the familiar object in each experiment was also as expected and in agreement with prior studies that used a similar one-trial object recognition design (e.g. Ainge et al., 2006; Berlyne, 1950; Ennaceur & Delacour, 1988).

In prior studies, novelty preference behaviour was found in a 3-minute testing session (T2) after as little as 20 seconds contact with a familiar object during the training session (T1), with a 1-minute ITI (Ennaceur and Delacour, 1988). Ennaceur and Delacour (1988) suggested the presence of novel object recognition is dependent upon the amount of object exploration during T1 as well as the duration of the interval

between training and testing. Novelty preference was also observed after a 3-hour ITI when both the training and testing sessions were concluded upon reaching 30 seconds total contact time (Kosten, Lee & Kim, 2007). Other studies have found novelty preference in trials of a set time of 3-5 minutes after training trials of the same period, while examining various ITIs (Berlyne, 1950; Sutcliffe et al., 2007). An object recognition study of memory that exposed animals to 2 of the same objects for 3 minutes in T1, with a 5-minute ITI before a 3-minute test trial with one novel and one familiar object, found that rats spent more time interacting with the novel object during only the first 2 minutes of the test trial (Dix & Aggleton, 1999). However, in our first three NOR tests, novelty preference behaviour, defined as contacting a novel object more than a familiar object, was robust enough to be measurable in a 10-minute trial after a 2-minute ITI. This robustness of response in our study was most likely a result of different experimental procedures, as the animals were exposed to the familiar objects during T1 for a period of 10-minutes compared to the 5-minute T1 used by Dix and Aggleton (1999). Albasser and colleagues (2009) found a positive relationship between the time spent in contact with familiar objects during a training session and novel object discrimination in the test trial after a 24-hour interval. Although there are differences in ITIs, perhaps a similar relationship is occurring with the 10-minute trial even after only a 2-minute ITI. Thus, it is not only the presence of novelty preference behaviour, but also the robustness of the response that are functions of the amount of contact with the familiar objects during the training session and the duration of the ITI.

As future studies were planned that would examine novelty preference behaviour in adolescent animals, the last NOR experiment with adult animals used 5-minute sessions, as prior studies had been successful at detecting a preference for a

novel object in this time. In the repetition study, the preference for novelty not only was apparent in the first 5-minute test session (T2), but also showed intersession reliability, becoming stronger in additional novel trials (T3-T4). Since the preference for novelty was robust in the 10-minute trial and novelty preference continued to occur in repeated 5-minute trials after 2-minute ITIs, future studies could explore the maximum trial length before the animals habituated to novelty and the maximum number of trials where novelty preference is still observed. Based on the findings of these experiments and of prior studies, one 5-minute training session (T1) and one 5-minute test session (T2), with a 2-minute ITI, would be sufficient to detect novelty preference behaviour in the next series of experiments with adolescents.

Although there were no sex differences in total object contact in any of the experiments, there were differences in total object contact due to the novel objects used. In each of the experiments, some of the objects used elicited either higher (e.g., plastic blocks) or lower (e.g., KONG) contact from the animals, perhaps due to the physical properties of the object. While exploring the role of ethanol withdrawal in object exploration, Chemero and Heyser (2005), found differences in exploratory behaviours between objects, which they concluded were attributable to differing *affordances* of the objects. Affordances (introduced by Gibson, 1979) are based on the propensity of an object to elicit naturally occurring behaviours, such as climbing or touching, because of the properties of the objects. An object which is flat, therefore, may elicit more interaction than an irregular shaped object, as an animal may perceive it as easier to climb. Upon further investigation, Chemero and Heyser (2005) suggested potential methodological issues with object exploration research, indicating that different affordances of objects used in prior studies may have confounded the results. In the 116 articles they surveyed, Chemero and Heyser found

that approximately 44% (52 articles) did not provide details of the properties of the objects used in the study, an additional 28% (32 articles) used objects with differing affordances, leaving 28% (32 articles) that both provided sufficient object information and were of equivalent affordances (Chemero & Heyser, 2005).

Addressing the concerns about object affordances, Ennaceur (2010) agrees the properties of objects used must be considered when deciding which objects to use in the NOR task, but argues that an experimental design that includes counterbalancing should help to mitigate the influence of affordances. In our examination of object preference, the first NOR test used only 2 different items as novel objects: short, flat-topped, plastic blocks and an irregularly shaped clay brick. The results indicated higher levels of novelty preference when the blocks were novel objects compared to when the brick was the novel object. Although time spent climbing on the object is not considered when calculating object interaction, the affordances of the object were still more attractive to the animal, causing higher levels of contact with the preferred than the non-preferred object. In the second set of experiments, when more than two novel objects were used, counterbalanced among the animals and between trials, the differences in contact between novel objects were diminished.

In addition to counterbalancing, the ratio of animals to objects must be considered, as the responses to the different objects could add variation or 'noise' to the analyses, perhaps masking preferential behaviours. Using total object contact as a covariate during analysis can help alleviate the influence of object response differences and confirmed the presence of preference for the novel object over the familiar object. Although the results were not reported, a pilot study was completed to examine the amount of interaction with a series of different object by adult rats. For our subsequent tests examining responses to novel objects during development,

discussed in more detail in the next chapters, the objects used in the NOR task were those that elicited similar amounts of interaction in the pilot study. Therefore, although Ennacuer (2010) argues that novelty preference and object preference are sometimes confused, by analyzing animal responses to different objects and by using objects in the NOR test that elicit similar responses, any preferences exhibited for the novel object during testing can be attributed to a preference for novelty.

There are several advantages to using the NOR test to assess novelty preference in rats. Including a 10-minute familiarization session to habituate the animals to the testing apparatus, with two 5-minute testing trials with the objects and an ITI of 2-minutes, the total time required for each testing session, and thus the time the animal was kept out of its home cage, was reduced to less than 30-minutes per animal. Additionally, the responses to the objects are due a natural tendency for animals to approach and explore novel objects more than familiar objects, and are not dependent on food or water restriction (Berlyne, 1950; Dember, 1956; Thompson, 1954). Since our next set of experiments assess developmental as well as sex differences in the NOR task, taking less time to complete the testing session (i.e., one-day testing vs. multi-day) will decrease the possible effects due to naturally occurring developmental changes, and the lack of dietary restriction will not affect the normal development process.

Chapter 4: Adolescent sex differences in novel object recognition

4.0 Introduction

Adolescence is period of transition, with both physiological changes occurring at puberty, and emotional changes while moving toward independence (Spear, 2000; Steinberg & Morris, 2001). In human studies, sensation seeking levels were generally highest during the adolescent years than at younger or older ages (e.g. Kelley, Schochet, & Landry, 2004; Roth, Schumacher & Brähler, 2005; Zuckerman, 2006). In addition, males were reported to engage in more sensation seeking behaviours than females across all age categories, while both males and females showed decreased sensation seeking as they age (Magaro, Smith, Cionini & Velicogna, 1979; Trimpop, 1998; Zuckerman, Eysenck & Eysenck, 1978). These data collectively suggest that age and sex differences in sensation-seeking are relatively robust.

Several studies, with ages tested ranging from 7 to 19 years old, have also reported males as higher than females in sensation-seeking, risk-taking or venturesomeness, whereas females are typically rated higher in empathy or experience-seeking during these ages (e.g. Arnett, 1992; Eysenck, Easting & Pearson, 1984; Russo, Lahey, Christ et al., 1991; Zuckerman, Eysenck & Eysenck, 1978). Studies of juveniles, however, are limited, perhaps due to the nature of the questions on the various measures (Zuckerman, 1994). A children's version of the SSS was developed by Russo and colleagues (1991, revised 1993), which showed increased total sensation seeking from ages 7 to 12 years of age and from 9 to 14 years of age, with males higher than females in TAS and total scores. Similar finding were reported in an earlier study by Kafry (1982), which indicated an increase between 6 and 10 years of age, although males were higher than females only in the older age

groups. Additional research is needed, however, to understand the pattern of sensation seeking expression from early childhood through adolescence.

As in humans, rodents exhibit both physiological and behavioural changes as they transition through developmental stages from birth to adulthood. Rat pups remain with their lactating dam during a period of infancy until weaning, which typically occurs when they reach the age of 21 days (postnatal day, pnd, 21). The adolescent period ranges from weaning at pnd 21 through early adulthood at pnd 60, with puberty occurring between pnd 28-42, earlier in females than males (Spear, 2000; Tirelli, Laviola, & Adriani, 2003). Studies of novelty preference behaviour in weanlings (pnd 18-23) have encountered problems with animals failing to explore, suggesting extrinsic motivations may be stronger at this age (Anderson et al, 2004; Reger, Hovda, & Giza, 2009). Additionally, since sex differences in human sensation seeking become apparent in adolescence, animal studies of age and sex differences in novelty seeking typically begin at this stage of development.

We previously examined sex differences in the performance of adult rodents on the NOR task, and found no differences in novel object preference between adult males and females when short (2-minute) inter-trial-intervals were used. These findings were in agreement with other studies examining sex differences in adult rodents in NOR (Ghi, Orsetti, Gamalero & Ferretti, 1999; Ricceri, Colozza & Calamandrei, 2000; Sutcliffe, Marshall & Neill, 2007). Most studies of novelty preferences in rodents examine this behaviour in male, adult animals. In order to understand the ontogeny of sex differences in responses to novelty, both males and females will be tested from adolescence through adulthood. Through the use of animal models, it is possible to get a better understanding of the physiological

mechanisms that may be influencing age and sex differences in sensation seeking in humans.

Two studies of mice have examined age and sex differences in performance on the NOR task, comparing adolescents (pnd 45 or 46) with younger (pnd 28) and older age groups (pnd 70 or 90) (Calamandrei, Rufini, Valanzano, & Puopolo, 2002; Ricerri, Colozza, & Calamandrei, 2000). In the earlier study, Ricerri and colleagues (2000) examined the ontogeny of spatial and object discrimination in CD-1 mice in a 7-session modified open-field task. Each session was 4-minutes in duration, with session 1 a familiarization trial without objects, sessions 2-4 habituation trials with 4 objects present, session 5 with one of the four objects in a different location, session 6 a repeat of session 5, and session 7 with a novel object introduced to replace one of the four objects. The ITI in all cases was 2-minutes. To measure object discrimination, time spent with the novel object in session 7 was compared to the time spent with the object in the same position in session 6, with the difference considered the measure of novel object exploration. Novel object exploration was then compared to the difference between session 6 and session 7 mean combined exploration times of the 3 remaining objects. Both male and female mice were tested at pnd 18, 28, 46 and 90 (n = 8-10 per group). In session 7, mice spent more time exploring the novel object compared to the familiar objects at pnd 28, 46 and 90, but not at pnd 18. No significant sex differences were found in any of the age groups. Although comparisons between age groups were not reported, the response to the novel object is elevated at pnd 46 and 90 compared to pnd 18 and 26.

Calamandrei and colleagues (2002) examined novelty discrimination in CD-1 mice over the 7-session spatial and object recognition task while testing the effects of prenatal administration of zidovudine (AZT) or saline to pregnant dams. The

offspring of the treated (AZT) or control (saline) mice were tested at pnd 28, 45 or 70 (n = 8 per group). At each age, in both control and treated groups, mice spent more time interacting with the novel object in session 7. No sex differences were apparent, but there was an elevated preference for the novel object over the familiar objects in pnd 45 control animals compared to the treated animals when male and female data were pooled. Both studies suggest that, following a 2-minute inter-trial delay, mice interact with the novel object more than the familiar object during the final stage of the task, and that the strength of the preference for the novel object in the final test phase peaks at adolescence (Calamandrei, Rufini, Valanzano, & Puopolo, 2002; Ricerri, Colozza, & Calamandrei, 2000). However, both studies also used a 7-sesssion design, with object novelty not tested until the last session, and a decrease in locomotion and total object exploration due to habituation or fatigue may have contributed to differences in novel object preferences (Bâ & Seri, 1995; Chapillon & Roullet, 1997; Leussis & Bolivar, 2006).

In contrast, a recent study of male rats reported no difference in strength of preference for the novel object during Trial 2 between weanlings (pnd 20-23), adolescents (pnd 29-40), and young adults (pnd 50+) after a 15-minute inter-trial interval (Reger, Hovda, & Giza, 2009). Reger and colleagues (2009) used a design similar to that of Ennaceur and Delacour (1988) to examine the ontogeny of recognition memory in male Sprague Dawley rats. Three age groups (weanling: pnd 20-23, n = 26; juvenile: pnd 29-40, n = 17; and young adult: pnd 50+, n = 13; ns for 15-minute ITI) were given three 10-minute habituation sessions over 3 days, and tested in the NOR task on the fourth day. During testing, the animals were exposed to 2 identical objects in the first trial, then after ITIs of either 15 minutes, 1 hour, 24 hours, or 48 hours, the animals were reintroduced to the apparatus for the second trial

with one of the familiar objects now replaced by a completely different object. For each age group, Reger et al. used a different testing apparatus, scaled in size relative to the size of the subject, with the weanling apparatus similar in size to the home cage. Although weanlings were given longer test trials (5-minutes) compared to the older animals (3-minutes), only the first 3 minutes of exploration was considered for comparison. After the 15-minute ITI, the researchers reported that each age group spent a significantly greater amount of time exploring the novel object compared to the familiar object in the second trial, with no difference found across the ages. However, due to the broad range of ages in each group (e.g. pnd 29-40 for adolescents), rapid developmental changes, such as the overproduction of dopamine receptors (Andersen & Teicher, 2000), may have had an effect on performance in the NOR task.

Although no age differences were reported in the study by Reger et al. (2009), several weanlings were excluded from the analyses as they failed to explore the objects. Anderson et al. (2004) had a similar problem when comparing NOR performance in pre-weanling (pnd 18) and adult (pnd 90) male Long Evans rats after an ITI of either 1 minute or 120 minutes. No difference was indicated in preference for the novel object between the age groups at the 1-minute ITI (pnd 18, $n = 14$; pnd 90, $n = 14$). However, of the pre-weanlings tested ($n = 49$), almost half (23) were excluded from analyses for non-exploration based on the criteria of reaching 5 seconds total object contact (minimum of 1 second each object) during testing, perhaps confounding the results (Anderson et al., 2004). The contact criteria were established *post hoc* in order to reduce the variability arising from the individual differences in exploration (Anderson et al., 2004). The minimum contact criteria of 5 seconds of total contact with the objects in Trial 1 and at least 1 second contact with

either object in both Trial 1 and Trial 2 was kept consistent across all age groups, and is comparable to the minimum contact time used in studies of recognition memory (Ainge et al., 2006; Kosten, Lee & Kim, 2007).

Evidence for age differences in performance on the NOR task is thus very sparse and contradictory. In this study, we examined the performance of male and female Lister hooded rats in the NOR task with a 2-minute ITI at early adolescence (pnd 28), mid-adolescence (pnd 40) or early adulthood (pnd 80). The age groups in the current study were selected to facilitate comparison with previous research that examined physiological changes that occur during adolescence, specifically dopamine receptor density overproduction and subsequent pruning (Andersen & Teicher, 2000). The age of pnd 28 was selected as it was exactly one week after weaning, and would be an indicator of early adolescent behaviour. In addition to collecting data on interactions with the objects during Trial 1 and Trial 2, we collected data on locomotor activity in the arena, as age and sex differences in locomotion could potentially influence object interactions. Based on the physiological changes in the male dopaminergic system, males are expected to show an increase in novelty preference from pnd 28 to pnd 40, followed by a decline in early adulthood. As there are few studies of female adolescent rats, the pattern of performance in the NOR task expected between the female age groups is less clear, and preferences may be similar at each age.

4.1 Novel Object Recognition

4.1.0 Methods

Subjects and housing

The subjects were 36 male and 36 female Lister-hooded rats bred in-house from stock supplied by Harlan, U.K. All animals were housed in cages (measuring 25cm x 45cm x 15cm) with *ad libitum* access to soy-free rodent pellets and water. Housing rooms were controlled for temperature (20 ± 1ºC) and humidity (55 ± 5%), and maintained on a 12-hour light:dark cycle (lights on 7am). From pnd 17, pups were handled once per day and were weaned into same-sex sibling groups at pnd 21. At pnd 28, animals were housed as same-sex sibling pairs.

Each subject underwent behavioural testing only once, with different animals used in each age group. Subjects were tested at pnd 28 (*n* = 12 males, 12 females), pnd 40 (*n* = 12 males, 12 females) or pnd 80 (*n* = 12 males, 12 females). The subjects were taken from 19 litters, and littermates and cagemates were distributed as evenly as possible among all the age groups. Any animal that did not exhibit a minimum of 5 seconds of total contact with the objects in Trial 1, and at least 1 second contact with either object in both Trial 1 and Trial 2, was excluded from the study. One pnd 40 female was excluded on these criteria and was replaced by a new subject. A pilot study with pnd 21 animals found that over half of the animals at this age failed to meet the criteria, mostly due to a lack of object contact in the Trial 2. These weanling subjects were therefore not included in the study. All appropriate guidelines and requirements were adhered to, as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85-23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986.

