EXPLORING THE BRAIN-BEHAVIOUR INTERFACE: THE ROLE OF JUVENILE PLAY EXPERIENCES

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ABSTRACT

In laboratory rats, juvenile play behavior has been shown to influence the
development of the medial prefrontal cortex (mPFC) and the experience of interacting
with multiple partners has been shown to influence the orbital frontal cortex (OFC).
Several studies in this thesis further explored these relationships. Two main findings
arose. 1). The play-induced changes to the mPFC and the partner-induced changes to the
OFC differ in their longevity. The neural remodeling of the mPFC remains relatively
unchanged into adulthood, whereas that of the OFC decreases over time, suggesting that
these two areas of the prefrontal cortex serve different roles in social behavior. 2) Though
wild rats play in a similar manner to domesticated rats, the play-induced changes to the
mPFC are not present, suggesting that complex patterns of play fighting have evolved
independently of their role in the development of the mPFC. These findings shed new
light on play.

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LIST OF ABBREVIATIONS

AID   dorsal agranular insular cortex
ANOVA   analysis of variance
BLA   basolateral nuclei
CA1   cornu ammonis 1
Cg1   cingulate area 1
Cg3   cingulate area 3
CO   control-mother and siblings group
CPP   conditioned place preference
Fr1   frontal area 1
IL   infralimbic cortex
LE   Long-Evans rats
LO   lateral orbital area
MO   mother-only group
mPFC   medial prefrontal cortex
NPN   no play-nicotine group
NPS   no play-saline group
OFC   orbital frontal cortex
PAG   periqueductal gray
PFC   prefrontal cortex
PL   prelimbic cortex
PN   play-nicotine group
Pnd   postnatal day
PS   play-saline group
SD   Sprague-Dawley rats
SO   sibling-only group
VO   ventral orbital area
WWCPS   Wild Warsaw Captive Pisula Stryjek rats

Chapter 1: General Introduction

For most young mammalian species, one of the most prevalent forms of social interaction is engaging in rough-and-tumble play with peers (e.g., Burghardt, 2005; Fagen, 1981; Pellis & Pellis, 2009). While there is considerable research on the social play behavior of humans (e.g., Pellegrini, 2009; Smith, 2010), the vast majority of our understanding of the neurobiology of social play is derived from studies on laboratory animals, especially rats (e.g., Siviy & Panksepp, 2011; Pellis & Pellis, 2009; Vanderschuren & Trezza, 2014). As for many animals, the occurrence of social play in rats develops early, beginning shortly before weaning and continuing into adulthood; however, levels of play peak during the juvenile period (e.g., Baenninger, 1967; Bolles & Wood, 1964; Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1990, 1997). Thus, play is generally referred to as a juvenile-typical behavior and it is to the understanding of the emergence and function of juvenile play in rats to which this thesis is directed.

Play in rats involves the attack and the defense of the nape, which is nuzzled if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). Once the nape is contacted, rats will defend themselves using a variety of tactics, including rotating to supine or simply running away and evading the attack (Pellis, Pellis & Whishaw, 1992). Although sometimes argued to be an immature form of aggression (e.g., Hurst, Barnard, Hare, Wheeldon, & West, 1996; Taylor, 1980), the target competed over during play is not the same of those attacked during aggression, which involve bites directed at rump and lower flanks (Blanchard, Blanchard, Takahashi, & Kelley, 1977). Rather, the nape is contacted by the male during sexual encounters, suggesting that play in rats is a simulation of

sexual behavior, but not aggression (Pellis, 1993). Irrespective of the origins of social play, and debate about the involvement of aggression (Cheng, Taravosh-Lahn & Delville, 2008), the production and regulation of play involves some distinct neural circuitry (Panksepp, 1998; Panksepp & Biven, 2012). Moreover, it serves functions that are different to those of either aggression or sex (Pellis, Pellis & Himmler, 2014; Vanderschuren & Trezza, 2014).

The experience of play in rats is pleasurable and rewarding, as they emit 50-kHz or 'happy' vocalizations both in anticipation of social play (Knutson, Burgdorf, & Panksepp, 1998) and while playing (Burgdorf, Panksepp, Beinfeld, Kroes, & Moskal, 2006; Himmler, Kisko, Euston, Kolb, & Pellis, 2014a; Kisko, Himmler, Himmler, Euston, & Pellis, 2015). Moreover, play is a sufficient reward for the conditioned place preference paradigm (Calcagnetti & Schechter, 1992; Siviy, 1998; Trezza, Damsteegt, & Vanderschuren, 2009) and also for maze learning (Humphreys & Einon, 1981; Normansell & Panksepp, 1990).

In addition to being rewarding, social play in the juvenile period has been shown to provide beneficial influences on the development of social, emotional and cognitive skills. For example, rats that have experienced play display better regulation of their emotional responses to fearful and stressful situations (e.g., Arakawa 2002, 2003; da Silva, Ferreira, Carobrez, & Morato, 1996) and are better at following social rules when interacting with other rats (van den Berg et al., 1999; Von Frijtag et al., 2002). Furthermore, they exhibit better impulse control and decision-making (Baarendse, Counotte, O'Donnell, & Vanderschuren, 2013). With regard to the brain, playing activates various cortical and subcortical brain regions (e.g., van Kerkhof, Trezza,

Mulder, Gao, Voorn, & Vanderschuren, 2013; van Kerkhof, Damsteegt, Trezza, Voorn, & Vanderschuren, 2013; Gordon, Burke, Akil, Watson, & Panksepp, 2003; Northcutt & Nguyen, 2014). Over the longer term, the experience of juvenile-typical play behavior influences the development of the dendritic plasticity of cells in prefrontal cortex (PFC) (Bell, Pellis, & Kolb, 2010; Himmler, Pellis, & Kolb, 2013b). The experience of play itself prunes the dendritic arbor of the medial prefrontal cortex (mPFC). In contrast, social experience with a diverse range of partners increased the dendritic arbors of the orbital frontal cortex (OFC), and this experience need not be with playful partners. It should be noted, however, that once weaned, adults do not interact much with young (Cramer, Thiels & Alberts, 1990), making interactions with peers the most common source of social interaction (Thiels, Alberts, & Cramer, 1990). Also, they are highly motivated to play at this age (Varlinskaya, Spear, & Spear, 1999) and will do so with both familiar and unfamiliar peers (Panksepp, 1981; Pellis & Pellis, 1990). Therefore, it is highly likely that under natural, colony conditions, play is likely to be an important source for experiencing social interactions with multiple partners, and so, on the development of the OFC (Pellis & Pellis, 2009).

Given that play behavior has been shown to activate numerous subcortical regions, I previously investigated whether the play-induced plasticity seen in the PFC also extended into subcortical regions. I focused on two areas, the basolateral amygdala (BLA) and area CA1 of the hippocampus, which are known to have strong connections with the OFC and mPFC and are involved in the fear circuitry. There were no play-induced changes in the dendritic arbor of either area, suggesting that the play-induced structural changes to the neurons may be limited to the prefrontal cortex, specifically to the OFC and the mPFC.

Additionally, I investigated if the play-induced changes to the mPFC allow the brain to be more flexible, or display greater neural plasticity, to experiences occurring later in life. As previously mentioned, juvenile play experiences induce the pruning of the dendritic arbor of cells in the mPFC (Bell et al. 2010; Himmler et al., 2013b). However, other experiences, such as exposure to psychostimulants (e.g., amphetamine, nicotine), will induce proliferation in cells in the mPFC (Robinson & Kolb, 2004). Therefore, in my MSc, I both attempted to replicate the findings from Bell et al. (2010) and assess whether rats with prior play experience would show a greater proliferation in dendritic arbor to later nicotine exposure.

There were two main findings: 1) the previous findings of play-induced pruning of the mPFC were replicated and 2) that the mPFC neurons of rats with juvenile play experience had a bigger response to nicotine. However, the previous finding on the effect of multiple partner-induced proliferation of the OFC was not replicated. Therefore, based upon these findings, there were two outstanding major questions that remained from my MSc work. The first question is why we were able to replicate the previous findings of the play-induced pruning of the mPFC, but not the partner-related changes to the OFC. The second question is whether the dendritic remodeling by nicotine administration in rats with previous play experience results in a behavioral change to nicotine exposure. Thus, section one of my thesis will include chapters that are built around attempting to answer these two questions.

1.1 Section 1

In the second chapter, I explore the first question of why we were able to replicate the play-induced pruning to the mPFC, but not the partner-induced proliferation to the OFC. In the Bell et al. (2010) study, the rats were paired with either three age-and-sex matched partners (play group) or with an adult female (non-play group). Adult females will not readily engage in play with juveniles, but will engage juveniles in other social interactions, such as huddling, licking and grooming (Einon, Morgan, and Kibbler, 1978; Pellis & Pellis, 1997). By being paired with three peers, the rats would experience not only the social interactions experienced by the juvenile housed with an adult female, but would also have ample opportunity to engage in play behavior and do so with multiple partners. They remained in these groups until they were 60 days of age, before their brains were harvested. However, in my MSc study, our rats remained in these groups until they were 90 days old, with their brains being harvested at around 100 days. Given that with age there is a gradual pruning of neuron number and complexity (Koss et al., 2013), it is possible that the lack of an effect on the OFC in my MSc may have resulted from the difference in the age when the animals' brains were examined. In order to test this, rats were reared with three partners or in a pair and were either sacrificed at 60 or 100 days of age.

Although the mPFC and OFC of the rat have several subregions, specifically Zilles' IL, PL, and Cg3 in the mPFC and LO, VO, and AID in OFC (Zilles, 1985), I decided to focus on Cg3 and AID for two reasons (Figure 1.1). First, these regions are the largest in the respective zones and thus there are more Golgi-stained cells to draw. Second, lesions

specific to these regions disrupt play behavior, although in different ways (Bell et al., 2009; Pellis et al., 2006).

1.1.1 Hypothesis: The lack of proliferation seen in the OFC of 100 day-old rats is due to an age-related pruning of cells.

1.1.2 Prediction: Rats reared in quads should display a proliferation in the dendritic complexity of cells in the OFC at 60 days but should not display a comparable proliferation at 100 days of age.
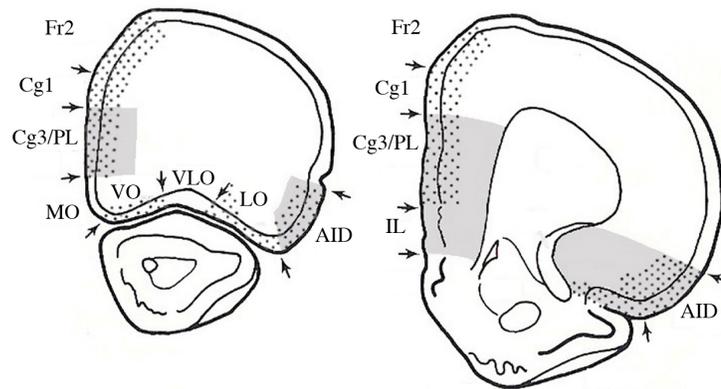
Figure 1.1
A coronal schematic showing the location of the two subareas (Cg3 and AID) focused on in this thesis.

The third chapter is designed to determine whether the neural changes due to nicotine exposure of animals with previous play experience are associated with changes in the behavioral and physiological response to nicotine. There is some evidence that suggests that the priming that play behavior has on the plasticity of the mPFC to the subsequent exposure to nicotine may serve as a neural protectant against the effects of nicotine. For example, early experiences, such as tactile stimulation, can attenuate the behavioral and

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anatomical effects of another psychostimulant drug, amphetamine (Muhammad, Hossain, Pellis, & Kolb, 2011). Additionally, animals that are deprived of social interactions as juveniles are more vulnerable to becoming addicted to psychostimulant drugs (Whitaker, Degoulet, & Morikawa, 2013). It is therefore reasonable to propose that other early experiences, such as play behavior, would also attenuate the effects of nicotine. In this thesis, the behavioral and physiological response to nicotine exposure was tested in rats that had previous play experience. These responses were assessed by overall activity induced by nicotine (i.e., sensitization) and by the voluntary intake of nicotine.

1.1.3 Hypothesis: Juvenile play experience will attenuate the behavioral and physiological response to later nicotine exposure.

1.1.4 Prediction: If rats have prior play experience they should display less nicotine sensitization in the activity box directly following nicotine injections and also consume less nicotine than rats without juvenile play experiences.