Apparatus

The NOR testing apparatus was a wooden, light grey-painted square chamber, measuring 67cm x 67cm x 45cm (l x w x h), with a solid floor constructed of the same

material. The chamber was raised 28cm above the ground on a metal stand. Three objects were used during the experiment (yellow rubber toy, glass jar filled with rocks, blue plastic bottle filled with sand) and were chosen to deter climbing and chewing. A pilot study with adult male and female rats showed that, from a range of objects, the amount of time spent interacting was very similar for these items.

The apparatus was surrounded by a black curtain, and a video camera attached to the ceiling relayed images to a computer. All tests were conducted between 09:00-14:00 hours in the same testing room under dim, white light (approximately 25 lux), and a white noise generator was used to mask external sounds.

Experimental design

At the beginning of the test session, a subject was brought to the testing room in a carrying box measuring 42cm x 26cm x 13cm (l x w x h). The subject was placed into the empty apparatus and given a 10-minute familiarization session. The animal was then returned to the carrying box for a period of 2 minutes while the apparatus was cleaned with a 70% ethanol solution and allowed to air dry. Two objects were placed into the apparatus in adjacent quadrants, 15cm apart and 8cm from the wall (**Figure 19**), after which the animal was placed into an empty quadrant, facing away from the objects. During this session, Trial 1 (T1), which lasted 5 minutes, the subject had the opportunity to interact with the two objects. At the end of Trial 1, the animal was again removed to the empty carrying box for an inter-trial interval of 2 minutes, during which the apparatus and objects were cleaned with the ethanol solution and one of the objects was replaced by a novel object. The animal was then reintroduced to the apparatus and allowed to interact with the objects for 5 minutes, Trial 2 (T2). The object that remained from the first trial was considered the familiar object, and

the new object was considered the novel object. At the end of Trial 2, the subject was immediately returned to the home cage, and all objects and apparatus were cleaned with an ethanol solution in preparation for the next subject. The objects used in each trial were counterbalanced across subjects and between age groups, and whether the left-hand or right-hand object was replaced in Trial 2 was also counterbalanced.

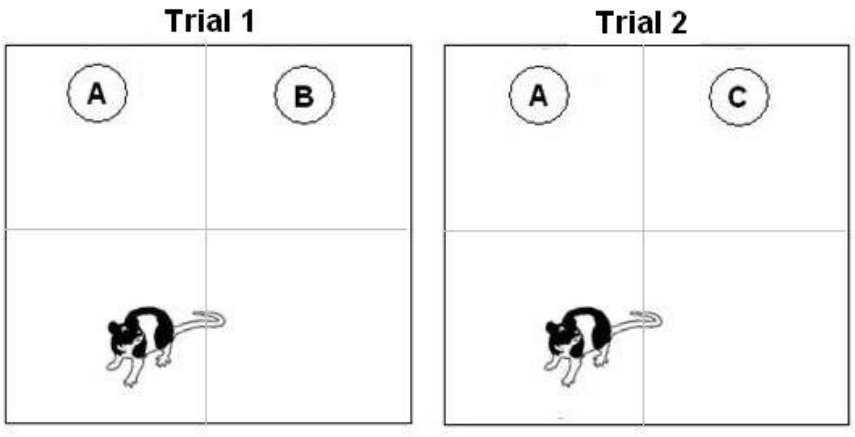

Figure 19. A diagram of object position and animal placement in the novel object recognition task during Trial 1 and Trial 2. Grey lines denote quadrant boundaries but are not present in the apparatus.

Behavioural measures

All sessions were digitally recorded directly onto the computer for later observation. Measures of object interaction were recorded manually using in-house software, while locomotor activity was analysed using EthoVision XT 5.0 software (Noldus Information Technology, Netherlands, 2008).

During the two trials, locomotor activities measured included and *total distance moved*. Behavioural measures collected during the Trials 1 and 2 included the *amount of time spent moving* and the *time spent interacting with each object*. Object interaction was defined as the nose being in contact with an object, which excluded behaviours such as backing into an object, tail only contact, or time resting

next to an object without direct interaction. Any animal that did not exhibit a minimum of 5 seconds of total contact with the objects in Trial 1, and at least 1 second contact with either object in both Trial 1 and Trial 2 was excluded from the study (one female at pnd 40).

Two measures of novelty preference were calculated. The first measure, referred to as *preference for novelty*, was calculated as the proportion of time spent interacting with the novel versus the familiar object in Trial 2, converted to a percentage [(Time with novel – Time with familiar)/(Time with novel + Time with familiar) * 100]. A positive value indicates a preference for the novel object, while a negative value indicates a preference for the familiar object, and a score of zero indicates equal preference for the two objects.

The second measure, referred to as *preference change*, takes into account any initial left/right side-bias shown by the subject. To take into account any such bias and also any individual object preference bias, a side preference was calculated for both Trials 1 and 2 [(Time with right object – Time with left object)/(Total time with both objects) * 100], with a negative value representing a left-side preference, and a positive value indicating a right-side preference. *Preference change* was then calculated as the change in object contact times from Trial 1 (T1) to Trial 2 (T2), [(T2Right – T2Left)/(T2Right + T2Left)]*100 – [(T1Right – T1Left)/(T1Right + T1Left)] *100. The preference change value was adjusted to positive (+) if contact changed toward the novel object, or to negative (–) if more toward the familiar object. Therefore, if the preference ratio increased between trials in the direction of the novel object, this score would have a positive value, and *vice versa*. An example of this measure is given in the previous chapter on adult NOR performance.

Statistical analyses

One-sample *t*-tests were used to examine whether animals showed a significant preference for the novel object compared to chance as indicated by a score significantly greater than zero. Analysis of variance (ANOVA) was used to examine whether the percentage of contact with the novel object (the dependent variable) differed with sex and age (the independent variables). Locomotor activity, object interaction, and novelty preference measures were compared using Pearson correlations. The amount of time spent moving was selected as the preferred locomotion indicator, as we predicted it would be less influenced by differences in sizes between the ages and sex of the subjects than would total distance moved. If a significant correlation was found between novelty-preference and another behavioural measure, an analysis of covariance (ANCOVA) was carried out to see if any sex and age differences remained. Repeated measures ANOVA were also used to analyse locomotor and object contact measures for sex and age differences, with Greenhouse-Geisser correction if needed.

An α value of .05 was used for all comparisons, and Bonferroni pairwise comparisons were used to investigate sex and age differences. All data were analysed using SPSS 17.0 for Windows (2009) software package. Effect size (partial-eta squared, η_p^2) and power (β) values for ANOVAs were calculated by SPSS, and Cohen's *d* and power for *t*-tests were calculated with G*Power Version 3.0.8.

4.1.1 Results

Locomotion

Between the two trials, there was a significant decrease in movement from Trial 1 to Trial 2 ($F_{1, 66} = 15.40$, $p < .001$, $\eta_p^2 = .19$, $\beta = .97$; **Figure 20**). Although neither the

main effect of age ($F_{2, 66}$ = 2.49, p = .091, η_p^2 = .07, β = .48) nor the trial by age interaction were significant ($F_{1, 66}$ = 1.99, p = .145), the difference between trials was influenced mainly by differences in the pnd 28 and 40 age groups (ps ≤ .018) rather than the difference at pnd 80 (p = .483). There was no significant main effect of sex ($F_{1, 66}$ = 1.42, p = .238), nor were there significant interactions between sex and age or sex and trials (Fs$_{1\text{-}2, 66}$ ≤ 2.07, ps ≥ .134), although there was an age difference for males between pnd 28 and 40, (p = .038), and a sex difference at pnd 28 (p = .024; **Table 4**).

Total amount of contact with objects

There was a significant main effect of age on the total amount of time spent in contact with the objects across both trials ($F_{2, 66}$ = 11.27, p < .001, η_p^2 = .25, β = .99; **Figure 20**), with comparisons indicating increases from pnd 28 to 40 (p = .037) and pnd 28 to 80 (p < .001), but no difference between pnd 40 and 80 (p = .101). The total amount of time spent in contact tended to decrease between T1 and T2 ($F_{1, 66}$ = 3.65, p = .060, η_p^2 = .05, β = 50). Although the trial by age interaction was not significant, ($F_{1, 66}$ = 2.21, p = .118), the differences between all the age groups were evident mainly in T2 (ps ≤ .020) rather than in T1, where an age difference was only observed between pnd 28 and 80 (p = .043) and not between the other ages (ps ≥ .257). There was no significant main effect of sex ($F_{1, 66}$ = .44, p = .509), nor were there significant interactions between sex and age or sex and trials (Fs$_{1\text{-}2, 66}$ ≤ 1.21, ps ≥ .304; **Table 4**).

As there was a significant correlation between total object contact in T2 and locomotion (r_{72} = .39, p = .001), the object contact data were also analysed using locomotion as a covariate. Although locomotion did have a tendency to influence

contact ($F_{1, 65}$ = 3.39, p = .070, η_p^2 = .05, β = .44), the age difference remained significant ($F_{2, 65}$ = 13.17, p < .001, η_p^2 = .29, β = 1.00). However, pairwise comparisons now indicated a trend for an increase in object contact from pnd 28 to 40 (p = .073) and a significant increase from pnd 40 to 80 (p = .008), as well as the difference between pnd 28 and 80 (p < .001). There was still no significant main effect of sex ($F_{1, 66}$ = .005, p = .941), nor was there a significant interaction between sex and age ($F_{2, 66}$ = .30, p = .744).

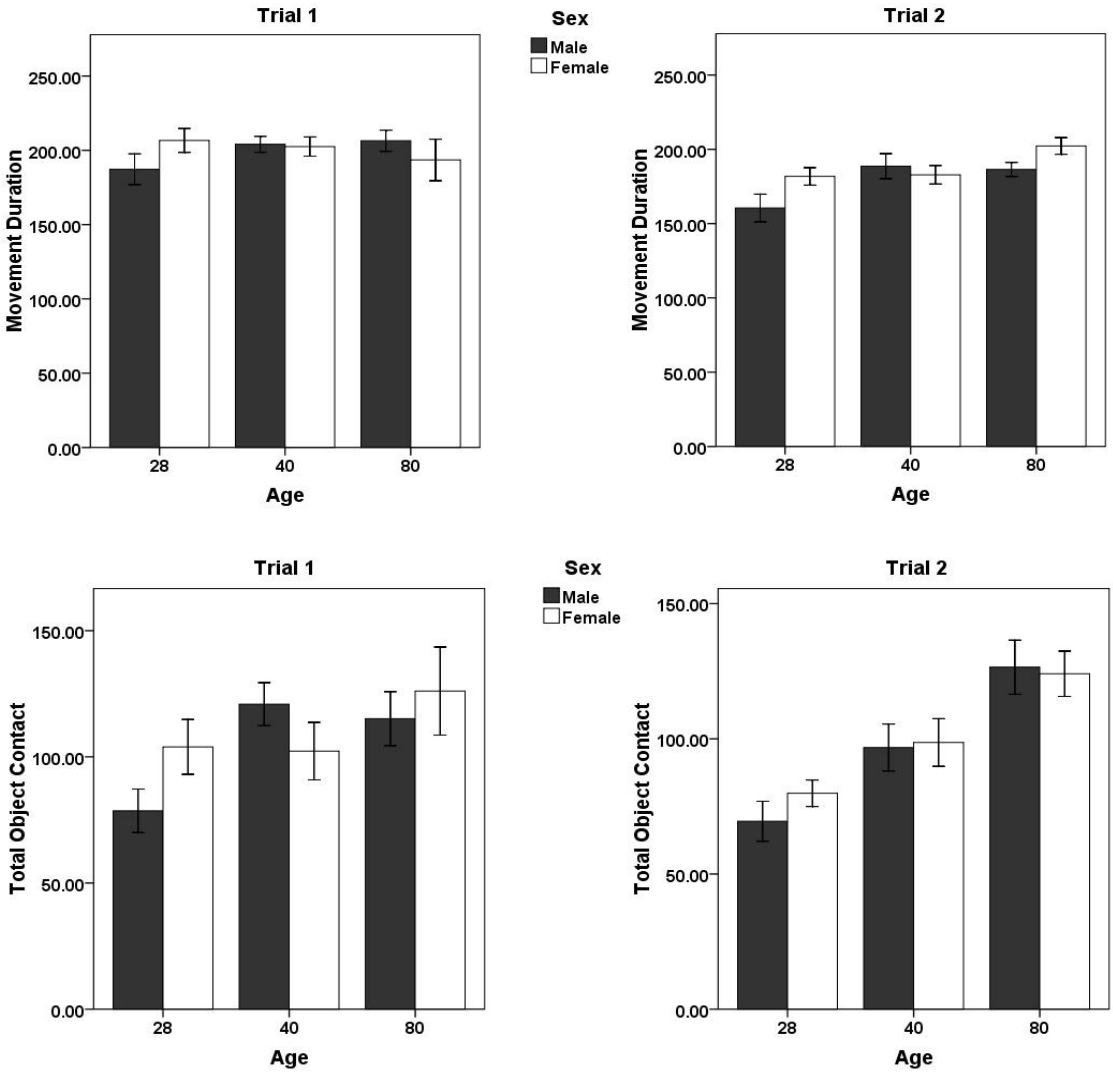

Figure 20. **Top:** Mean movement duration, in seconds, and **Bottom:** Mean total object contact, in seconds, during Trial 1 and Trial 2 by sex and age group. Error bars represent +/- 1 standard error of the mean.

Preference for novelty

When all subjects were combined, a one sample *t*-test verified that the subjects exhibited a significant preference for novelty in Trial 2 ($t_{71} = 4.21$, $p < .001$, $d = .50$, $\beta = .99$), with animals spending approximately 60% of contact time with the novel object and 40% with the familiar object. Males showed a preference for novelty at pnd 40 ($t_{11} = 3.07$, $p = .011$, $d = .89$, $\beta = .80$) but not at pnd 28 ($t_{11} = 1.16$, $p = .272$) or pnd 80 ($t_{11} = 1.45$, $p = .175$). Females exhibited a significant preference for the novel object at pnd 28 ($t_{11} = 2.40$, $p = .035$, $d = .69$, $\beta = .59$) and pnd 80 ($t_{11} = 2.86$, $p = .015$, $d = .83$, $\beta = .74$), but not pnd 40 ($t_{11} = .37$, $p = .720$).

As a Pearson correlation indicated a significant negative relationship between time spent moving in T2 and novelty preference ($r_{72} = -.24$, $p = .043$), this locomotor measure was used as a covariate in the analyses. An ANCOVA showed a significant sex by age interaction in preference for novelty, ($F_{2, 65} = 4.47$, $p = .015$, $\eta_p^2 = .12$, $\beta = .75$; **Figure 21**), with the effect of locomotion significant, ($F_{1, 65} = 12.42$, $p = .001$, $\eta_p^2 = .16$, $\beta = .94$). Pairwise comparisons indicated that males exhibited greater novelty preference than females at pnd 40 ($p = .039$), and females higher than males at pnd 80 ($p = .049$). There was no sex difference at pnd 28 ($p = .320$). Males showed an increase in novelty preference from pnd 28 to pnd 40 ($p = .043$), with a non-significant decrease from pnd 40 to pnd 80 ($p = .797$), and no difference between pnds 28 and 80 ($p = .439$). Females exhibited no change in novelty preference between pnd 28 and pnd 40 ($p = 1.00$), and a significantly higher novelty preference at pnd 80 than at pnd 28 ($p = .048$) and pnd 40 ($p = .013$). The main effect of sex was not significant ($F_{1, 65} = .29$, $p = .589$; **Table 4**).

Neither total object contact in T1 nor total object contact in T2 correlated with novelty preference ($rs_{72} \leq .08$, $ps \geq .507$). However, given that object contact significantly increased across the age groups, an additional ANCOVA was performed that also included object contact in T1 and object contact in T2 as covariates. The sex by age interaction remained significant ($F_{2, 63} = 3.83$, $p = .027$, $\eta_p^2 = .11$, $\beta = .68$), the main effect of age difference was reduced ($F_{2, 63} = 2.50$, $p = .090$), and the main effect of sex remained non-significant ($F_{2, 63} = .27$, $p = .607$). Neither T1 total object contact ($F_{1, 63} = .63$, $p = .432$) nor T2 total object contact ($F_{1, 63} = .06$, $p = .801$) significantly influenced the results, but the influence of T2 movement continued to be significant ($F_{1, 63} = 12.19$, $p = .001$, $\eta_p^2 = .16$, $\beta = .93$).