1.2 Section 2

Whereas the first section of my thesis was based on answering the outstanding questions derived from my MSc thesis, the second section of my thesis was aimed at further investigating the potential benefits derived from juvenile play-induced pruning of the neurons of the mPFC. To this end, chapter 4 investigates how the mPFC may contribute to the organization of complex social interactions.

Rats that have played with peers as juveniles are better at regulating their physiological and behavioral responses to stressful situations (e.g., Von Frijtag et al., 2002; Arakawa 2002, 2003; da Silva et al., 1996; Lukkes et al., 2009a,b) and adopting suitable coping strategies when confronted by dominant animals (van den Berg et al.,

1999). At least some of these improved abilities may be mediated by changes to the mPFC, as some of the same behavioral disturbances seen in animals deprived of play behavior are present in rats with lesions of the mPFC (Holson, 1986; Shah & Treit, 2003). Recently, it has been shown that the mPFC is involved in orchestrating more complex movements during social interactions, as rats with mPFC lesions have a preference for using simpler defensive tactics during social play (Bell et al., 2009). One hypothesis for this shift from using more complex to simpler tactics is that the mPFC has a role in the coordination of the performer's movements with those of a partner (Bell et al., 2009).

In order to test this hypothesis, adult rats with lesions of the mPFC were evaluated in the food robbing and dodging task. In this task, one rat (i.e., the dodger) attempts to protect a food item from another rat (i.e., the robber) (Field, Whishaw, & Pellis, 1996; Whishaw, 1988). To protect the food item, the dodger must coordinate its own movements with those of the robber in order to gain and maintain a specific distance away from the robber (Bell, 2014; Bell & Pellis, 2011). Given that lesions of the mPFC have been shown to impair the ability to protect food items in the robbing and dodging paradigm successfully (Whishaw, 1988), it seems likely that this decrement in performance arises from a reduced ability to maintain a safe distance from the robber.

1.2.1 Hypothesis: The mPFC is important for integrating the performer's movements with those of the partner and so contributes to inter-animal coordination.

1.2.2 Prediction: Rats with mPFC lesions should be more likely to have their food items stolen and show a diminished ability to gain and maintain a consistent distance from the robber.

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1.3 Section 3

In the third section of the thesis, the role of domestication in the development of the mPFC and play is considered. To date, all the brain and behavior changes characterized between juvenile play experience and adult performance have been found in domesticated laboratory rats (Pellis & Pellis, 2009; Vanderschuren & Trezza, 2014). Whether these findings can be applied to non-domesticated animals needs to be determined, as domestication affects body composition, physiology, neural mechanisms and behavior (e.g., Albiach-Serrano, Brauer, Cacchione, Zickert, & Amici, 2012; Castle, 1947; Coppinger & Coppinger, 2001; Lockard, 1968; Pisula, Turlejski, Stryjek, Nałęcz-Tolak, Grabiec, & Djavadian, 2012). Behavioral and anatomical changes arise rapidly following selective breeding and domestication (Trut, 1999). Given that rats have been used in the laboratory for well over 100 years (Sławiński, 1991), it is not surprising that a number of anatomical, physiological and behavioral changes have been documented in domestic rats as compared to wild ones (e.g., Keeler, 1947; Lockard, 1968; Barnett & Hocking, 1981; Blanchard & Blanchard, 1994 Kruska, 2005). The effects of domestication may change the frequency and form of social play and also the effects that play in the juvenile period may have on the development mPFC-mediated skills. Therefore, in the third section of the thesis, I will investigate, 1) whether domesticated rats play similarly to wild rats in the juvenile period and 2) whether wild rats also display the play-induced pruning to the mPFC seen in domesticated rats.

The wild-type of rat used in both of these studies is the Wild Warsaw Captive Pisula Stryjek (WWCPS) strain of *Rattus norvegicus*. This strain of rat was derived in 2006 from genetic material obtained from five different colonies of wild rats that were caught

in Warsaw, Poland (Stryjek & Pisula, 2008). The WWCPS rats used were from the F2-5 generations and were housed and bred in captivity. However, these rats are housed and handled in a way which reduces human contact (Stryjek 2008, 2010) in order to maintain the integrity of the non-domesticated rat.

Chapter 5 will compare the juvenile-typical play behavior between wild rats (WWCPS) and a domesticated strain (Long-Evans) using the same housing paradigms used in previous studies. Domestication typically involves animals reaching sexual maturity earlier, which results in the retention of juvenile-typical features such as play behavior. As a result, domesticated animals engage in play behavior more often (Budiansky, 1999; Burghardt, 1984; 2005) and are often less hostile than their wild counterparts. Some researchers suggest that play behavior is an immature form of serious fighting (e.g., Hurst, Barnard, Hare, Wheeldon, & West, 1996; Silverman, 1978; Taylor, 1980), however, given that the target of attacks differ between serious fighting and play behavior (Pellis & Pellis, 1987), this is unlikely to be the case. In fact, the targets of playful attacks resemble the target for courtship behaviors; thus, juvenile play in rats is considered a sexually based behavior (Pellis & Pellis, 1993; Pellis & Pellis, 1998). However, because domestication involves an earlier onset of sexual maturity, it may be the case that the play behavior in domesticated rats resembles courtship behaviors, whereas wild rats combine both courtship and aggressive behavior in their play. Nonetheless, where direct comparisons between domesticated animals and their wild counterparts have been examined in detail, such as the social play of wolves and dogs (Mech, 1970; Bekoff, 1972; Abrantes, 2005), the basic organization of the play appears similar.

1.3.1 Hypothesis: While the motivation to play in wild rats may be less than in domesticated rats, I suspect that the organization of play fighting in wild and domesticated rats is the same – both involve attack and defense of the nape. That is, I hypothesize that, as in domesticated rats, the play of wild rats is a simulation of sexual not aggressive behavior.

1.3.2 Prediction: Play in the peak juvenile period should reveal the same pattern of attack of defense of the nape in wild rats as it does in domesticated rats.