Preference change

Although side biases were not apparent overall ($ts_{71} \leq .97$ $ps \geq .337$), there was an effect of trial by sex interaction on side biases ($F_{2, 66} = 5.23$, $p = .025$, $\eta_p^2 = .07$, $\beta = .62$). Females tended to show some changes in side bias between the trials ($p = .061$), whereas males did not ($p = .190$).

T-tests revealed similar findings as with novelty preference except that, in females, preference for novelty was no longer significant at pnd 28 and only tended towards significance at pnd 80 ($t_{11} = 2.10$, $p = .059$, $d = .61$, $\beta = .48$). The main effects of age ($F_{2,65} = 1.84$, $p = .167$) and sex ($F_{1,65} = .25$, $p = .617$) were not significant.

A Pearson correlation indicated a trend for a positive relationship between T2 total object contact and preference change ($r_{72} = .21$, $p = .078$), but no significant correlations between either T1 total contact ($r_{72} = .15$, $p = .212$) or T2 movement ($r_{72} = -.18$, $p = .133$) and preference change. However, for consistency in analyses between novelty preference and preference change, similar ANCOVAs were

performed examining first the influence of T2 movement alone and then the influence of both T1 and T2 total contact along with T2 movement. Analysis of the preference change measure with locomotion as a covariate, the sex by age interaction was significant ($F_{2, 65} = 3.61$, $p = .033$, $\eta_p^2 = .10$, $\beta = .65$; **Figure 21**), as was the influence of locomotion, ($F_{1, 65} = 5.22$, $p = .026$, $\eta_p^2 = .07$, $\beta = .61$). The preference change was higher for males than females at pnd 40 ($p = .019$), but no longer at pnd 80 ($p = .168$). There were still no sex differences at pnd 28 ($p = .930$). Age differences in males remained with an increase between pnds 28 and 40 ($p = .047$), and a decreasing trend between pnds 40 and 80 ($p = .072$). There was still no difference in males between pnds 28 and 80 ($p = 1.00$). Females, however, no longer exhibited significant differences between any age groups ($ps \geq .345$). The main effects of sex ($F_{1, 65} = .25$, $p = .617$) and age ($F_{2, 65} = 1.84$, $p = .167$) were not significant (**Table 4)**.

In the additional ANCOVA with T1 and T2 total object contact and T2 movement as covariates, the sex by age interaction remained significant ($F_{2, 63} = 4.22$, $p = .019$, $\eta_p^2 = .12$, $\beta = .72$), and the main effects of both age and sex remained non-significant ($Fs_{1, 63} \leq 1.41$, $ps \geq .252$). There was a significant influence of both T2 locomotion ($F_{1, 63} = 9.03$, $p = .004$, $\eta_p^2 = .13$, $\beta = .84$) and T2 total object contact ($F_{1, 63} = 6.21$, $p = .015$, $\eta_p^2 = .09$, $\beta = .69$), but T1 total object contact did not have a significant effect ($F_{1, 63} = .16$, $p = .694$). Age and sex comparisons were similar to the findings in the first ANCOVA, with an age difference in males ($F_{2, 63} = 5.03$, $p = .009$, $\eta_p^2 = .14$, $\beta = .80$), but not in females ($F_{2, 63} = .39$, $p = .676$), and a sex difference indicated only at pnd 40 ($p = .014$).

There was a significant positive correlation between novelty preference and preference change ($r_{72} = .61$, $p < .001$), and no significant difference between the two measures ($t_{71} = .31$, $p = .760$).

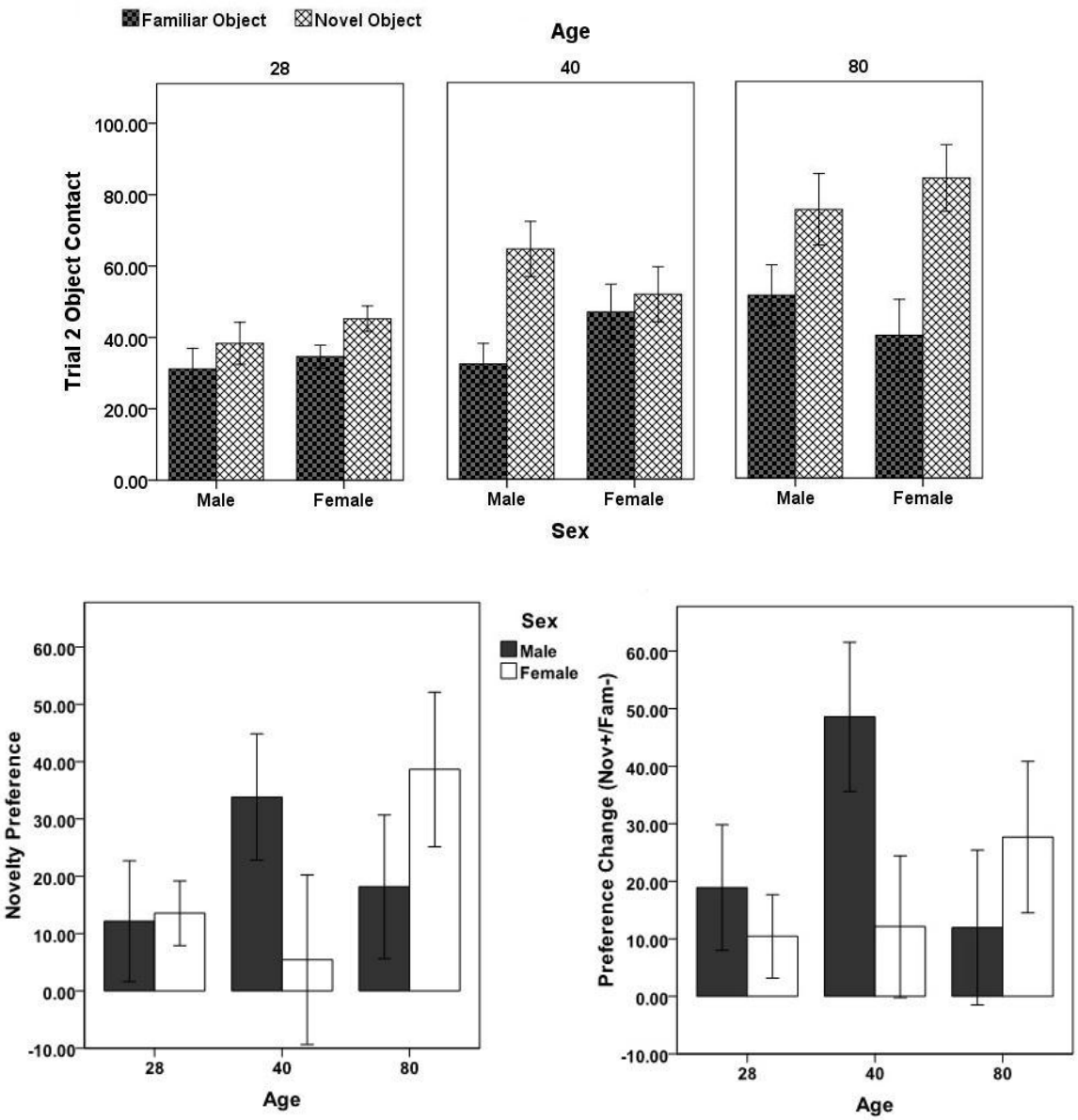

Figure 21. **Top:** Mean contact time, in seconds, with Familiar and Novel objects in Trial 2 by age and sex. **Bottom:** Mean novelty preference (Left) and mean preference change (Right) during Trial 2 by sex and age group. Error bars represent +/- 1 standard error of the mean.

Table 4

Means, in seconds, and standard error of mean of behavioural measures and preference scores by sex and age group (n = 12 per group).

		Males	Females	Totals
Movement Duration				
Trial 1	PND 28	187.25	206.67	196.96
		(10.37)	(8.10)	(6.75)
	PND 40	204.06	202.51	203.29
		(5.33)	(6.47)	(4.10)
	PND 80	206.43	193.51	199.97
		(7.12)	(13.96)	(7.78)
Trial 2	PND 28	160.53	181.78	171.15
		(99.31)	(5.86)	(5.82)
	PND 40	188.66	182.86	185.6
		(8.45)	(6.22)	(5.17)
	PND 80	186.43	202.27	194.35
		(4.73)	(5.62)	(3.95)
Total Object Contact				
Trial 1	PND 28	78.60	103.93	91.26
		(8.61)	(10.86)	(7.28)
	PND 40	120.89	102.26	111.57
		(8.52)	(11.38)	(7.22)
	PND 80	115.13	126.06	120.60
		(10.70)	(17.46)	(10.08)
Trial 2	PND 28	69.51	79.87	7.69
		(7.41)	(4.89)	(4.47)
	PND 40	96.78	98.64	97.71
		(8.69)	(8.82)	(6.06)
	PND 80	126.49	124.05	125.27
		(10.01)	(8.38)	(6.39)
Trial 2 Novel Object Contact				
	PND 28	38.34	45.25	41.79
		(5.91)	(3.57)	(3.26)
	PND 40	64.48	51.79	58.14
		(7.74)	(7.71)	(5.50)

PND 80	75.28	84.07	79.68
	(10.01)	(9.36)	(6.76)
Trial 2 Familiar Object Contact			
PND 28	31.17	34.63	32.90
	(5.79)	(3.21)	(3.26)
PND 40	32.30	46.85	39.57
	(5.71)	(7.76)	(4.95)
PND 80	51.21	39.98	45.59
	(8.52)	(10.08)	(6.56)
Novelty Preference			
PND 28	12.15	13.54	12.85
	(10.51)	(5.65)	(5.84)
PND 40	33.82	5.43	19.62
	(11.01)	(14.78)	(9.49)
PND 80	18.17	38.62	28.40
	(12.55)	(13.49)	(9.26)
Preference Change			
PND 28	18.91	10.42	14.67
	(10.93)	(7.27)	(6.48)
PND 40	48.59	12.11	30.35
	(12.97)	(12.33)	(9.54)
PND 80	11.95	27.69	19.82
	(13.45)	(13.17)	(9.35)

Note. Numbers in parentheses are standard error of mean.

4.2 Discussion

This study examined the ontogeny of response to novel objects in both male and female Lister hooded rats from adolescence to adulthood, using the novel object recognition task with a short inter-trial interval. The results indicated the strength of preference for the novel object in Trial 2 of the task exhibited a significant sex difference only at mid-adolescence (pnd 40), with males showing a higher novelty-preference than females. This sex difference was not present at early adolescence (pnd 28), and, while the opposite pattern of results was observed at early adulthood, the

adult sex difference was only present when calculated as preference for novelty, and not when calculated as preference change, suggesting that the adult sex difference is not robust. In contrast, other measures did not exhibit significant age by sex interactions. The total time spent in contact with objects gradually increased, and latency to contact objects decreased, across the age groups, while neither measure differed between the sexes. These results indicate that the sex difference in behaviour at mid-adolescence was specific to situations involving choice of novelty. The amount of time spent moving during the task also did not exhibit a significant age by sex interaction, although locomotion tended to increase across the age groups. In summary, these results provide evidence that mid-adolescent rats exhibit a sex difference in behaviour when provided with the opportunity to interact with a novel versus a familiar object that is not seen at younger or older ages.

Consistent with prior findings in mice (Calamandrei et al., 2002; Ricceri et al., 2000), our study found that male and female rats from pnd 28 through to adulthood spent a greater amount of time interacting with the novel object over the familiar object. However, although the overall novelty preference scores were significant at each age, a significant novelty preference was indicated only in pnd 40 males, and pnd 28 and 80 females, with female preferences at both ages reduced when side biases were considered. Our finding that mid-adolescent male rats exhibit stronger novelty preference than females has not been reported previously. In males, novelty preference was low during early adolescence, peaked at mid-adolescence and tended to decrease into adulthood, while, in females, preference for novelty was relatively low during early and mid-adolescence and rose in adulthood. Prior studies in our laboratory did not find sex differences in open field or elevated plus-maze behaviour at mid-adolescence, suggesting that our results are not related to sex differences in

anxiety-like responses at this age (Lynn & Brown, 2009, 2010). While the two previous studies of mice have reported that the strength of the preference for the novel object in the final test phase peaks at adolescence (Calamandrei et al., 2002; Ricceri et al., 2000), neither reported a sex difference at this age despite testing subjects of both sexes. In the study by Ricerri and colleagues (2000), the lack of sex difference in NOR performance in mice at varying ages, including adolescence, was potentially due to small sample sizes (n = 4-5 per sex per age group). Calamandrei and colleagues (2002) also used a smaller sample size (n = 8 per sex per age group) than in our study.

The difference in results between our study and previous reports could also be due to inherent differences between mice and rats or other methodological differences. For example, in contrast to our study, the studies on mice used a seven session behavioural task to assess both spatial and object novelty responses and included four objects for discrimination. In the 7-session design used by Calamandrei et al. (2002) and Ricceri et al. (2000), novel object preferences were not tested until session 7 and habituation or fatigue could influence the findings. Reger and colleagues (2009) examined age differences in NOR task in rats, but only in males. These researchers were primarily assessing memory and used various inter-trial intervals, the shortest of which was 15 minutes. At this delay, no age differences were found in the proportion of interactions with novel versus familiar objects between their adolescent (pnd 29-40) and adult groups (pnd 50+). In comparison, our study did find an age difference in males, with novelty-preference higher at pnd 40 compared with pnds 28 and 80. The inconsistency in findings between the two studies could be attributable to the broad age classifications used by Reger and colleagues (2009), which could have masked more subtle age differences. In line with previous reports that adult female rats exhibit a stronger preference for the novel object than

males in the NOR task (Ghi et al., 1999; Sutcliffe et al., 2007), our study reported that females (pnd 80) spend more time interacting with the novel object than males in Trial 2, but that this sex difference disappeared when side-biases were taken into account, suggesting that the adult sex difference is not robust. The absence of a sex difference in preference change at pnd 80 is in agreement with our prior study, which also did not find a sex difference in adults.

In contrast to our findings on novelty preference, no sex differences were apparent at any age in other measures of object interaction, including total contact with objects and latency to first contact an object. Therefore, it does not appear that novelty-preference differences at mid-adolescence were related to differences in attending towards novel objects. Our study supports previous studies that have reported a lack of sex differences in total contact with novel objects in adult rats (e.g. Thor, Harrison, Schneider, & Carr, 1988; Renner, Bennett, & White, 1992). The object interaction measures gradually increased from early adolescence into adulthood, in support of previous studies (e.g. Renner et al., 1992), and, similarly, the time spent moving tended to increase across the age groups. The locomotor results are consistent with previous studies from our laboratory that have shown a gradual increase in locomotor activity in novel environments from early adolescence to adulthood, and also an increase in the time spent in relatively more aversive areas of novel apparatus with age (Lynn & Brown, 2009, 2010). The increase in time spent in contact with objects across age groups could result from the increase in locomotor activity and/or a decrease in anxiety-like response with age. Given that the increase in time spent in contact with the objects with age was still present when locomotion was used as a covariate, the age difference is more likely to result from a decrease in anxiety-like responses from adolescence to adulthood. The decrease in time spent

interacting with objects and time spent locomoting between Trials 1 and 2 could result from habituation effects or from physical tiredness in subjects. The decrease in these measures was greatest in the younger age groups (pnd 28 and 40), suggesting that habituation or tiredness effects were most pronounced in adolescents.

Interactions between the developing gonadal hormone system and dopamine neurotransmitter system could potentially underlie the sex differences in novelty-preference observed in mid-adolescent rats. During adolescence, gonadal hormones levels are increasing due to pubertal changes, and evidence from adult rodents suggests that gonadal hormones influence NOR performance. For example, adult gonadectomized male rats given testosterone spent more time with the novel object in a NOR task compared with those given estradiol supplements and those without replacement hormones (Aubele, Kaufman, Montalmant, & Kritzer, 2008). An additional difference between males and females during adolescence can be found in the dopaminergic system. Males show a much larger increase in dopamine receptor density, specifically D_2 receptors, up to pnd 40 compared with females, along with a subsequent pruning to adulthood (Andersen & Teicher, 2000). Although D4 receptor density has a similar developmental change in males (Tarazi & Baldessarini, 2000), a similar study has not been done in females. In one study, D4 receptor knockout mice spent less time exploring novel objects than wild-type mice (Dulawa, Grandy, Low, Paulus, & Geyer, 1999), suggesting a link between D4 and novelty-seeking behaviour. Alternately, rather than the adolescent sex-difference in novelty-preference behaviour being attributable to changes in males, it could be that physiological differences in females are suppressing the expression of this behaviour.