Chapter 6 investigates if wild rats show the same play-induced changes to the mPFC. Irrespective of whether play in wild rats mostly involves simulation of sexual or aggressive behavior, the issue of whether such playful experience in the juvenile period leads to changed social skills and brain mechanisms remains. Indeed, studies on species of monkeys in which play involves attack and defense of aggressive targets have shown that depriving them of the opportunity to engage in play as juveniles leads to impoverished social skills and impoverished regulation of stressful responses (Kalcher-Sommersguter, Preuschoft, Crailsheim, & Franz, 2011; Kempes, Gulickx, van Daalen, Louwerese, & Sterk, 2008).

There is growing evidence that the mPFC is important for the development of social and emotional skills. For example, the mPFC is anatomically and functionally linked to cognitive and emotional systems (Euston, Gruber, & McNaughton, 2012). Thus, selective lesions of the mPFC have been shown to influence how rats interact socially (Bell et al., 2009). Furthermore, rats with lesions to the PFC will react inappropriately to stressful or fearful situations (Holson, 1986; Shah & Treit, 2003). Given the link between the mPFC and the development of these skills, it is important to know if the effect of juvenile play

experiences on the development of the mPFC is due to play behavior rather than an association between juvenile play experiences and the development of the mPFC that has arisen as a byproduct of the domestication process.

1.3.3 Hypothesis: Play is a critical juvenile experience that refines mPFC-mediated social skills irrespective of domestication.

1.3.4 Prediction: Wild rats that have experienced play should have a reduction in the length, branching and spine density of mPFC cells as compared to wild rats that have not experienced play.

1.4 Section 4

In the fourth section of this thesis, I will investigate the experiences needed for the development of juvenile-typical play. As previously mentioned, playful experiences during the juvenile period are important for developing the skills necessary for becoming a competent adult (e.g., Arakawa 2002, 2003; Baarendse et al., 2013; da Silva, Ferreira, Carobrez, & Morato, 1996; van den Berg et al., 1999; Von Frijtag et al., 2002). However, what remains unknown is if play experience before the juvenile period is important for developing social competency during the juvenile period. Play behavior develops in a piecemeal fashion starting around 15-17 days of age (e.g., Baenninger, 1967; Bolles & Woods, 1964; Thiels, Alberts, & Cramer, 1990) and does not reach the juvenile-typical form until 28-30 days (Pellis & Pellis, 1997). Previous studies investigating the use of defensive tactics across different rat strains revealed that strains differ in the frequency of use of defensive tactics (Himmler et al., 2013c; Himmler et al., 2014c). The playful defense of the two strains with the greatest difference between them - Sprague-Dawley (SD) and Long-Evans (LE) rats - was further compared by testing how these rats

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modified their tactics when playing with a peer of the opposite strain (Himmler et al., 2014b). If the SD and LE strains were cross-housed from 24-30 days, they changed their strain-typical defenses and displayed defensive patterns of play that were intermediate between the two (Himmler et al., 2014b), suggesting that practicing play behavior in an immature form may be necessary for the maturing of play that is typical for juveniles. In order to test this, peer-peer play experiences in the peri-weaning period was denied to young rats.

1.4.1 Hypothesis: Peri-weaning playful experiences with peers are necessary for developing the skills needed for juvenile-typical play behavior.

1.4.2 Prediction: Rats reared without peer play during the peri-weaning period will have atypical patterns of play as juveniles.

1.5 Summary: The objectives for the thesis

This thesis is composed of 4 sections that are designed to further our understanding of how juvenile social play influences the development of the brain and behavior.

- The first section investigates whether the play-induced changes in the mPFC and OFC are stable or whether they gradually erode during adulthood and also whether the play-induced changes in the mPFC confer resistance to the physiological and behavioral effects of psychoactive drugs.

- The second section investigates the role that the mPFC has on inter-animal coordination.

- The third section investigates the potential role of domestication on the play that is performed and whether play modifies the mPFC in wild rats.

- The fourth section investigates the experiences that are necessary for the development of juvenile-typical social play behavior.

Chapter 2: Juvenile social experience and differential age-related changes in the dendritic morphologies of subareas of the prefrontal cortex in rats

2.1 Introduction

The experience of juvenile social play is important for the development of social, emotional, and cognitive skills (e.g., Pellis, Pellis, & Himmler, 2014; Vanderschuren & Trezza, 2014). If deprived of these social experiences, rats will react inappropriately to fearful and stressful social and nonsocial situations (da Silva, Ferreira, Carobrez, & Morato, 1996; Von Frijtag, Schot, van den Bos, & Spruijt, 2002; Einon & Potegal, 1991) and fail to behave submissively when confronted by a dominant rat (van den Berg, Hol, Van Ree, Spruijt, Everts, & Koolhaas, 1999). Furthermore, they are less competent in solving cognitive tasks (Einon, Humphreys, Chivers, Field, & Naylor, 1981) and display higher levels of impulsivity (Baarendse, Counotte, O'Donnell, & Vanderschuren, 2013). In part, these changes are likely due to the changes to the prefrontal cortex (PFC) that arise from social experiences gained in the juvenile period (Pellis, Pellis & Bell, 2010).

The PFC is an area crucial for executive functions, which include monitoring behavior, behavioral inhibition, planning, decision making (*for a review see* Dalley et al., 2004), as well as impulse control (Baarendse et al., 2013). Furthermore, selective lesions to different parts of the PFC result in deficits that are similar to those seen in adult rats deprived of juvenile social play behavior (e.g., Holson, 1986; Shah & Treit, 2003; Rudebeck, Walton, Millette, Shirley, Rushworth, & Bannerman, 2007). For example, damage to the medial prefrontal cortex (mPFC) leads to difficulties in coordinating movements with social partners in both playful (Bell, Pellis, & Kolb, 2009) and non-playful (Himmler, Bell, Horwood, Harker, Kolb, & Pellis, 2014d) interactions. In contrast, damage to the orbital frontal cortex (OFC) leads to an inability to modulate

responses when interacting with different partners in both playful and non-playful social interactions (Pellis et al., 2006). Juvenile social experiences influence the development of the dendritic plasticity of cells of the PFC (Bell, Pellis, & Kolb, 2010; Himmler, Pellis, Kolb, 2013b). However, the influences on the PFC differ depending on the form of the juvenile social experience involved and the area of PFC examined.