Our study has shown that adolescent male rats exhibit a particularly strong preference for novelty during mid-adolescence compared both to females and to

males of other ages. While adolescence in rats encompasses the range from weaning, pnd 21 to early adulthood, pnd 60, the onset of puberty typically occurs during mid-adolescence, from pnd 33-44, and is characterized by an increase in the release of gonadal hormones as in humans (Becú-Villalobos et al., 1997; Spear, 2000; Tirelli et al., 2003). Therefore, our next study will examine the role of gonadal hormones in mid-adolescent rats (pnd 40) in sex differences on the NOR task.

Chapter 5: Influence of gonadal hormonal levels in novel object recognition in mid-adolescence

5.0 Introduction

One of the more recognized signs of adolescence is the onset of puberty, triggered by the circulation of gonadotrophin-releasing hormone (GnRH), which by stimulating the release of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH), leads to the production of testosterone in males and oestrogen in females (Sisk & Foster, 2004). Studies in humans linking sex differences in sensation seeking to gonadal hormones levels have had mixed findings. Sensation seeking and pubertal stage were positively associated in two studies, either in both male and female adolescents (age 11-14 years) (Martin et al., 2002) or only in males (age 10-30 years) (Steinberg et al., 2008). Daitzman et al. (1978, 1980) found a positive association between androgens and estrogens in young adult males (age 17-23 years) and the Disinhibition (Dis) subscale of the Sensation Seeking Scale (SSS; Zuckerman, 1994). In adult females (mean age 31 years), Balada et al. (1993) found an 'inverted U shape' relationship with FSH and total SSS score, as well as lower scores on the Thrill and Adventure Seeking (TAS) subscale of the SSS in participants with higher levels of 17β–estradiol. However, a more recent study did not find a relationship between testosterone and sensation seeking in either adult males (mean age 22 years) or females (mean age 22 years), but did find an inverse relationship with cortisol and sensation-seeking in males (Rosenblitt et al., 2001). Dabbs et al. (1990) also did not find a correlation between SSS-Dis and testosterone in either adult male or female undergraduate students (ages not reported).

Novelty seeking behaviour in rodents has been compared with sensation seeking in humans as similar biological systems are activated in animals exposed to novel situations (Bardo, Donohew & Harrington, 1996; Dellu et al., 1996). In our previous experiments, we examined sex differences in the performance of adult Lister hooded rats on the NOR task, and found no difference in novel object preference between males and females when short (2-minute) inter-trial intervals were used. These findings were in agreement with other studies examining sex differences in adult rodents in NOR (Ghi, Orsetti, Gamalero & Ferretti, 1999; Ricceri, Colozza & Calamandrei, 2000; Sutcliffe, Marshall & Neill, 2007). We then examined the ontogeny of the response to novel objects in male and female rats at three different ages (early adolescence: pnd, 28; mid-adolescence: pnd 40; early adulthood: pnd 80) and found that mid-adolescent males exhibited an increased preference for the novel object compared to males of other ages and to mid-adolescent females (Cyrenne & Brown, 2011).

The aim of this next study was to examine the influence of gonadal hormones in the sex difference found in mid-adolescent animals in the response to novel objects on the NOR task. In rats, the onset of puberty typically occurs during mid-adolescence, from pnd 33-44, and is characterized by an increase in the release of gonadal hormones (Becú-Villalobos et al., 1997; Spear, 2000; Tirelli et al., 2003). As discussed in the previous chapter, changes to the dopaminergic system also occur during mid-adolescence, with dopamine receptor densities increases observed in male rats, but not in females (Andersen & Teicher, 2000; Nair & Mishra, 1995; Tarazi and Baldessarini, 2000). However, Andersen and colleagues (2002) found that gonadectomy did not affect the overproduction and subsequent pruning in males, and ovariectomy did not increase receptor production in female rats, suggesting receptor

density changes were independent of changing hormone levels in adolescence. Alternately, given that gonadal hormone receptors, especially androgen receptors, are present in dopaminergic neurons, gonadal hormones could directly mediate dopaminergic processes (Ernst, Romeo & Andersen, 2009; Kritzer & Creutz, 2009; Kuhn et al., 2010). As performance on the NOR task in adult rodents has been associated with changes in dopamine levels (Dellu et al., 1996; Dere et al., 2007), changes in gonadal hormones during development could contribute to the sex difference we found in mid-adolescent animals, when male rats showed higher levels of novel object preference than females..

Several rodent studies have examined the link between gonadal hormone levels during adolescence and activity levels, social interaction, aggression and even sensory systems (Beatty, 1979; Broida & Svare, 1984; Lipska & Weinberger, 1994; Palanza et al., 2001; Pellis, Pellis & Kolb, 1992; Primus & Kellogg, 1990). However, few studies have examined the association of gonadal hormones to novelty preference as measured in the NOR task, and most of those were in ovariectomized (OVX) adult females rats while investigating the effect of hormones on memory (e.g. Frye, Llaneza & Walf, 2009; Luine, Jacome & MacLusky, 2003; Wallace, Luine, Arellanos & Frankfurt, 2006). Using a variation of the NOR task designed by Ennaceur and Delacour (1988), in each study a rat was first exposed to two identical objects during a training or sample trial (T1) for a period of 3-minutes. Following an inter-trial interval of 4 hours, the animal was reintroduced to the testing apparatus for an additional 3-minute trial (T2), where one of the objects had been replaced by a novel object.

In one lab, NOR responses were investigated in adult female Long Evans rats (approx. pnd 55 when received), OVX 1 week prior to behavioural testing, after

administration of hormones or vehicle immediately after (T1). All the animals given estradiol, progesterone, oestrogen receptor agonists or a progesterone metabolite, but not the animals given vehicle only, exhibited a preference for the novel object in T2 (Frye et al., 2009; Walf, Rhodes & Frye, 2006). Similar results were found in a different lab which examined the effects in female Sprague Dawley rats, age 55-60 days old when received, that had been ovariectomized either by the vendor (Harlan Sprague Dawley, Inc., Indianapolis, IN) one week prior to delivery (Inagaki et al., 2010; Jacome et al., 2010; Luine et al., 2003), or after an initial NOR test in order assess pre-surgical performance on the task (Wallace et al., 2006). While novelty preferences were observed prior to surgery, deficits were seen in only OVX animals post-surgery. Novelty preferences were observed in animals given oestrogen replacements, and oestrogen receptor β (ERβ) agonists, but the effects were found to be time-sensitive (i.e. not effective at 2-hours post T1) and dose dependent. The control animals given vehicle only did not exhibit a preference for the novel object over the familiar object in any of the conditions (Inagaki et al., 2010; Jacome et al., 2010; Luine et al., 2003).

In studies of object recognition memory, OVX females given hormone replacement generally displayed a stronger preference for the novel object over the familiar object in the NOR task compared to control animals (OVX females administered vehicle only) in which novelty preference response was absent. However, in these studies, only the effects of oestrogen or progesterone replacement, and oestrogen receptor agonists, not testosterone, were considered, and neither performance in castrated males nor sex differences were examined.

Although gonadectomized (GDX) male rats have shown deficits in spatial memory and extra-dimensional set-shifting on operant tasks testing measuring

cognitive function (Kritzer et al., 2007), only one study has examined the effects of gonadectomy and hormone replacement on novel object recognition (Aubele, Kaufman, Montalmant & Kritzer, 2008). Four groups of adult, male Sprague Dawley rats (weight 200-250g at surgery) were either gonadectomized (n = 8, GDX), gonadectomized and given testosterone propionate (n = 7, GDX-TP), gonadectomized and given 17β–estradiol (n = 8, GDX-E) or received a sham-operation as the control group (n = 8). Following a 21-day recovery period, the animals were tested in the NOR task (as previously described) with an ITI of 1.5 hours, then, 2 days later, retested in the NOR with a 4 hour ITI. Although the trials were for 3-minutes, analyses were spit into 90-second periods. During the first 90 seconds of T2 following the 1.5-hour ITI, a preference for the novel object over the familiar object was displayed by control and testosterone-supplemented animals, but not in those left untreated or treated with estradiol. There were no differences between the groups during the second 90-second period. In the NOR task T2 after a 4-hour ITI, there were no significant differences between the groups, although GDX males in the first 90 second block did not display a preference for the novel object. Unlike the studies in females, the results with males suggest that novel object preference is androgen sensitive but oestrogen insensitive (Aubele et al., 2008). However, in prior studies, improvements in cognitive tasks due to testosterone or estradiol replacement in gonadectomized male rats have been task dependent, and therefore, the hormones may be acting on different cognitive areas (Gibbs, 2005).

Researchers examining the influence of gonadal hormones on behaviour typically gonadectomize animals, then compare the behaviours of animals treated with gonadal hormones to those left untreated. As comparisons are typically between gonadectomized animals, whether treated or untreated, intact animals are less

frequently considered. However, there are several limitations in using castration or ovariectomy to study the influence of hormones in adolescent rats. Any surgery on animals requires recovery time, for example 21 days in Aubele's (2008) study, which would hinder the ability to test behaviour during the desired developmental period of mid-adolescence. Additionally, although gonadectomy eliminates steroidal hormone release from the testes and ovaries, steroid-independent mechanisms can still influence production of GnRH, and thus LH and FSH, potentially affecting behaviour (Ojeda & Urbanski, 1994; Sisk & Foster, 2004; Urbanski & Ojeda, 1987). An alternative method, chemical castration through GnRH antagonism, allows for the cessation of androgens and oestrogens, as well as LH and FSH, and is reversible if needed (Habenicht, Schneider & El Etreby, 1990; Wallen et al., 1991). A sufficient dose of Antide administered prior to the onset of puberty is effective at stopping hormone production through mid-adolescence (Habenicht et al., 1990).

In this study, we examined sex differences in the response to novel objects on the NOR task in mid-adolescent rats, pnd 40, after administering a GnRH antagonist, Antide, at pnd 28, prior to the onset of puberty. In the previous experiment, sex differences in the NOR task were not evident prior to puberty, but were present at mid-adolescence. If the responses to novel objects are influenced by gonadal hormones, the results expected are either that males treated with Antide will show lower levels of novelty preference, comparable to control females, or females treated with Antide will exhibit an increase in novelty preference, comparable to control males. As with previous testing NOR testing, the ITI was kept at 2-minutes, which would reduce the effect of differences in memory (see chapter 3). In addition to collecting data on interactions with the objects during the first and second trials, we

collected data on locomotor activity in the arena, as age and sex differences in locomotion could potentially influence object interactions.

5.1 Antide study

5.1.0 Methods

Subjects and housing

The subjects were 24 male and 24 female Lister-hooded rats bred in-house from stock supplied by Harlan, U.K. All animals were housed in cages (measuring 25cm x 45cm x 15cm) with *ad libitum* access to soy-free rodent pellets and water. Housing rooms were controlled for temperature (20 ± 1ºC) and humidity (55 ± 5%), and maintained on a 12-hour light:dark cycle (lights on 7am). From postnatal day (pnd) 17, pups were handled once per day and were weaned into same-sex sibling groups at pnd 21. At pnd 28, animals were housed as same-sex sibling pairs.

The subjects were taken from 16 litters, with no more than one individual in each experimental group taken from a single litter. All appropriate guidelines and requirements were adhered to, as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85-23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986.

Experimental design

On pnd 28, experimental animals (12 males, 12 females) were treated with a gonadotrophin releasing hormone (GnRH) antagonist, Antide (Bachem Distribution Services, Germany; dissolved in 1:1 mixture of propylene glycol:saline) via subcutaneous injection and administered in a volume of 2 ml/kg body weight to achieve a final dose of 6 mg/ml per animal (based on Habenicht et al., 1990;

Takeyoshi et al., 2002). Control animals (12 males, 12 females: cage-mates of Antide-treated subjects) were administered with a subcutaneous injection of the vehicle solution at pnd 28. For all subjects, body weight and ano-genital distance were measured at pnd 21, 28, 35 and 40. Ano-genital distance (AGD) was measured from the centre of the anus to the centre of the genital orifice, by use of calipers. AGD is greater in males, lengthens in both sexes during development, and has been positively associated with levels of androgens (Clemens, Gladue & Coniglio, 1978). Behavioural testing was conducted on pnd 40. Immediately after testing, subjects were humanely euthanized with an inter-peritoneal (i.p.) injection of sodium pentobarbital ('Dolethal', Univet Ltd., Bicester, Oxon, UK; 200 mg/ml). Male testes, from the scrotal sac if descended or abdominal cavity if undescended, and female uterine horns from the abdominal cavity were removed and weighed (see **Figure 23** in Results for comparison). Blood for hormonal analysis was also collected at this time, by means of cardiac puncture just prior to transcardial perfusion. Blood samples were allowed to clot for at least 4 hours, before the samples were spun in a centrifuge (10 minutes) and the serum aliquoted into tubes. The serum was stored at -80˚C prior to assay.

Hormone assays

Serum samples from male subjects were analysed using a testosterone ELISA assay kit (Assay Designs, Enzo Life Sciences, U.K.). Samples were diluted (1:10) and run in duplicate. This kit has a lower limit of detection of 5.67 pg/ml, an inter-assay coefficient of variation of 11.3%, and an intra-assay coefficient of variation of 10.0%. Serum samples from female subjects were analysed using a progesterone ELISA assay kit (Assay Designs, Enzo Life Sciences, U.K.). Samples were diluted (1:100)

and run in duplicate. This kit has a lower limit of detection of 8.57 pg/ml, an inter-assay coefficient of variation of 8.3%, and an intra-assay coefficient of variation of 5.4%. Pilot assays were completed with serums samples from a previous study in this lab in order to learn technique and assure assays were sensitive to testosterone and progesterone levels of rats. Assays for this study were completed by Dr. Gillian Brown to ensure accuracy of results, as serum quantities were limited.

Apparatus

The testing apparatus was a wooden, light grey-painted square chamber (67cm x 67cm x 45cm, l x w x h) with a solid floor constructed of the same material. Five objects (yellow rubber toy, glass jar filled with rocks, blue plastic bottle filled with sand, orange plastic toy watering can, multi-coloured LEGO® Duplo blocks tower) of similar size (approximately 15cm high x 6cm diameter) were used in the experiment. Objects were chosen that would deter climbing and chewing. A pilot study with male and female adult rats showed that, from a range of objects, the amount of time spent interacting was very similar for all of these items. The chamber was surrounded by a black curtain, and a video camera attached to the ceiling relayed images to a computer. All tests were conducted between 09:00-14:00 hours in the same testing room under dim, white light (approximately 25 lux), and a white noise generator was used to mask external sounds.

Behavioural testing

At the beginning of a session, a subject was brought to the testing room in a carrying box (42cm x 26cm x 13cm, l x w x h) and placed into the empty apparatus for a 10-minute familiarization session. The animal was then returned to the carrying box for

a period of 2 minutes while the apparatus was cleaned with a 70% ethanol solution and allowed to air dry. Two objects were placed into the apparatus in adjacent quadrants, 15cm apart and 8cm from the wall (**Figure 22**), after which the animal was placed into an empty quadrant, facing away from the objects. During this session, Trial 1, which lasted 5 minutes, the subject had the opportunity to interact with the two objects. At the end of Trial 1, the animal was again removed to the empty carrying box for an inter-trial interval of 2 minutes, during which the apparatus and objects were cleaned with the ethanol solution and one of the objects was replaced by a novel object. The animal was then reintroduced to the apparatus and allowed to interact with the objects for 5 minutes, Trial 2. The object that remained from the first trial was considered the familiar object, and the new object was considered the novel object. At the end of Trial 2, the subject was immediately returned to the home cage, and the apparatus and all objects were cleaned with the ethanol solution in preparation for the next subject. The objects used in each trial were counterbalanced across subjects and between treatment groups, and whether the left-hand or right-hand object was replaced in Trial 2 was also counterbalanced.

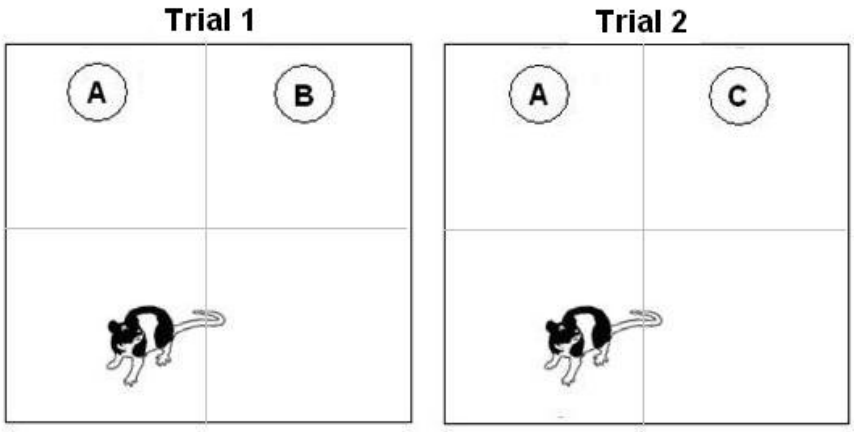

Figure 22. A diagram of object position and animal placement in the novel object recognition task during Trial 1 and Trial 2. Grey lines denote quadrant boundaries but are not present in the apparatus.

Behavioural measures

All sessions were digitally recorded direct to a computer and were analysed using EthoVision XT 5.0 software (Noldus Information Technology, Netherlands, 2008). During Trials 1 and 2, the software recorded the amount of time spent moving by the subject. By delineating an area around each object (an additional 2cm beyond the object) and by tracking the position of the animal's nose, the software was also able to calculate the time spent interacting with each object during Trials 1 and 2 (walking past the object, backing into an object and tail-only contact were thus excluded). We confirmed that the EthoVision measure of time spent interacting with an object strongly correlated with data collected by a human observer ($r_{99} = .74$, $p < .001$). Therefore, for this experiment, the data from EthosVision was used for all analyses.

Time spent with the novel and familiar objects in Trial 2 was converted to a measure of preference for novelty, calculated as the proportion of time spent interacting with the novel versus the familiar object in Trial 2, converted to a percentage [(Time with novel – Time with familiar)/(Time with novel + Time with familiar)*100]. A positive value indicates a preference for the novel object, while a negative value indicates a preference for the familiar object, and a score of zero indicates equal preference for the two objects. Any animal that did not exhibit a minimum of 5 seconds of total contact with the objects in Trial 1, and at least 1 second contact with either object in Trial 2, was excluded from the study. No animals were removed based on these criteria.

Statistical analyses

T-tests and repeated-measures analyses of variance (ANOVAs) were used to analyse hormone data and physical measurements. Repeated-measures ANOVAs were used to examine behavioural data, with sex and treatment group as independent variables and trial as the repeated measure. Three-way interactions were only reported when significant. One-sample *t*-tests were used to examine whether subjects showed a significant preference for the novel object in Trial 2 (preference values were compared to zero, indicating no preference). Pearson's correlations were used to examine relationships between the behavioural measures and, where significant, analyses of co-variance (ANCOVAs) were carried out. To examine whether individual responses to objects influenced preference scores, object identity was included as a random factor in the analyses. An α value of .05 was used for all comparisons, and Bonferroni pairwise comparisons were used to investigate sex and treatment group differences. All data were analysed using SPSS 17.0 for Windows (2009) software package. Effect size (partial-eta squared, η_p^2) and power (β) values for ANOVAs were calculated by SPSS, and Cohen's *d* and power for *t*-tests were calculated with G*Power (Version 3.0.8).

5.1.1 Results

Hormone levels and physical measures

Serum testosterone levels were significantly lower in Antide-treated males (mean ± *SEM*: .60 ± .04 ng/ml) than control males (1.82 ± .42 ng/ml; $t_{22} = 5.00$, $p < .001$, $d = 2.04$, $\beta = 1.00$), and serum progesterone levels were significantly lower in Antide-treated females (.58 ± .23 ng/ml) than control females (12.30 ± 2.76 ng/ml; $t_{22} = 6.15$, $p < .001$, $d = 2.51$, $\beta = 1.00$). Testes weights were significantly lower in Antide-

treated males (.22 ± .05g) than control males (1.24 ± .04g; t_{22} = 15.44, p < .001, d = 6.30, β = 1.00), and uterine weights were significantly lower in Antide-treated females (.09 ± .02g) than in control females (.21 ± .03g; t_{22} = 2.95, p = .007, d = 1.20, β = .80; **Figure 23**).

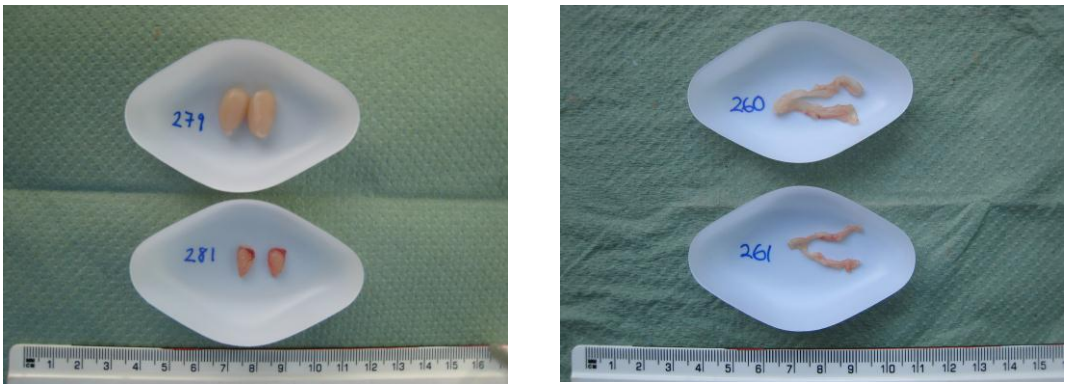

Figure 23. Comparison of Antide treated (bottom in both pictures) and control (top) male testes (left) and female uterine horns (right) at posnatal day 40.

There was a significant interaction between sex, age and treatment group for ano-genital distances ($F_{3, 132}$ = 9.15, p < .001, η_p^2 =.17, β = 1.00). Antide-treated males had smaller ano-genital distances at pnd 35 (mean ± *SEM*: 19.60 ± .33 mm) and pnd 40 (22.5 ± .54 mm) than same-aged control males (pnd 35 = 23.39 ± .83 mm; pnd 40 = 27.42 ± .61 mm; *p*s < .001), but not at pnd 21 or 28, i.e., before treatment (data not shown; *p*s ≥ .158). Antide-treated females and control females did not differ in ano-genital distance at any age (data not shown; *p*s ≥ .586).

For body weight, there was a significant interaction between sex, age and treatment group ($F_{3, 132}$ = 11.26, p < .001, η_p^2 = .20, β = 1.00). Control males were heavier than Antide-treated males at pnd 40 (p = .034), but not at any other ages (*p*s ≥ .435). Antide-treated females did not differ from control females at pnd 40 (p = .103)

or any other ages ($ps \geq .511$). Control males were heavier than control females at pnd 35 ($p = .006$) and pnd 40 ($p < .001$) only (other ages, $ps \geq .343$; **Table 5**).

Table 5

Means and standard error of mean of animal weights, in grams, by sex, age and treatment group (n = 12 per group).

	Males		Females	
Age	Control	Antide	Control	Antide
PND 28	60.01	59.45	57.37	57.44
	(2.21)	(2.21)	(1.80)	(1.50)
PND 35	100.42	97.33	89.18	91.77
	(3.55)	(2.74)	(2.25)	(2.33)
PND 40	136.36	125.41	111.58	119.92
	(4.48)	(4.03)	(2.51)	(2.75)

Note. Numbers in parentheses are standard error of mean.

Locomotion

Between the two trials, there was a significant increase in the duration of movement from Trial 1 to Trial 2 ($F_{1, 44} = 45.68$, $p < .001$, $\eta_p^2 = .51$, $\beta = 1.00$; **Figure 24**), with all subjects spending almost twice as long moving in Trial 2 compared to Trial 1. There were no significant differences by either sex or treatment group between the trials ($Fs_{1, 44} \leq .06$, $ps \geq .815$), nor did the interaction of both differ by trial ($F_{1, 44} = .06$, $p = .812$; **Table 6**). Overall movement duration for both trials also did not differ by either sex or treatment group ($Fs_{1, 44} \leq .57$, $ps \geq .456$), nor the interaction of both ($F_{1, 44} = .05$, $p = .830$).

Although the interaction of sex and treatment group did not differ by trial, in Trial 2 the pairwise comparisons indicated there was a significant difference between the Antide treated and control males in movement duration ($F_{1, 44}$ = 6.28, p = .016, η_p^2 = .13, β = .70), with control males spending more time moving than Antide treated males.

Total amount of contact with objects

Total amount of time spent in contact with the object did not differ between trials ($Fs_{1, 44}$ = .07, p = .796), and there were no significant main effects of sex ($Fs_{1, 44}$ = .12, p = .736) or treatment group ($Fs_{1, 44}$ = .03, p = .871). All interactions were also non-significant (treatment group and trial: $Fs_{1, 44}$ = .50, p = .485; treatment group and sex: $Fs_{1, 44}$ = .92, p =.343; trial and sex: $Fs_{1, 44}$ = .26, p = .615; **Figure 24**). Subjects spent around 110-130 seconds interacting with objects during each 5-minute trial (**Table 6)**.

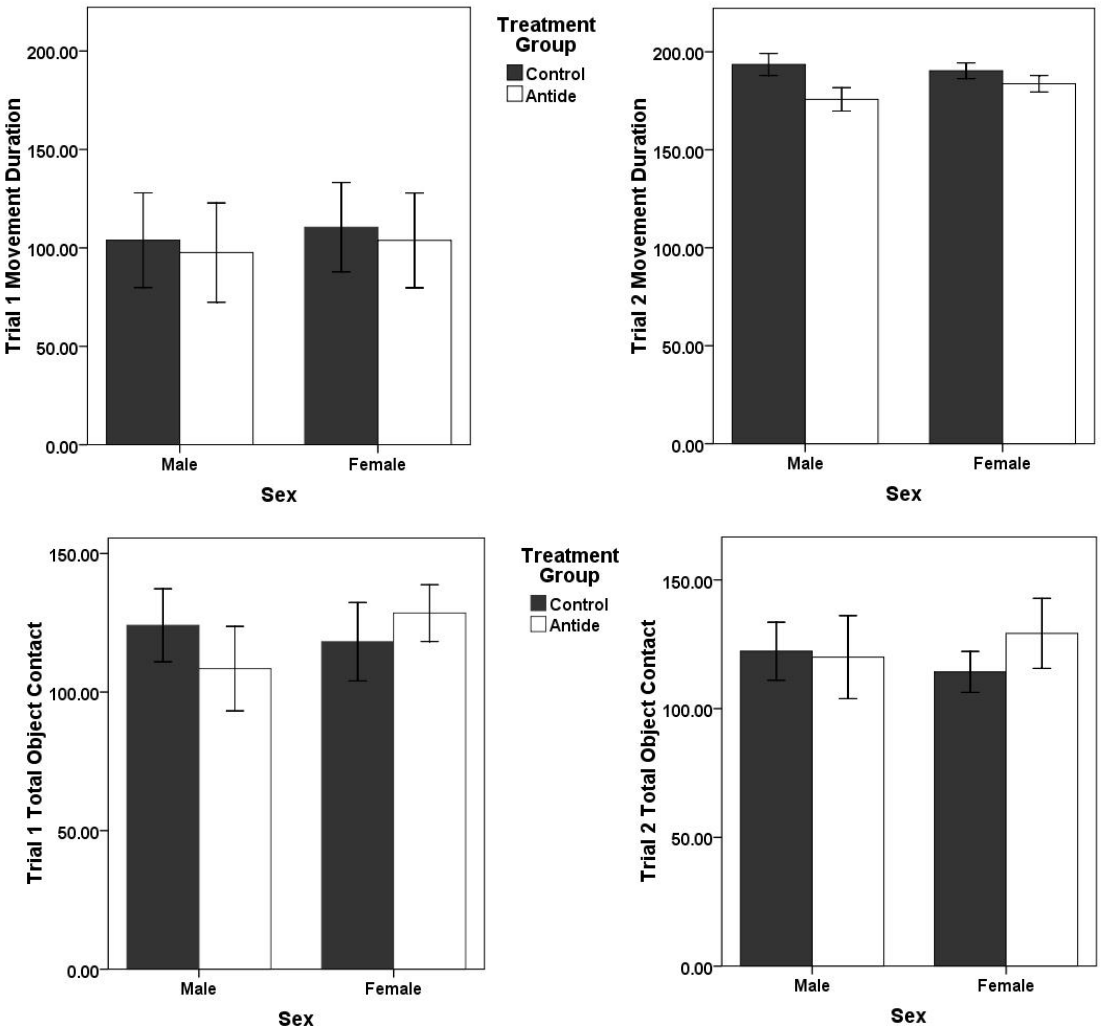

Figure 24. **Top:** Mean movement duration, in seconds, and **Bottom:** Mean total object contact in Trial 1 and Trial 2, in seconds, by sex and treatment group. Error bars represent +/- 1 standard error of the mean.

Preference for novelty

When all subjects were combined, a one-sample *t*-test indicated that animals did show a significant preference for the novel object ($t_{47} = 6.74$, $p < .001$, $d = .97$, $\beta = 1.00$), spending approximately 68.5% of the time with the novel object during the novel object trial, or approximately 37% more time with the novel object than with the familiar object. When each group was examined separately, a significant preference for the novel object was indicated in three groups: Antide males ($t_{11} = 2.34$, $p = .039$,

d = .67, β = .57), control males (t_{11} = 7.08, p < .001, d = 2.04, β = 1.00), and Antide females (t_{11} = 3.92, p = .002, d = 1.13, β = .95), with a trend toward a significance preference in control females (t_{11} = 1.99, p = .073, d = .57, β = .44).

A Pearson correlation revealed no significant relationships between movement duration and total object contact with novelty-preference ($rs_{48} \leq .18$, $ps \geq .220$), therefore no measures were considered as covariates when examining for group differences. There were also no significant side biases present in either of the two object trials ($ts_{48} \leq 1.01$, $ps \geq .319$), which indicated animals had no biased preference for either the left or right objects in T1 and T2 based on the location of the object in the apparatus, so no correction was needed to preference scores to compensate for biases.

The ANOVA to examine group differences in novelty-preference indicated a significant interaction of sex with treatment group ($F_{1, 44}$ = 4.84, p = .033, η_p^2 = .10, β = .58). A significant sex difference consistent with prior research was found in the control animals (p = .015), with males exhibiting greater novelty-preference than females. No sex difference was found between the Antide treated animals (p = .573). In males, control animals displayed significantly higher novelty-preference than those treated with Antide (p = .027), but there was no difference between control and Antide treated females (p = .416). Neither main effect of treatment group ($F_{1, 44}$ = 1.08, p = .305) or sex ($F_{1, 44}$ = 1.95, p = .169) indicated significant differences between groups (**Figure 25**; **Table 6**).

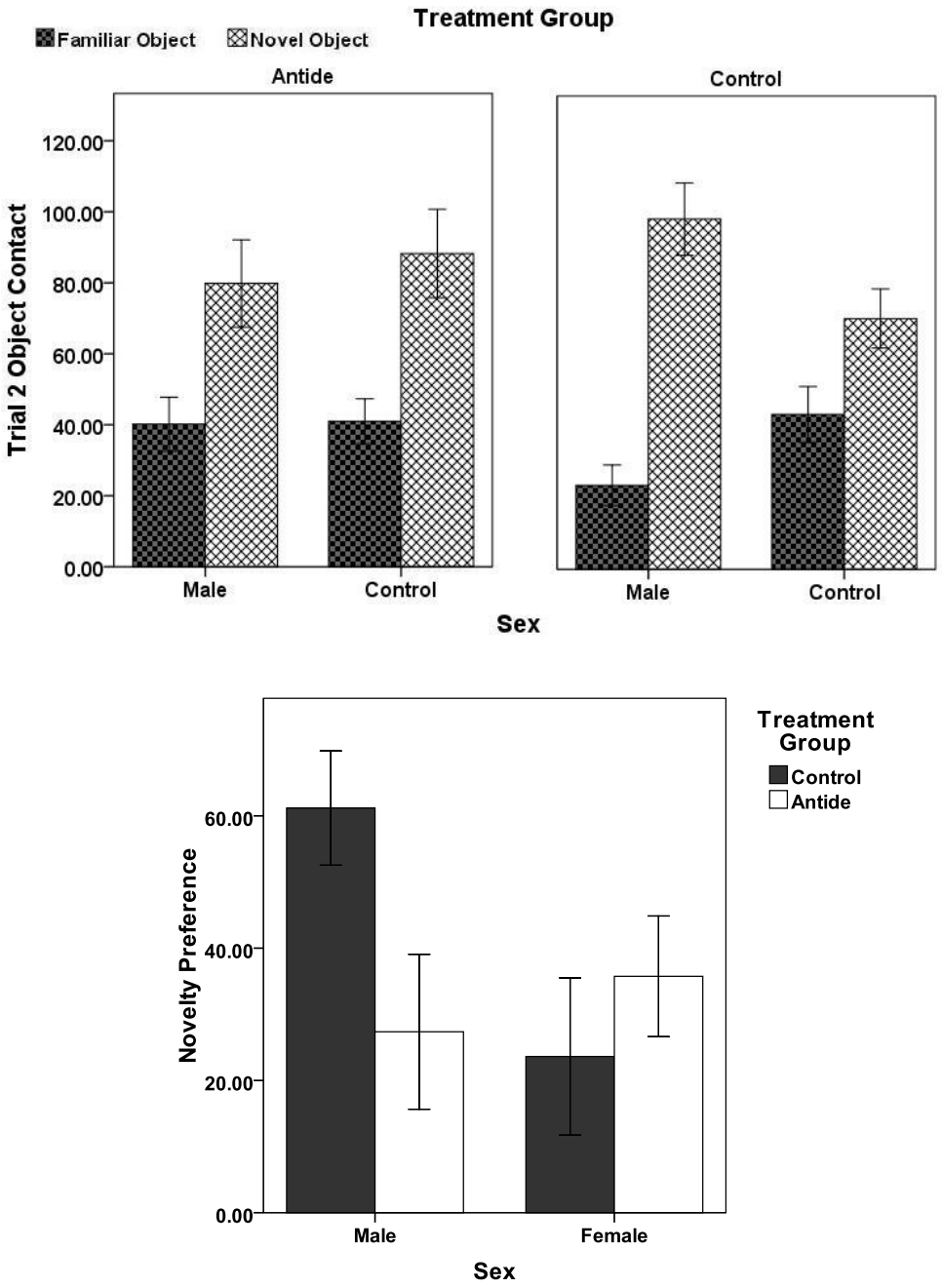

Figure 25. **Top:** Mean contact time, in seconds, with Familiar and Novel objects in Trial 2 by sex and treatment group. **Bottom:** Mean novelty preference by sex and treatment group. Error bars represent +/- 1 standard error of the mean.