In Bell et al., (2010), rats were housed with either three age-and-sex matched partners or with a single age-and-sex matched peer (play groups), or alternatively, with three adult females or with a single adult female (non-play groups). Adult females will not readily engage in play with juveniles, but will engage juveniles in other social interactions, such as huddling, licking and grooming (Einon, Morgan, and Kibbler, 1978; Pellis & Pellis, 1997). When paired with peers, rats would experience not only the social interactions experienced by the juvenile housed with an adult female, but also would have ample opportunity to engage in play behavior. Whether housed with one or three peers, the dendritic branching of the mPFC was significantly pruned compared to being housed with one or more adults. In contrast, the OFC showed proliferation of dendritic branching when housed with three partners, whether peers or adults, compared to when housed with a single partner of either age (Bell et al., 2010). In a subsequent study, rats housed with 3 peers or with a single adult were compared. Like the Bell et al. (2010) study, there was play-induced pruning of the mPFC, but unlike the previous study, the partner-induced proliferation to the OFC was not present (Himmler, 2011; Himmler et al., 2013b).

One possibility for this lack of replication for the effects on the OFC could have arisen from the different ages at which the animals were sacrificed and the brains compared. In Bell et al., (2010), the rats were sacrificed at 60 days of age, shortly after

sexual maturity, in early adulthood, whereas, in the subsequent studies (Himmler, 2011; Himmler et al., 2013b), the rats were sacrificed at around 100 days of age, when fully adult. Brain development generally involves an overproduction of neurons, dendrites and synapses at an early age followed by natural pruning, which continues throughout life, but at varying rates (Kolb, Mychasiuk, Muhammad, Li, Frost, & Gibb, 2012; Kolb, Mychasiuk, & Gibb, 2014; Koss, Belden, Hristov, & Juraska, 2014; Markham, Morris, & Juraska, 2007; Milstein, Elnabawi, Vinish, Swanson, Enos, Bailey, Kolb, & Frost, 2013). The largest overproduction of synapses and slowest pruning occurs in the PFC (Elston, Oga, & Fujita, 2009). The prefrontal cortex in rats continues to develop into adulthood (Van Eden & Uylings, 1985a,b) and this development is generally associated with a reduction of PFC volume (Van Eden & Uylings, 1985a) and laminar specificity (Van Eden & Uylings, 1985b). In addition, both major subareas of the PFC, the mPFC and OFC, undergo cellular and dendritic pruning between adolescence and adulthood (Markham, et al., 2007; Koss et al., 2013; Milstein et al., 2013). Therefore, it is possible that the juvenile social experiences that induce the retention of dendritic complexity in the OFC may show a greater age-related pruning than is the case for the play-induced changes in the mPFC which already undergoes pruning. If so, the absence of a multiple partner effect on the OFC in Himmler (2011) could be due to the older age at which the brains were examined.

The present study investigates the effects of post-juvenile development on the maintenance of multiple partner-induced complexity of the OFC. This was tested by housing juvenile rats with age-and-sex matched peers in quads (multiple partners) or in pairs (single partner), and their brains were compared at 60 days or at 100 days. For the

OFC, it was predicted that there should be both a rearing and an age effect, so that subjects should have significantly more complex cells at 60 days if reared in quads and that this complexity should diminish significantly by 100 days. Given that in both housing conditions the subjects had the opportunity to play with peers, then the mPFC of all rats should show pruning of dendritic complexity as previously reported, and, if our hypothesis is correct, this should remain relatively unchanged with age (Bell et al., 2010; Himmler et al., 2013b).

2.2 Methods

2.2.1 Subjects

A total of 32 female Long-Evans hooded rats were used in this study. All rats were born in the vivarium at the University of Lethbridge, Canadian Centre for Behavioral Neuroscience from 14 mothers originally obtained from Charles River Laboratories (St. Constant, Quebec). Subjects were weaned at 22 days old and were housed in quads or pairs. Half of the rats from these rearing conditions were sacrificed at 60 and the other half at 100 days. It should be noted that the pups that were housed together were as closely age matched as possible (i.e., no more than three days difference in age) and all group mates were derived from different litters. For the groups reared in quads, the rats were housed in a 55.9 cm x 43.2 cm x 20.3 cm polyethylene tubs, whereas the groups reared in pairs were housed in standard 46 cm x 25 cm x 20 cm polyethylene tubs. All groups were maintained at a constant 21-23 °C on a 12:12 light-dark cycle and food and water were provided *ad libitum*. All rats were handled and cared for in accordance with the Canadian Council for Animal Care (CCAC) regulations.

2.2.2 Histology

Depending on the experimental group, on either PND day 60 or 100, subjects were deeply anesthetized using 0.6 ml of 3.4% sodium pentobarbital and were then perfused with 9% saline and their brains were collected. All brains were prepared using the modified Golgi-Cox procedure (Gibb & Kolb, 1998). The brains were placed in Golgi-Cox solution for 14 days and were then placed in 30% sucrose solution for seven days. The brains were then cut into 200 micron (μm) sections using a vibrating microtome, placed on 2% gelatin-dipped glass slides, placed in an airtight and darkened container for three days, stained, cover slipped and left to dry for approximately 2 weeks.

2.2.3 Anatomy

In order to quantify neuronal morphology, cells were traced onto paper using a *camera lucida* at a magnification of 250X. A total of three-to-five cells were selected from each hemisphere in each area from each subject (for a total of 6-10 cells per brain) with the mean score of each measure being used for analysis. In order for cells to be selected for analysis, they needed to meet two criteria: (1) the cell was fully impregnated with stain, and (2) the cell was not overlapping other cells. No brain for which less than three cells from each hemisphere could be sampled was used.

2.2.4 Areas and Quantification

Layer III pyramidal neurons were traced from Zilles (Zilles, 1985) area Cg3 (mPFC), and layer II/III from AID (OFC). For area Cg3, both apical and basilar dendrites were drawn. However, for AID, only basilar dendrites were drawn due to lack of intact apical fields. Two methods of analysis were used to obtain information of dendritic morphology; Sholl analysis and branch order analysis. Sholl analysis (Sholl, 1956) was

used to determine the total dendritic length by overlaying a transparency of concentric circles onto the drawing of the neuron and counting the number of dendrites which crossed each circle (16 circles). In order to estimate the complexity of branching of each dendrite, branch order analysis (Coleman & Riesen, 1968) was used, where complexity is calculated by counting the number of bifurcations on each specific dendrite.

Table 2.1: The total number of brains that were used to compare across conditions for both the OFC and the mPFC.

Rearing Condition	Age	Total Number of Brains used for Analysis	
		OFC	*mPFC*
Quads	*60*	8	8
Quads	*100*	6	4
Pairs	*60*	7	5
Pairs	*100*	7	6

2.3 Statistical Analysis

All the rats were used for brain extraction and analysis, yielding 8 animals per condition. However, due to poor staining or the inability to meet the minimum criterion for using a brain (*see* above), not all conditions had the full complement of subjects (Table 2.1). The data were first analyzed using a paired t-test for hemisphere, however since hemisphere failed to reveal any significant differences for any of the measures, it was not considered to be a factor in any further analyses. The data were then analyzed with a two-way analysis of variance (ANOVA), with age (60 or 100 days) and rearing condition (quads or pairs) as independent variables. For the mPFC measurements,

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separate ANOVA tests were conducted for each field (apical and basilar), whereas, for the OFC comparisons were limited to the basilar field. For *post hoc* pair wise comparisons the least significant difference test was used. Differences were considered significant for *p* values ≤ 0.05.

2.4 Results

2.4.1 mPFC

2.4.1.1 Sholl Analysis

There were no significant main effects for age or rearing condition for either dendritic field (p > 0.05) (Table 2.2).

2.4.1.2 Branch Order

There were no significant main effects for age or rearing condition for either dendritic field (p > 0.05) (Table 2).

Table 2.2: Mean ± SEM for all dendritic measurements for both the apical and basilar fields for cells in the mPFC. For comparative purposes in microns, the mean and SEM for dendritic length should be multiplied by 20.

Rearing Condition	Age	Dendritic Length		Branch Order	
		Apical	*Basilar*	*Apical*	*Basilar*
Quads	*60*	54.73±2.54	56.16±2.11	18.55±1.08	25.04±0.72
Quads	*100*	53.48±3.59	56.75±2.99	18.51±1.52	26.34±1.02
Pairs	*60*	56.62±3.21	57.56±2.67	19.62±1.36	25.73±0.91
Pairs	*100*	54.05±2.93	52.87±2.44	19.28±1.24	23.31±0.83

2.4.2 OFC

2.4.2.1 Sholl Analysis

A 2 x 2 ANOVA revealed a significant main effect for age, $F(1,52) = 20.075$, $p = 0.001$, with rats sacrificed at 60 days having longer dendrites. There was a trend for an effect of rearing condition, $F(1,52) = 3.835$, $p = 0.056$, and a significant interaction between age and rearing condition, $F(1,52) = 7.367$, $p = 0.009$. The interaction showed that there was more complexity at 60 days for the groups reared in quads ($p < 0.05$) with the difference disappearing by 100 days (Figure 2.1A). This pattern was confirmed with independent t-tests, which revealed a significant condition difference at 60 days ($t(28) = 3.344$, $p = 0.001$), but not at 100 days ($t(24) = -0.533$, $p = 0.599$).

2.4.2.2 Branch order

A 2 x 2 ANOVA revealed a significant main effect for age, $F(1,52) = 8.590$, $p = 0.005$, with more branching occurring in the 60 day age group. There was no significant main effect for rearing condition ($p > 0.05$) or a significant interaction ($p > 0.05$) (Figure 2.1B).

A.   B.
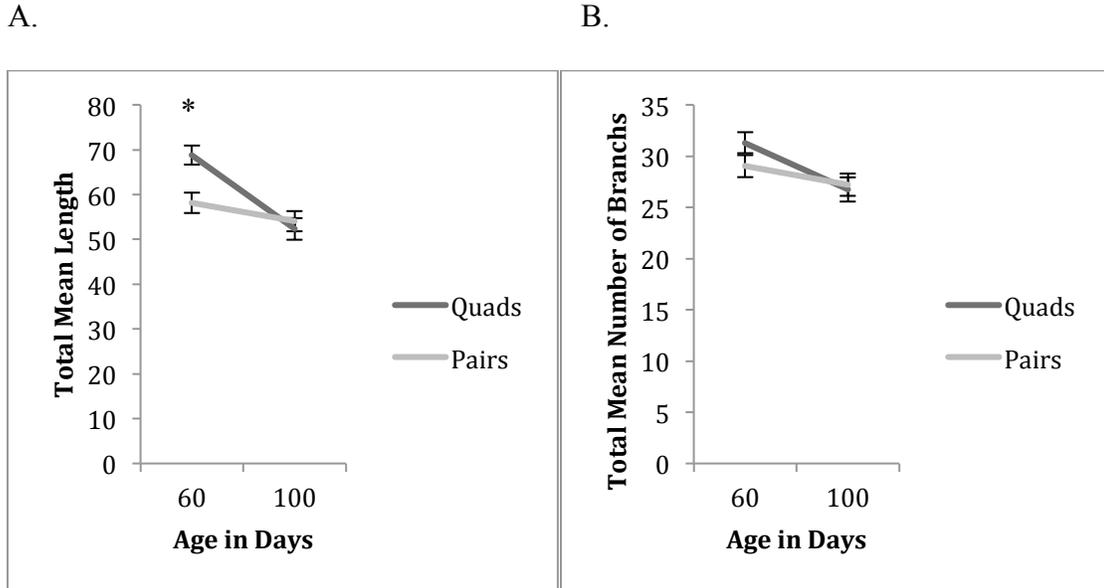
Figure 2.1
The mean ($\pm$SEM) total length of basilar dendrites are shown for the OFC for the two ages sampled and for the two rearing conditions (a), and the mean ($\pm$SEM) total amount of branching of basilar dendrites for the two ages sampled and for the two rearing conditions (b).