Table 6

Means, in seconds, and standard error of mean of behavioural measures and preference scores by sex and treatment group (n = 12 per group).

	Males		Females	
	Control	Antide	Control	Antide
Movement Duration				
Trial 1	103.91 (24.03)	97.61 (25.24)	110.53 (22.69)	1103.82 (24.08)
Trial 2	193.56 (5.61)	175.78 (5.95)	190.35 (4.00)	183.74 (4.21)
Total Object Contact				
Trial 1	124.09 (13.18)	108.44 (15.25)	118.17 (14.16)	128.50 (10.24)
Trial 2	122.31 (11.29)	120.01 (16.10)	114.24 (7.95)	129.25 (13.58)
Trial 2 Novel Object Contact	98.67 (10.21)	79.83 (12.31)	70.65 (8.33)	88.24 (12.48)
Trial 2 Familiar Object Contact	23.65 (5.75)	40.18 (7.61)	43.61 (7.88)	41.01 (6.35)
Novelty Preference	61.18 (8.64)	27.36 (11.71)	23.62 (11.89)	35.74 (9.12)

Note. Numbers in parentheses are standard error of mean.

5.2 Discussion

The present study examined sex differences in the response to novel objects on the NOR task in mid-adolescent rats, pnd 40, after administering a GnRH antagonist, Antide, at pnd 28, prior to the onset of puberty. The sex difference found in the previous study at pnd 40 was replicated in this study, as control males displayed higher novelty-preference than control females. However, novelty-preference was lower in males treated with the GnRH antagonist, Antide, compared with control males. A similar effect of treatment was not observed in the female rats. Antide

treatment animals spent somewhat less time in motion during the novel object trial, but this did not influence object contact, as there was no effect of treatment on object interaction. Treatment did not affect any other behavioural measures. The lack of sex differences in other behavioural measures was consistent with prior findings for this age group (Lynn & Brown, 2009, 2010).

A significant increase in locomotion from Trial 1 to Trial 2 was observed in all groups, a result opposite to our prior findings in the NOR task. However, since the duration of movement in Trial 2 was comparable to the prior study with pnd 40 animals, the difference is an indication of decreased movement in Trial 1. Although the housing conditions were consistent in both studies, all the animals in this study were given a subcutaneous injection, either vehicle or Antide, at pnd 28, twelve days prior to testing, and ano-genital distances were measured at regular intervals. The differences in handling could result in the animals in this study exhibiting an increased fear response through decreased exploration of the apparatus when the objects were first presented in Trial 1 (Hughes, 1997; Montgomery, 1955). Although movement duration was decreased in Trial 1 in this study compared to the previous study of adolescents, total object contact in both Trial 1 and Trial 2 was comparable to our prior findings in pnd 40 animals.

The physical effects of treatment were apparent in blood serum testosterone and progesterone levels as well as in post-mortem gonadal weights. Both sexes had underdeveloped reproductive organs as a result of absent luteinising hormone (LH) and follicle-stimulating hormones (FSH), leading to suppression of testosterone in males, and oestrogen in females, thereby arresting puberty. Additionally, after administration of the drug, there was less sexual dimorphism in body weights,

indicative of the potential influence of gonadal hormones on factors affecting weight change during adolescence (Gabriel, Roncancio, & Ruiz, 1992).

Previous research found that novel object preference in the NOR task was absent in both ovariectomized female (e.g. Frye et al., 2009; Luine et al., 2003; Walf et al., 2006) and castrated male rats (Aubele et al., 2008), which was reversed by hormone replacement. In contrast, in this study Antide treated animals of both sexes continued to show a preference for the novel objects during the test trial, as did the control animals (although only a trend in control females). Differences in the experimental designs could contribute to the dissimilar findings between studies, as a longer ITI was used in studies of gonadectomized animals (1.5 to 4 hours) rather than a short ITI (2-minutes) in this study. A short ITI was utilized in this study in order to reduce the effects of differences in memory performance so that performance in the NOR task could be attributed to novel preferences rather than memory ability.

A difference that should be noted between this study and prior studies is the amount of time the subjects interacted with the objects during both the learning trial (T1) and the test trial (T2). Total object exploration in T1, when noted, ranged from 4.7 ± 1.0 seconds (Walf et al., 2006) to 14.13 seconds (Inagaki et al., 2010) in a 3-minute trial, or a maximum of 16% of trial time spent exploring objects; whereas total exploration time in this study was greater than 100 seconds for each group in a 5-minute trial, or at least 34% of the total trial time. In T2, novelty preference ratios in prior studies were calculated based on interaction time with novel object over familiar object in the 3-minute trial with total contact as low as 6 seconds (e.g. 5.7 ± 1.3 novel vs. 1.2 ± .3 familiar, Frye et al., 2009). In this study, total object contact did not decrease from T1 to T2, remaining over 100 seconds in the 5-minute trial. Ennaceur and Delacour (1988) found that a preference for novel objects was dependent upon

the amount of contact the animals had with the objects. Normal adult males did not exhibit a novelty preference after a 4-hour ITI when total exploration time was limited to 20 seconds (avg session length approx. 167 sec.) (Ennceur & Delacour, 1988). Therefore, although there were design differences in trial lengths and inter-trial intervals, more likely there were additional factors contributing to the discrepancies in object exploration, perhaps locomotor or attentional deficits as a result of gonadectomy.

Deficits on the NOR task with a long ITI in adult gonadectomized animals could be a result of differences in activating oestrogen and androgen receptors in the hippocampus, a neural area implicated in aspects of memory formation (Aggleton & Brown, 2001). Although lesions to the hippocampus generally have little effect on preference for the novel object in the NOR task (Ainge et al., 2006), animals with partial hippocampal lesions did not display a novelty preference in ITIs beyond 10 minutes when object exploration time was limited to 10 seconds, but did display a preference when contact time was increased. This would support the findings of Ennacuer and Delacour (1988), indicating a minimum amount of object exploration time is required to exhibit novelty preference behaviour, and could contribute to why our GnRH antagonized animals continued to show preference for the novel object, but gonadectomized animals did not.

Age differences between the animals in the gonadectomy studies and this study should also be considered. In the prior NOR studies, subjects were post-pubertal upon ovariectomy or castration, and, therefore, hormone dependent changes would have been at or near completion (Becú-Villalobos et al., 1997; Spear, 2000). Gonadal hormone dependent changes are time-sensitive during development, with neuronal organizational effects occurring postnatally in juveniles and again in early

adolescence (Becú-Villalobos et al., 1997; Debeljuk et al., 1972a, 1972b). The developmental timing of gonadectomy can either affect the physiological structure of the brain if done in prepubertal adolescents (e.g. dopaminergic structures), or the activational effects of gonadal hormones if done on postpubertal adults (e.g. LH and FSH levels on receptors). Therefore, deficits on the NOR task with a long ITI in gonadectomized animals could be a result of differences in activating oestrogen and androgen receptors in the adult hippocampus, whereas the reduction of novelty seeking in the males in our study could be due to a different mechanism.

Since dopamine receptor levels rise and fall more in males than females during mid-adolescence, differences in the dopaminergic system would seem a potential mechanism for our findings (Andersen et al., 1997; Tarazi and Baldessarini, 2000). However, as prepubertal gonadectomy did not influence the dopamine receptor overproduction and subsequent pruning (Andersen et al., 2002) this is an interesting area for further exploration. Since the behavioural effects of neonatal lesions of the frontal cortex and hippocampus were not seen until young adulthood (Flores et al., 1996), perhaps organization of the dopamine receptors is triggered by gonadal hormones earlier in development.

Hippocampal growth during puberty peaks earlier in human females than males before decreasing to adult levels (Bramen et al., 2010; Giedd et al., 1999; Huttenlocher & Dabholkar, 1997). Hippocampal volume is larger in adolescent males, while amygdala volume is greater in females (Bramen et al., 2010; Giedd, 2004; Giedd et al., 1997). Therefore, the decrease in male NOR performance in our study may be a result of the organizational effect of gonadal hormones on the hippocampus. Differences in the organizational effects of hormones have been

associated with sex differences in social behaviours, anxiety-related behaviours and cognition (see Schultz et al., 2009 for review).

This experiment was designed to examine sex differences in the response to novel objects on the NOR task in mid-adolescent rats, pnd 40, after administering a GnRH antagonist, Antide, at pnd 28, prior to the onset of puberty. Novelty-preference was lower in males treated with the GnRH antagonist, Antide, compared with control males, but a similar effect of treatment was not observed in the female rats. These findings support the hypothesis that androgens contribute to the sex difference in response to novel objects in adolescent rats. In order to find the specific mechanism of effect, i.e., if the effect is due to the activational or organizational properties of gonadal hormones, what specific neural area is affected, and if the neural change is time-sensitive, additional research will need to be conducted. The next steps in understanding the sex and age differences include selectively reintroducing gonadal hormone to GnRH antagonized animals to determine the mechanism of the sex difference in mid-adolescent animals. From there, dopamine agonists and antagonists can be administered to examine the influence of dopamine, as well as the interaction of dopamine and gonadal hormones, on response to novelty.

Chapter 6: Conclusion

6.0 Summary of results

The aim of this research was to take a closer look at age and sex differences in sensation seeking and explore the physiological mechanisms that could lead to an understanding of why males, especially adolescents, show higher levels of this behaviour than females. Although data suggest gonadal hormone changes during adolescence and differences in the dopaminergic reward system are the bases for why some people exhibit sensation seeking behaviour while others do not, causal relationships between physiology and behaviour are difficult to establish in humans. Although novelty seeking may be associated only with the TAS and ES aspects of human sensation seeking measures, novelty seeking is also a characteristic of the general sensation seeking trait. Since animal responses to novelty can be measured and compared to human responses to novelty, the behaviour of animals in a novel task can potentially model aspects of human sensation seeking.

Sensation seeking in humans is measured through the use of self-report questionnaires, which have their limitations; however, determining the motivation for animals to explore novel stimuli is more difficult. Based on the review of the various exploratory tests, both 'forced' and 'free', the CPP and NOR tests may be better at eliciting behaviour in animals that is comparable to sensation seeking in humans. Therefore, this research examined animal responses to novelty in two designs, the conditioned place preference test (CPP) and the novel object recognition test (NOR), and assessed the benefits and limitations of each. Additionally, most studies of novelty preferences in rodents examine this behaviour in male, adult animals only, with few examining either the responses in both sexes, or any changes that occur

during development. In order to understand the ontogeny of sex differences in responses to novelty, both males and females were tested from adolescence through adulthood.

While no sex difference in novelty preference was found in adult animals in the NOR test, mid-adolescent males spent a greater ratio of exploration time with the novel object in the NOR test than either younger or older males, or females at each stage of development. The results of these studies agreed with the previous findings in animal studies, and support the hypothesis that novelty preference tests are a successful method to examine intrinsically motivated exploratory behaviour in rodents. Since gonadal hormones levels are elevated beginning at puberty, a GnRH antagonist was used to remove the influence of gonadal hormones in early adolescence before again examining responses on the NOR test at mid-adolescence. Gonadal hormone suppression from early adolescence onwards eliminated the sex difference in the NOR test at mid-adolescence by reducing the male response to novelty, while no difference was measured in the female animals. These findings suggest that gonadal hormones play a role in the development of response to novelty, especially in males.

The rest of this chapter will present an overview of the findings from the studies in the previous chapters, followed by potential application of the research as well as any limitations. Finally, future directions of study will be proposed.

Measuring novelty preference in animals

Behavioural assessments of exploratory behaviour in animals can be divided into two basic categories: 'forced' tasks that require entry to a novel environment or measure interaction with a single, novel object, and 'free' tests of exploration that provide the

animal a ‘choice’ of novel versus familiar objects or environments (Welker, 1957). Although ‘forced’ novelty tests may be useful measures of locomotion, it is difficult to ascertain if the animal’s behaviour was a result of intrinsic motivation to explore the environment or just looking for a means to escape due to fear (Hughes, 1997). Of the behavioural tests that offer a ‘choice’ to animals, conditioned tasks are considered to be stronger indicators of the appetitive properties of novelty preference, similar to the rewarding aspects of drugs of abuse leading to addiction (Bevins & Bardo, 1999). Since sensation seeking in humans has been associated with increased levels of substance abuse (Zuckerman, 1994), the conditioned place preference task was selected as the first test to investigate sex differences in adult rats.

Over a series of four experiments, both the apparatus and the experimental design were refined to minimize the influence of extraneous variables to ensure the novel objects were sufficient to elicit place preference conditioning. However, when the change in preference from the familiarization session to the preference test was examined, neither males nor females exhibited a preference for the side paired with the novel object during the conditioning trials. These results did not replicate the findings of prior studies, which were able to measure a novelty induced place preference (Besheer et al., 1999; Bevins & Bardo, 1999; Bevins et al., 2002; Douglas et al., 2003).

In the next series of experiments, the novel object recognition task was assessed for its reliability at eliciting a preference for novel objects over familiar objects in adult rats, while also evaluating sex differences in novelty preference behaviour. While the NOR task has been used in prior studies of both novelty preference and memory, the design of the task varied across studies as to the quantity and type of objects used in each of the trials, the inter-trial-interval lengths, the

duration of both the trials and familiarization sessions, as well as in the number of trials needed to determine if an object preference was present.

A preference for the novel object over the familiar object was observed in adult rats in each of the NOR experiments, with any sex differences, if present, eliminated when differences in total object contact are controlled. No sex differences in the NOR task were expected in adult rats when a 2-minute inter-trial interval (ITI) was used, and thus, our absence of sex differences in novelty preference is similar to the findings of prior researchers (Ghi et al., 1999; Ricceri, Colozza & Calamandrei, 2000; Sutcliffe, Marshall & Neill, 2007). The preference for the novel object over the familiar object in each experiment was also as expected and in agreement with prior studies that used a similar one-trial object recognition design (Ainge et al., 2006; Berlyne, 1950; Ennaceur & Delacour, 1988).

The absence of sex differences in adult animals in the NOR test is comparable with the findings in only one aspect of human sensation seeking studies. In humans, adult sex differences were consistently absent only on the experience seeking subscale of the SSS-V (Ridgeway & Russell, 1980; Wang et al., 2000; Zuckerman et al., 1978; Zuckerman, 1994). Zuckerman (1994, p. 31) describes the experience seeking factor as "seeking of novel experiences and sensations through the mind and senses…", and indicates that this factor had the highest genetic contribution to a general measure of sensation seeking based on the analysis of monozygotic and dizygotic twins as well as environmental interactions (Fulker, Eysenck & Zuckerman, 1980; Jinks & Fowler, 1970; Zuckerman, 1994). While the definition of ES in humans can be compared to novelty seeking in animals, high heritability suggests the possibility of a biological link to both sensation seeking and novelty seeking behaviour.