2.5 Discussion

The experience of juvenile social play has been shown to influence the dendritic pruning of neurons in the mPFC, whereas experiences with multiple partners during the juvenile period, whether involving play or not, increases the dendritic complexity in the cells of the OFC (Bell et al., 2010; Himmler et al., 2013b). Whereas the play-induced pruning of the mPFC has been replicated multiple times (Bell et al., 2010; Himmler et al., 2013b), the partner-induced proliferation of complexity in the OFC has yielded conflicting results. In the study showing the partner-induced increase of dendritic complexity, the brains of the rats were harvested at 60 days (Bell et al., 2010), whereas, in the study not showing this effect, the brains were harvested at 100 days (Himmler, 2011). Given that the brain, including the PFC, goes through a developmental increase in

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synaptic density at an early age and then a more gradual pruning with age (Kolb et al., 2012; Kolb et al., 2014; Koss et al., 2014; Markham, et al., 2007; Milstein et al., 2013), the present study tested the hypothesis that the lack of replication in the findings on the OFC is due to an age-related pruning that by 100 days leads to the loss of the complexity gained from the juvenile experiences. The data supported this prediction. The rats living with more partners during the juvenile period had more complex dendrites at 60 days, but that complexity was diminished with age so that by 100 days the cells of the OFC did not differ between the rats that were reared in quads or pairs. There was no comparable age-related decline in complexity in the mPFC, thus confirming that the experiences in the juvenile period that prune the dendrites of these neurons have a lasting effect, an effect that can be measured either shortly after the end of the juvenile period (Bell et al., 2010) or well into adulthood (Himmler et al., 2013b).

The question that arises is why the juvenile-induced changes in the mPFC (i.e., play-induced pruning) remain unchanged in adulthood, whereas, the juvenile-induced changes in the OFC (i.e., partner-induced proliferation) erode over time? Many of the developmental improvements in social, emotional and cognitive skills that are associated with the experience of play in the juvenile period (e.g., da Silva, et al., 1996; Einon & Potegal, 1991; Einon et al., 1981; Von Frijtag et al., 2002) has been hypothesized to be derived from play-induced changes of the mPFC (Baarendse et al., 2013; Pellis, Pellis, & Bell, 2010; Pellis, Pellis, & Himmler, 2014). Given that these skills, once fully established, are likely of similar importance at all ages, I propose that the neural circuitry would remain unchanged, as shown here for dendritic complexity. The situation is different for the skills that are dependent on the OFC.

The OFC is critically linked to animals being able to modify their responses based on the identity of the partner (Pellis et al., 2006), and it is the number of partners experienced during the juvenile period that seems to influence the development of the dendritic complexity of the OFC (Bell et al., 2010; present study). During early infancy, pups mostly interact with their mothers and littermates (Alberts, 2007; Champagne, Francis, Mar & Meaney, 2003), but once weaned their interactions with the mother diminish and most of their social interactions are with peers (Cramer, Thiels, & Alberts, 1990; Thiels, Alberts, & Cramer, 1990). Owing to the high motivation to play, juveniles will readily interact with both littermates and similar aged, unrelated, peers (Varlinskaya, Spear & Spear, 1999). However, once the rats reach sexual maturity they interact with adults of both sexes, many of whom they are unlikely to know, especially for males, which may move to other colonies (Barnett, 1975; Calhoun, 1962). Given the time spent in social investigation among adults, it is clear that being able to identify partners is important (Barnett, 1975). Indeed, because social investigation involves sniffing the partner's mouth, flanks and the anogenital area, rendering adults anosmic leads to a greater likelihood of escalating encounters to aggression, even among rats that were previously familiar with one another (Flanelly & Thor, 1976; Thor & Flanelly, 1976). In contrast, social investigation is not increased when rats are highly motivated to play, even when interacting with an unfamiliar peer (Panksepp, 1981), and anosmia does not inhibit social play (Thor & Holloway, 1982). Thus, in adulthood, rats are likely to need to adapt to interacting with a different suite of individuals. If the changes in the OFC found in rats that live with multiple partners leads to them being able to adapt their responses to those

individuals, then retaining that neural circuitry when encountering and having to adjust to novel individuals would seem maladaptive.

This then leads to the hypothesis that once a level of familiarity is reached with the rats they are reared with, the complexity of the OFC may be pruned, allowing for more proliferation to occur in response to confronting new partners. If so, this would suggest that the OFC in adult rats should increase in complexity when confronted with novel social partners. Some preliminary evidence supports this hypothesis. Adult rats were housed in pairs and every second day for 14 days they were removed from their cages which were cleaned, and then either returned to the cage with their established cage mate or paired with another rat. Rats with novel cage mates showed increased complexity of the dendritic arbor of the OFC compared to control rats given exposure to familiar colony members over the same time period (Hamilton, Silasi, Pellis, & Kolb, 2003). This study supports others showing naturally occurring pruning of the PFC (Koss et al., 2013; Markham, et al., 2007; Milstein et al., 2013), but suggests that the degree of such pruning depends of the functional demands of the brain areas involved.

Finally, our results have a more general importance for studies examining dendritic changes in response to a wide range of experiences such as drugs or leaning tasks. Clearly, the age and housing experience of the animals can influence the results.

Chapter 3: Juvenile play experience does not affect nicotine sensitization and

voluntary consumption of nicotine in adult rats[*]

3.1 Introduction

Social play behavior is the one of the earliest forms of non-mother directed social behavior in young mammals and peaks during the juvenile period (e.g., Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1990, 1997). This period is important for proper development as brain connections are still maturing and are particularly sensitive to experiences occurring during this period (Spear, 2000). If animals are deprived of social play interactions during the juvenile period, they exhibit inappropriate behavioral responses to a variety of social and non-social contexts (e.g., Arakawa, 2003; Einon & Morgan, 1977; van den Berg, Hol, Van Ree, Spruijt, Everts, & Koolhaas, 1999). Such findings support the theory that juvenile social play increases an animal's adaptability to unpredictable circumstances (Pellis, Pellis, & Bell, 2010; Špinka, Newberry, & Bekoff, 2001).

Rats that have experienced play as juveniles cope better in stressful (Von Frijtag, Schot, van den Bos, & Spruijt, 2002) and fearful situations (Arakawa, 2003; Da Silva, Ferreira, Carobrez Abe, & Morato, 1996; Lukkes, Mokin, Sholl, & Forster, 2009). In addition, when compared to play-deprived rats, those with prior play experience exhibit appropriate coping strategies when confronted with dominant animals (van den Berg, Hol et al., 1999; Von Frijtag et al., 2002) and also show better impulse control and

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decision making (Baarendse, Counotte, O'Donnell, & Vanderschuren, 2013). These experiences are so important, that even instability in social interactions during the juvenile period results in a reduced ability to regulate behavioral responses later in life to fearful situations and social interactions (Green, Barnes, & McCormick, 2012). Furthermore, animals with prior play experience are better able to modulate physiological reactions to a variety of stimuli, such as regulating the corticotropin-releasing factor in response to stressful situations (Lukkes, Summers, Scholl, Renner, & Forster, 2009).