There are several advantages to using the NOR test to assess novelty preference in rats. The experimental design of this study included a 10-minute familiarization session to habituate the animals to the testing apparatus, two 5-minute testing trials with the objects and an ITI of 2-minutes, thereby keeping the total time required for each testing session, and thus the time the animal is kept out of its home cage, to less than 30-minutes per animal. Additionally, the responses to the objects are due a natural tendency for animals to approach and explore novel objects more than familiar objects, and are not dependent on food or water restriction (Berlyne, 1950; Dember, 1956; Thompson, 1954). Since the next set of experiments would be assessing developmental as well as sex differences in the NOR task, taking less time to complete the testing session decreased the possible influence of naturally occurring developmental changes (e.g. fluctuating gonadal hormone levels during puberty), and the lack of dietary restriction would not affect the normal development process.

Age and sex differences

Evidence for age differences in performance on the NOR task is very sparse and contradictory. In this study, we examined the ontogeny of response to novel objects in both male and female Lister hooded rats in the NOR task with a 2-minute ITI at early adolescence (pnd 28), mid-adolescence (pnd 40) or early adulthood (pnd 80). A preference for the novel object over the familiar object was observed in all the animals, but a significant sex difference occurred only at mid-adolescence (pnd 40), with males showing a higher novelty-preference than females. This sex difference was not present at early adolescence (pnd 28), and, while the opposite pattern of results was observed at early adulthood, the adult sex difference was only present when calculated as preference for novelty, and not when calculated as preference change,

suggesting that the adult sex difference is not robust. In contrast, other measures did not exhibit significant age by sex interactions. The total time spent in contact with objects gradually increased, and latency to contact objects decreased, across the age groups, while neither measure differed between the sexes. These results indicate that the sex difference in behaviour at mid-adolescence was specific to situations involving choice of novelty.

Consistent with prior findings in mice (Calamandrei et al., 2002; Ricceri et al., 2000), our study found that male and female rats from pnd 28 through to adulthood did display a greater amount of time interacting with the novel object over the familiar object. Our finding that mid-adolescent male rats exhibit stronger novelty preference than females had not been reported previously. The sex difference found in this study is comparable to what is found in human adolescents, since several studies have shown males exhibit higher risk taking and sensation seeking behaviours relative to females at this age (Arnett, 1992; Butković & Bratko, 2003; Eysenck, Easting & Pearson, 1984; Zuckerman, Eysenck & Eysenck, 1978).

Influence of gonadal hormones

The previous study of age differences in the NOR task indicated a sex difference in novelty-preferences at mid-adolescence, pnd 40, that was not evident prior to puberty, and, therefore, could have been influenced by pubertal changes in circulating hormone levels. In this study, we examined sex differences in the response to novel objects on the NOR task in mid-adolescent rats, pnd 40, after administering a GnRH antagonist, Antide, at pnd 28, prior to the onset of puberty. The sex difference at pnd 40 was replicated in this study, as control males displayed higher novelty-preference than control females. However, novelty-preference was lower in males treated with

the GnRH antagonist, Antide, compared with control males. A similar effect of treatment was not observed in the female rats. Treatment did not affect any other behavioural measures. The lack of sex differences in other behavioural measures was consistent with our prior findings for this age group as well as other studies in our lab (Lynn & Brown, 2009, 2010). However, sex differences in the NOR task after hormone manipulation during adolescence had not been previously examined.

6.1 Conclusions and future research

The implications of these studies and why the results we found may differ from others on sex and age differences in novelty seeking are discussed in more detail at the conclusion of each chapter. Briefly, considerations for future studies can include the examination of specific neural areas based on the findings that gonadal hormone dependent changes are time-sensitive during development, with neuronal organizational effects occurring postnatally in juveniles and again in early adolescence (Becú-Villalobos et al., 1997; Debeljuk et al., 1972a, 1972b). Perhaps organization of the dopamine receptors is also triggered by gonadal hormones earlier in development, which could further explore the age and differences found in dopaminergic receptors (Andersen et al., 1997; Tarazi and Baldessarini, 2000). An additional area of interest may look further at the gonadal hormone effects on hippocampal and amygdala neurogenesis (Galea et al., 2008).

Throughout this thesis, the discussion of physiological influences on sex and age differences has been approached using the organizational/activational hypothesis (Phoenix, Goy, Gerall, & Young, 1959; Schulz, Molenda-Figueira, & Sisk, 2009). The organizational influences of gonadal hormones are generally understood to be permanent changes that occur either prenatally with sexual differentiation, or during

time-sensitive postnatal windows to masculinize or feminize the brain shortly after birth, and then trigger neuronal changes in adolescence. In puberty and then as adults, the activational effects of gonadal hormones regulate reproductive ability and behaviour. Data suggests organizational effects of hormones result in sex differences in social behaviours, anxiety-related behaviours and cognition (see Schultz et al., 2009 for review).

However, in addition to examining the interaction of gonadal hormones, dopamine and neural structures, and how changes within these areas affect novelty seeking and sensation seeking behaviour, another level to explore is the influence of genetics and epigenetics. The general statistical method of analysing genetic and environmental interactions was developed by and has been used to examine the degree of genetic influence on personality traits such as extroversion (42%), psychoticism (49%), and neuroticism (54%) (Eaves & Eysenck, 1975, 1977; Jinks & Fowler, 1970). An analysis of the genetic and environmental contributions to sensation seeking was performed using the responses of 422 pairs of twins to the SSS-IV. While controlling for mean differences due to age and sex, the researchers found 58% of the general sensation seeking trait was due to heritability. When males and females were considered separately, approximately 70% of the genetic variation in sensation seeking was common to both sexes, while the remaining 30% was due to inherent differences between males and females (Fulker, Eysenck & Zuckerman, 1980; Jinks & Fowler, 1970). Since the time of this study, genetic polymorphisms in dopamine and catechol-O-methyltransferase have been linked to differences in sensation seeking (Chen et al., 2004; Munafò, Yalcin, Willis-Owen & Flint, 2008). Although the results have been mixed, the studies in this area are still few, and could warrant further exploration.

Epigenetics is the study of changes in genes and the areas around the gene that influence expression of the gene by turning it on or off. An example affecting sex differences is in X inactivation in females triggered by SRY. If one of the two maternal X chromosomes is not inactivated, the results could lead to congenital disorders such as Klinefelter syndrome (XXY males), Turners syndrome (X only females), aneuploidy (XYY males) or Trisomy (XXX females). DNA changes can be caused by factors such as environmental influences, varying steroidal hormone levels produced by the gonad or even chemicals such as drugs of abuse. For example, a recent study found that epigenetic changes to oestrogen and progesterone receptors were mediated by estradiol, leading to sexual differentiation (Nugent, Schwarz & McCarthy, 2011). The study of epigenetic changes in the nervous system is an avenue of exploration that is still relatively new, but can help us understand sex differences in the brain and behaviour (McCarthy & Arnold, 2011; McCarthy, et al., 2009).

The aim of this research was to take a closer look at the age and sex differences in sensation seeking and explore the physiological mechanisms that could lead to an understanding of why males, especially adolescents, show higher levels of this behaviour. Based on this research, sexually dimorphic changes during puberty are influenced by gonadal hormones, leading to adolescent sex differences in sensation seeking behaviour, which are not present in adult animals. Additionally, the novel object recognition task was found to reliably elicit a novelty preference in a period of time that would minimize impact on juvenile animals. The next steps in understanding the sex and age differences include selectively reintroducing gonadal hormone to GnRH antagonized animals to determine the mechanism of the sex difference in mid-adolescent animals. From there, dopamine agonists and antagonists can be

administered to examine the influence of dopamine, as well as the interaction of dopamine and gonadal hormones, on novelty-seeking behaviour. The research conducted for this thesis provides a starting point for further research.

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Appendix 1

Publications resulting from the work presented in this thesis:

Cyrenne, D. M., & Brown, G. R. (2011). Ontogeny of sex differences in response to novel objects from adolescence to adulthood in lister-hooded rats. *Developmental Psychobiology*, *53*(7), 670-676. doi: 10.1002/dev.20542

Cyrenne, D. M., & Brown, G. R. (2011). Effects of suppressing gonadal hormones on response to novel objects in adolescent rats. *Hormones and Behavior, 60*(5), 625-631. doi: 10.1016/j.yhbeh.2011.08.015

De-Laine M. Cyrenne
Gillian R. Brown
School of Psychology
University of St Andrews
St Andrews, UK
E-mail: dc284@st-andrews.ac.uk

Ontogeny of Sex Differences in Response to Novel Objects from Adolescence to Adulthood in Lister-Hooded Rats

ABSTRACT: *In humans, novelty-seeking behavior peaks in adolescence and is higher in males than females. Relatively, little information is available regarding age and sex differences in response to novelty in rodents. In this study, male and female Lister-hooded rats were tested at early adolescence (postnatal day, pnd, 28), mid-adolescence (pnd 40), or early adulthood (pnd 80) in a novel object recognition task (n = 12 males/females per age group). Males displayed a higher preference for the novel object than females at mid-adolescence, with no sex difference at early adolescence. Adult females interacted with the novel object more than adult males, but not when side biases were removed. Sex differences at mid-adolescence were not found in other measures, suggesting that the difference at this age was specific to situations involving choice of novelty. The results are considered in the context of age- and sex-dependent interactions between gonadal hormones and the dopamine system. © 2011 Wiley Periodicals, Inc.* Dev Psychobiol 53: 670–676, 2011.

Keywords: *novelty; sex difference; adolescence; rats; novel object recognition*

INTRODUCTION

In human beings, adolescence can be associated with high levels of novelty- and sensation-seeking behavior (Arnett, 1992; Kelley, Schochet, & Landry, 2004; Zuckerman, 2006), and males are reported to engage in more sensation-seeking behavior than females across all age categories (Zuckerman, 2006). Attending to novelty during adolescence potentially allows maturing individuals to gain important information about the environment (Chambers, Taylor, & Potenza, 2003), while sex differences in novelty-seeking may result from sexual selection pressures favoring riskier strategies in males than females (Daly & Wilson, 1983; Spear, 2000). However, the biological mechanisms underlying age and sex differences in novelty-seeking are not well understood. The aim of this study was to examine age and sex differences in response to novelty in laboratory rats.

We used the novel object recognition (NOR) task (Berlyne, 1950; Ennaceur & Delacour, 1988), as this task forces rodents to confront novelty and also provides subjects with the opportunity to choose between a novel and a familiar stimulus. The procedure is to familiarize an animal to a novel arena, then place two objects into the arena and allow the animal to interact with the objects. During this first trial, Trial 1, the subject is ''confronted'' with novelty. One of these objects is then replaced with a completely novel item and, in Trial 2, the animal has the ''choice'' of interacting with the novel versus the familiar object. Rodents generally spend more time interacting with the novel than the familiar object in Trial 2 (Ennaceur & Delacour, 1988; Dere, Huston, & De Souza Silva, 2007).

The NOR task has been used extensively in rodent memory research; for instance, increasing the delay between the first and second trial to several hours has been shown to reduce the difference in response to the novel and familiar objects (Ennaceur & Delacour,

Received 15 September 2010; Accepted 2 February 2011
Correspondence to: De-Laine M. Cyrenne
Contract grant sponsor: Wellcome Trust;
Contract grant sponsor: School of Psychology, University of St Andrews.
Published online 31 March 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/dev.20542

1988; Şik, van Nieuwehuyzen, Prickaerts, & Blokland, 2003). However, the NOR task also allows researchers to investigate the mechanisms involved in novelty preference (Besheer, Short, & Bevins, 2001). Lesions to the mesolimbic dopaminergic system influence NOR task performance, although the effects pharmacological manipulations of the dopamine system are less consistent (Dere et al., 2007; Hughes, 2007; Woolley, Marsden, Sleight, & Fone, 2003). Using a variant of the task with a short interval between the two trials (e.g., 2 min) reduces the probability that age or sex differences in response to the novel versus familiar object will result from differences in memory ability.

Only three studies have previously investigated age differences in NOR task performance using short inter-trial intervals in rodents and have produced inconsistent results: two studies on mice reported that the strength of preference for the novel object in the choice trial peaks at adolescence (Calamandrei, Rufini, Valanzano, & Puopolo, 2002; Ricceri, Colozza, & Calamandrei, 2000), while a study of male rats reported no difference in the strength of preference for the novel object during Trial 2 between adolescents and adults (Reger, Hovda, & Giza, 2009). Similarly, studies of sex differences in NOR task performance have produced inconsistent results: adult male rats have been reported to spend a higher (Frick & Gresack, 2003; Kosten, Lee, & Kim, 2007) or a lower proportion of time (Ghi, Orsetti, Gamalero, & Ferretti, 1999; Sutcliffe, Marshall, & Neill, 2007) interacting with the novel object in Trial 2 than adult females.

In this study, we examined the performance of male and female Lister-hooded rats on the NOR task at early adolescence (postnatal day, pnd 28), mid-adolescence (pnd 40), or early adulthood (pnd 80; age categories are based on Tirelli, Laviola, & Adriani, 2003) using a 2-min inter-trial interval. The Lister-hooded rat is a pigmented, outbred strain that is widely used in cognitive and visual tasks in the UK and other parts of Europe (McDermott & Kelly, 2008). In addition to collecting data on interactions with the objects during Trials 1 and 2, we measured locomotor activity in the arena, as age and sex differences in locomotion could potentially influence object interactions.

METHODS

Subjects and Housing

The subjects were 36 male and 36 female Lister-hooded rats bred in-house (stock supplied by Harlan, UK). All animals were cage-housed (25 cm × 45 cm × 15 cm) with ad libitum access to soy-free rodent pellets and water. Housing rooms were controlled for temperature (20 ± 1°C) and humidity (55 ± 5%), and maintained on a 12-hr light:dark cycle (lights on 7 am). From pnd 17, pups were handled once per day and, at pnd 21, were weaned into same-sex sibling groups. At pnd 28, animals were housed as same-sex sibling pairs.

Each subject underwent behavioral testing only once, with different animals used in each age group. Subjects were tested at pnd 28 ($n = 12$ males, 12 females), pnd 40 ($n = 12$ males, 12 females), or pnd 80 ($n = 12$ males, 12 females). One additional female (pnd 40) that failed to reach criteria (Behavioral measurements and data analyses section) was excluded from the study. The subjects were taken from 19 litters, and littermates and cage-mates were distributed as evenly as possible among all the age groups. All appropriate guidelines and requirements were adhered to, as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85–23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986.

Apparatus and Experimental Design

The testing apparatus was a wooden, light grey-painted square chamber, measuring 67 cm × 67 cm × 45 cm (l × w × h), with a solid floor constructed of the same material. Three objects were used during the experiment (yellow rubber toy, glass jar filled with rocks, blue plastic bottle filled with sand) and were chosen to deter climbing and chewing. A pilot study with adult male and female rats showed that, from a range of objects, the amount of time spent interacting was very similar for these items. The apparatus was surrounded by a black curtain, and a video camera attached to the ceiling relayed images to a computer. All tests were conducted between 09:00 and 14:00 hr in the same testing room under dim, white light (approximately 25 lux), and a white noise generator was used to mask external sounds.

At the beginning of the test session, a subject was brought to the testing room in a carrying box (42 cm × 26 cm × 13 cm) and placed into the empty apparatus for a 10-min familiarization session. The animal was then returned to the carrying box for 2 min while the apparatus was cleaned with a 70% ethanol solution and allowed to dry. Two objects were placed into the apparatus in adjacent quadrants, 15 cm apart and 8 cm from the wall. The animal was placed into an empty quadrant, facing away from the objects, for a 5-min session, Trial 1, during which the subject had the opportunity to interact with the two objects. At the end of Trial 1, the animal was returned to the carrying box for an inter-trial interval of 2 min, during which one of the objects was replaced by a novel object. The apparatus and objects were cleaned as before, and the animal was reintroduced to the apparatus for another 5-min session, Trial 2. The object that remained from the first trial was considered the familiar object, and the new object was considered the novel object. At the end of Trial 2, the subject was returned to the home cage, and all objects and apparatus were cleaned again in preparation for the next subject. The objects used in each trial were counterbalanced across subjects and between age groups, and whether the left- or right-hand object was replaced in Trial 2 was also counterbalanced.

Behavioral Measurements and Data Analyses

All sessions were recorded directly onto the computer. Measures of object interaction were recorded manually using in-house software, while locomotor activity was analysed using EthoVision XT 5.0 software (Noldus Information Technology, Wageningen, The Netherlands, 2008).

Behavioral measures collected during Trials 1 and 2 included the *amount of time spent moving* and the *amount of time spent interacting with each object*. Object interaction was defined as the nose being in contact with an object, which excluded behaviors such as backing into an object, tail only contact, or time resting next to an object. Any animal that did not exhibit a minimum of 5 s of total contact with the objects in Trial 1 and at least 1 s contact with either object in Trials 1 and 2 was excluded from the study (one female at pnd 40). These criteria are comparable to those used in previous NOR studies (e.g., Anderson et al., 2004).

Two measures of novelty preference were calculated. The first measure, referred to as *preference for novelty*, was calculated as the proportion of time spent interacting with the novel versus the familiar object in Trial 2, converted to a percentage [(Time with novel − Time with familiar)/(Time with novel + Time with familiar) × 100]. A positive value indicates a preference for the novel object, while a negative value indicates a preference for the familiar object, and a score of zero indicates equal preference for the two objects.

The second measure, referred to as *preference change*, takes into account any initial biases by comparing the proportion of time spent with the two objects in Trial 1 to the proportion of time spent with the two objects in Trial 2. Previous research has reported that individual rats exhibit side-biases in behavioral tests and that rotational behavior differs between ages and sexes (e.g., Becker, Robinson, & Lorenz, 1982; Hyde & Jerussi, 1983; Schwarting & Borta, 2005). To take into account any biases that could affect the time spent with either object in Trial 1 (including individual preferences for a specific object), a side preference was calculated for both Trials 1 and 2 [(Time with right object − Time with left object)/(Total time with both objects) × 100], with a negative value representing a left-side preference, and a positive value indicating a right-side preference. *Preference change* was then calculated as the change in object contact times from Trial 1 (T1) to Trial 2 (T2), [(T2Right − T2Left)/(T2Right + T2Left)]×100 − [T1Right−T1Left)/(T1Right + T1Left)] × 100. The preference change value was adjusted to positive (+) if contact changed towards the novel object, or to negative (−) if contact changed towards the familiar object. Therefore, if the preference ratio increased between trials in the direction of the novel object, this score would have a positive value, and vice versa.

Statistical Analyses

Repeated-measures analyses of variance (ANOVAs) were used to examine age and sex differences locomotor and object contact measures across the two trials. Correlations between novelty-preference scores and these other behavioral measures were examined (Pearson correlations), and analyses of co-variance (ANCOVAs) were subsequently used to examine whether preference scores in Trial 2 differed with sex and age. One-sample *t*-tests were used to examine whether groups of animals showed a significant preference for the novel object, as indicated by a score significantly greater than zero. Bonferroni pairwise comparisons were used to investigate age and sex differences. An α value of .05 was used throughout. Data were analyzed using SPSS 17.0 for Windows (2009). Effect size (partial-eta squared, η_p^2) and power (β) values for ANOVAs were calculated by SPSS. Cohen's *d* and power for *t*-tests were calculated with G*Power Version 3.0.8.

RESULTS

Locomotion

The amount of time spent locomoting tended to increase with age ($F_{2,66} = 2.49$, $p = .091$, $\eta_p^2 = .07$, $\beta = .48$; pairwise comparisons non-significant, $ps \geq .116$; Fig. 1a). There was no significant main effect of sex ($F_{1,66} = 1.42$, $p = .238$), nor were there significant interactions between sex and age, age and trial, or sex and trial ($Fs_{1-2,\ 66} \leq 1.99$, $ps \geq .134$). Between the two trials, there was a significant decrease in movement from Trial 1 to Trial 2 ($F_{1,66} = 15.40$, $p < .001$, $\eta_p^2 = .19$, $\beta = .97$; Tab. 1).

Total Amount of Contact With Objects

There was a significant main effect of age on the total amount of time spent in contact with the objects across both trials ($F_{2,66} = 11.27$, $p < .001$, $\eta_p^2 = .25$, $\beta = .99$; Fig. 1b), with pairwise comparisons indicating increases from pnd 28 to 40 ($p = .037$) and pnd 28 to 80 ($p < .001$), but no difference between pnd 40 and 80 ($p = .101$). There was no significant main effect of sex ($F_{1,66} = .44$, $p = .509$), nor were there significant interactions between sex and age, age and trial, or sex and trial ($Fs_{1-2,\ 66} \leq 2.21$, $ps \geq .118$). The total amount of time spent in contact with objects tended to decrease between Trial 1 and Trial 2 ($F_{1,66} = 3.65$, $p = .060$, $\eta_p^2 = .05$, $\beta = .50$; Tab. 1).

Preference for Novelty

As a Pearson correlation indicated a significant negative relationship between time spent moving in Trial 2 and novelty-preference ($r_{72} = -.24$, $p = .043$), this locomotor measure was used as a covariate in the analyses. While the main effect of sex was not significant ($F_{1,65} = .29$, $p = .589$), the main effect of age was significant ($F_{2,65} = 3.59$, $p = .033$, $\eta_p^2 = .10$, $\beta = .65$). However, an ANCOVA also showed a significant sex by age interaction in preference for novelty ($F_{2,65} = 4.47$, $p = .015$, $\eta_p^2 = .12$, $\beta = .75$; Fig. 1c).

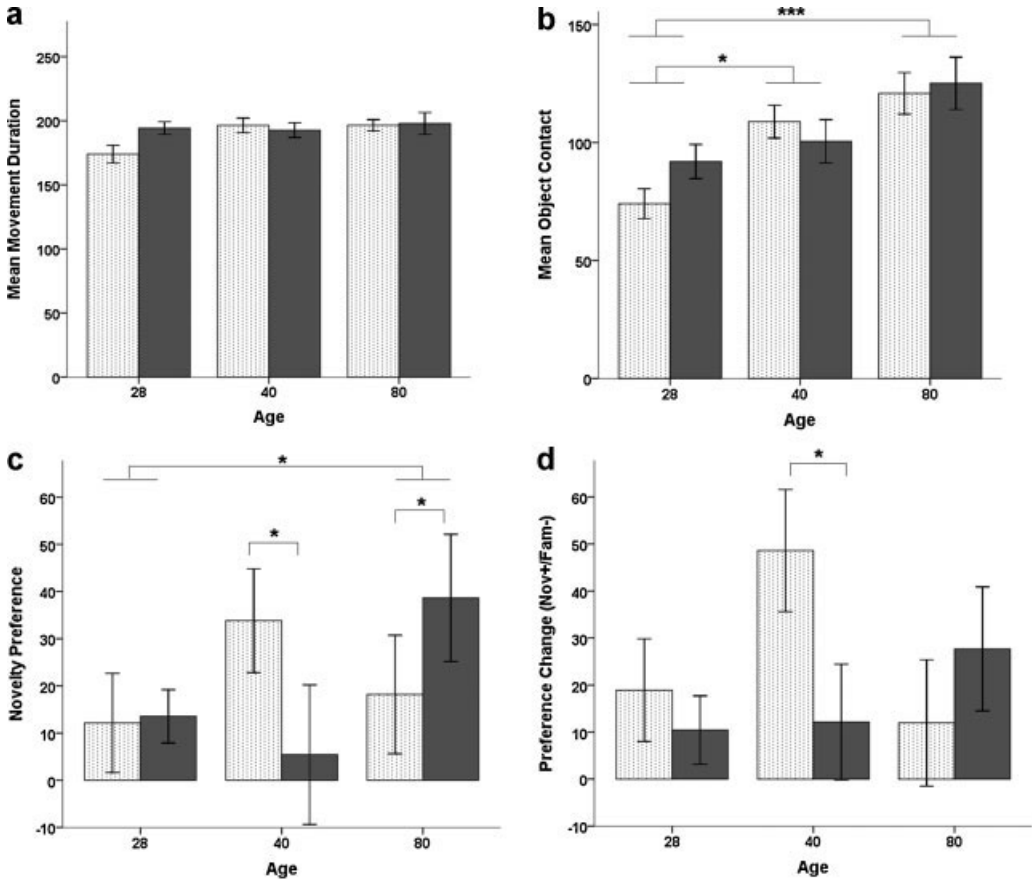

FIGURE 1 **a**: Amount of time spent moving (seconds) by age and sex for Trials 1 and 2 combined (means and SEMs). **b**: Total object contact (seconds) by age and sex across both Trials 1 and 2 (means and SEMs). **c**: Preference for novelty in Trial 2 by age and sex (means and SEMs). **d**: Preference change in Trial 2 by age and sex (means and SEMs). In all figures, stippled bars represent males, and grey bars represent females. Significant differences: $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

Pairwise comparisons indicated that males exhibited greater novelty-preference than females at pnd 40 ($p = .039$), and females higher than males at pnd 80 ($p = .049$). There was no sex difference at pnd 28 ($p = .320$). Males showed an increase in novelty-preference from pnd 28 to pnd 40 ($p = .043$), with a non-significant decrease from pnd 40 to pnd 80 ($p = .797$), and no difference between pnds 28 and 80 ($p = .439$). Females exhibited no change in novelty-preference between pnd 28 and pnd 40 ($p = 1.00$), and a significantly higher novelty-preference at pnd 80 than at pnd 28 ($p = .048$) and pnd 40 ($p = .013$).

In order to check whether the total amount of time spent interacting with objects in Trials 1 or 2 influenced preference for novelty, we carried out additional analyses. Neither total object contact in Trial 1 nor total object contact in Trial 2 correlated with novelty preference ($rs_{72} \leq .08$, $ps \geq .507$). However, given that object contact significantly increased across the age groups, an additional ANCOVA was performed that also included object contact in Trial 1 and object contact in Trial 2 as covariates. The sex by age interaction remained significant ($F_{2,63} = 3.82$, $p = .027$, $\eta_p^2 = .11$, $\beta = .68$), the main effect of age difference was reduced ($F_{2,63} = 2.50$, $p = .090$), and the main effect of sex remained non-significant ($F_{2,63} = .27$, $p = .607$).

When all subjects were combined, a one sample *t*-test verified that the subjects exhibited a significant preference for novelty in Trial 2 (i.e., preference scores were greater than zero; $t_{71} = 4.21$, $p < .001$, $d = .50$, $\beta = .99$), with animals spending approximately 60% of contact time with the novel object and 40% with the familiar object. Males showed a preference for novelty at pnd 40 ($t_{11} = 3.07$, $p = .011$, $d = .89$, $\beta = .80$) but not at pnd 28 ($t_{11} = 1.16$, $p = .272$) or pnd 80 ($t_{11} = 1.45$, $p = .175$). Females exhibited a significant preference for the novel object at pnd 28 ($t_{11} = 2.40$,

Table 1. Means, in Seconds, and Standard Deviations of Behavioral Measures by Sex and Age Group ($n = 12$ per Group).

	Males			Females			Totals		
Age	28	40	80	28	40	80	28	40	80
Trial 1: Movement duration	187.25 (35.91)	204.06 (18.47)	206.43 (24.66)	206.67 (28.07)	202.51 (22.42)	193.51 (48.35)	196.96 (33.04)	203.29 (20.11)	199.97 (38.11)
Trial 2: Movement duration	160.53 (32.24)	188.66 (29.27)	186.43 (16.39)	181.78 (20.28)	182.86 (21.56)	202.27 (19.48)	171.15 (28.49)	185.76 (25.32)	194.35 (19.37)
Trial 1: Total object contact	78.60 (29.84)	120.89 (29.50)	115.13 (37.05)	103.93 (37.63)	102.26 (39.43)	126.06 (60.48)	91.26 (35.64)	111.57 (35.36)	120.60 (49.37)
Trial 2: Total object contact	69.51 (25.68)	96.78 (30.12)	126.83 (32.66)	79.00 (17.47)	98.64 (30.55)	124.05 (29.03)	74.69 (21.92)	97.71 (29.68)	125.27 (31.30)

Note. Numbers in parentheses are standard deviations.

$p = .035$, $d = .69$, $\beta = .59$), and pnd 80 ($t_{11} = 2.86$, $p = .015$, $d = .83$, $\beta = .74$), but not pnd 40 ($t_{11} = .37$, $p = .720$).

Preference Change

Although side biases were not apparent overall ($ts_{71} \leq .97$ $ps \geq .337$), there was an effect of trial by sex interaction on side biases ($F_{2,66} = 5.23$, $p = .025$, $\eta_p^2 = .07$, $\beta = .62$): females tended to show some changes in side bias between the trials ($p = .061$), whereas males did not ($p = .190$). The preference change measure takes into account side biases by comparing the proportion of time spent with each of the objects in Trial 1 with the proportion of time spent with the objects in Trial 2. Using locomotion as a covariate, the sex by age interaction was significant for preference change ($F_{2,65} = 3.61$, $p = .033$, $\eta_p^2 = .10$, $\beta = .65$; Fig. 1d). The score was higher for males than females at pnd 40 ($p = .019$), but no longer at pnd 80 ($p = .168$). There were still no sex differences at pnd 28 ($p = .930$). Age differences in males remained with an increase between pnds 28 and 40 ($p = .047$), and a decreasing trend between pnds 40 and 80 ($p = .072$). There was still no difference in males between pnds 28 and 80 ($p = 1.00$). Females, however, no longer exhibited significant differences between any age groups ($ps \geq .345$). *T*-tests revealed similar findings as before, except that, in females, preference for novelty was no longer significant at pnd 28 and only tended towards significance at pnd 80 ($t_{11} = 2.10$, $p = .059$, $d = .61$, $\beta = .48$). The main effects of age ($F_{2,65} = 1.84$, $p = .167$) and sex ($F_{1,65} = .25$, $p = .617$) were not significant.

DISCUSSION

This study examined the ontogeny of response to novel objects in male and female Lister-hooded rats from adolescence to adulthood, using the NOR task with a short inter-trial interval. The results indicated that the strength of preference for the novel object in Trial 2 of the task exhibited a significant sex difference at mid-adolescence, with males showing a higher novelty-preference than females. This sex difference was not present at early adolescence, and, while the opposite pattern of results was observed at early adulthood, the adult sex difference was only present when calculated as preference for novelty, and not when calculated as preference change, suggesting that the adult sex difference is not robust. In contrast, other measures did not exhibit significant age by sex interactions, indicating that the sex difference in behavior at mid-adolescence was specific to situations involving choice of novelty. These results provide evidence that mid-adolescent rats exhibit a sex difference in behavior when provided with the opportunity to interact with a novel versus a familiar object that is not seen at younger or older ages.

Our finding that mid-adolescent male rats exhibit a stronger preference for novelty than females has not

been reported previously. While two rodent studies have reported that the strength of the preference for the novel object in the NOR task peaks at adolescence (Calamandrei et al., 2002; Ricceri et al., 2000), neither reported a sex difference at this age despite testing subjects of both sexes. In both of these studies, sample sizes were smaller than in the current study ($n = 4$–5 per sex per age group, Ricceri et al., 2000; $n = 8$ per sex per age group, Calamandrei et al., 2002; $n = 12$ per sex per age group, current study). These previous studies also used mice rather than rats, and used a different methodology that involved multiple tests of object interactions in one experiment. Reger et al. (2009) failed to find an age difference in NOR performance in male rats, but used broad age classifications (pnd 29–40 for adolescents; pnd 50+ for adults) that could have masked more subtle age effects.

In our study, no sex differences were found in the total amount of object contact at any ages, and the analyses of co-variance confirmed that the sex difference in novelty-preference at mid-adolescence was robust to any differences in object contact or locomotor activity. Previously, we have reported that Lister-hooded rats do not exhibit sex differences in open field or elevated plus-maze behavior at mid-adolescence (Lynn & Brown, 2009, 2010), suggesting that the current results are not related to sex differences in anxiety-like responses at this age and are unique to a test that presents a ''choice'' of novel and familiar stimuli. The total object contact and locomotor activity gradually increased from early adolescence into adulthood, in support of previous research (e.g., Lynn & Brown, 2009, 2010; Moore, Linsenbardt, Melón, & Boehm, 2010; Renner, Bennett, & White, 1992) and potentially due to psychomotor development. The decrease in object interactions and locomotor activity between Trials 1 and 2, particularly in adolescence, could have resulted from habituation or from physical tiredness in subjects.

This study has shown that adolescent male rats exhibit a particularly strong preference for novelty during mid-adolescence compared both to females and to males at other ages. Interactions between the developing gonadal hormone system and dopamine neurotransmitter system could potentially underlie this finding. Adolescent rodents exhibit a higher vulnerability than adults to the positive rewarding properties of psycho-stimulants and other drugs of abuse (Doremus-Fitzwater, Varlinskaya, & Spear, 2010). Researchers have recently begun to examine how male and female adolescent rodents differ in their response to drugs of abuse (e.g., Hensleigh, Smedley, & Pritchard, 2010; Walker et al., 2009). Understanding sex and age differences in the response of rodents to natural rewards, such as novel objects, could enhance our understanding of age and sex differences in drug-misuse in humans.

NOTES

This research was supported by the Wellcome Trust and the School of Psychology, University of St Andrews. We are grateful to Eric Bowman, Kevin Laland, and two anonymous reviewers for comments on the article.

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Effects of suppressing gonadal hormones on response to novel objects in adolescent rats

De-Laine M. Cyrenne, Gillian R. Brown *

School of Psychology, University of St Andrews, South Street, St Andrews, KY16 9JP, UK

Hormones and Behavior 60 (2011) 625-631
doi:10.1016/j.yhbeh.2011.08.015