In addition to behavioral flexibility and physiological adaptability, social play experiences influence changes in the brain. For example, if deprived of juvenile social play experiences cells in the medial prefrontal cortex (mPFC) exhibit a weakened sensitivity to dopamine (Baarendse et al., 2013). Moreover, social play experiences contribute to the apoptotic loss of mPFC neurons (Markham, Morris, & Juraska, 2007) and the pruning of dendritic arbor in some areas of the mPFC (Bell, Pellis, & Kolb, 2010). It has been hypothesized that the play-induced apoptosis and synaptic pruning would allow the mPFC to exhibit greater flexibility to subsequent plasticity inducing experiences and this appears to be the case (Himmler, Pellis, & Kolb, 2013b). When exposed to nicotine, a psychostimulant drug that alters plasticity in the mPFC (Brown & Kolb, 2001), rats that have had play experience as juveniles exhibit an increased dendritic arbor in the pyramidal neurons of the mPFC (Himmler et al., 2013b). While these findings suggest that there is play-induced priming of plasticity in the mPFC, whether there are associated changes in the behavior of such rats is unknown.

It has recently been shown that if animals receive pre-juvenile experiences,

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such as tactile stimulation in infancy, prior to exposure to the psychostimulant drug amphetamine, the behavioral effect of the drug is attenuated (Muhammad, Hossain, Pellis, & Kolb, 2011). Alternatively, there is evidence that suggests that if animals are deprived of social interactions during the juvenile stage, they are more vulnerable to addiction to psychostimulant drugs (Whitaker, Degoulet, & Morikawa, 2013).

Given that positive pre-juvenile experiences (i.e., tactile stimulation) attenuate the effects of psychoactive drugs, it is reasonable to predict that the experience of playful juvenile interactions would also attenuate the effects to later exposure to psychoactive drugs, such as nicotine. To test this hypothesis, two experiments were conducted. The first tested behavioral sensitization to injections of nicotine and the second examined the voluntarily consumption of nicotine. For the second experiment, both rats that had been pre-treated with nicotine injections and rats that had not were tested. This was done to determine whether the increased dendritic arbor in the neurons of the mPFC of rats with play experience when injected with nicotine (Himmler et al., 2013b) reflect compensatory changes in neural organization that provide enhanced protection against the effects of nicotine. If so, the rats with play experience that were injected with nicotine should ingest less nicotine voluntarily than either rats that did not have play experience or play experience and no pre-exposure to nicotine.

For both experiments, juvenile rats were reared in one of two social conditions: with three juvenile peers or with a single adult partner. The three peer condition provided ample opportunity for social play, whereas the adult-only condition provided opportunity for a variety of social experiences, such as grooming and huddling, but little play experience, as adults usually avoid engaging in play with

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juveniles (Einon, Morgan, & Kibbler, 1978). For several reasons females were used. First, given that males form dominance hierarchies (Adams & Boice, 1989) and these begin to form around puberty (Pellis & Pellis, 1991), females were used to avoid possible effects of dominance on subsequent brain-related changes. Second, using male juveniles to house with adult females would create problems around puberty as the males would likely be sexually attracted to the female (Pellis & Pellis, 1990), thus changing the experiences of the adult reared versus the juvenile reared rats. Third, adult males are less tolerant of unfamiliar juveniles than are adult females (Pellis, 2004, unpublished observations). Finally, using females in this paradigm has previously been shown to produce play-induced changes in the mPFC (Bell et al., 2010; Himmler et al., 2013b).

3.2 General Methods

3.2.1 Subjects and Experimental Groups

A total of 48 female Long-Evans hooded rats (LE) were used as experimental animals in these studies. Due to our experimental paradigm, an additional 24 LE rats served as the adult rearing partners for the no-play condition and an additional 72 LE rats served as the juvenile rearing partners for the play condition (see below). At 22 days of age, the weanlings were randomly selected for rearing in one of two conditions: a weanling was housed with three same-sex peers (the play experience condition) or with one adult female (the no-play experience condition). When subjects were 70 days old, half of each rearing group was randomly subdivided into two additional groups; a saline group and a nicotine group. Thus, in total, for both experiments, there were four separate groups; a no-play-saline group (NPS), a no-play-nicotine group (NPN), a

play- saline group (PS) and a play-nicotine group (PN). Each of the four groups had six subjects.

Although we did not formally measure the difference in the actual amount of play engaged in by the juveniles reared with an adult and the juveniles reared with other juveniles, casual inspection of the cages at various times during the day revealed that the rats in the all juvenile cages engaged in play, no play was ever seen in the cages composed of one juvenile and one adult. These observations are consistent with prior studies (Bell et al., 2010; Einon et al., 1978; Himmler et al., 2013b). Therefore, while we cannot conclude that the adult- reared juveniles never played, it is reasonable to conclude that the juveniles reared with other juveniles had much greater experience with play than did those reared with an adult.

3.2.2 Nicotine Administration

At 70 days of age, all experimental animals were either injected with nicotine (.3 mg/kg) or .9% saline subcutaneously into the nape of the neck, for 10 consecutive days. This dose was chosen as it has been shown to reliably produce dendritic remodeling in the mPFC (e.g., Gonzalez, Gharbawie, & Kolb, 2006; Hamilton & Kolb, 2005; Himmler et al., 2013b). All injections and subsequent behavior were tested during the rats' light phase of the day/night cycle.

3.3 Statistical Analyses

Data for both experiments were analyzed using repeated measures ANOVA using day as the repeated measure. For experiment 1, drug (nicotine or saline) and play (play or no play) were used as independent factors. For experiment 2, drug (nicotine or saline), play (play or no play) and nicotine concentration ingested (5 or 8 mg/ mL) were

used as independent factors. The least significant difference (LSD) post-hoc test was used for pair wise comparisons if further analysis of significant interactions were needed. Differences for all statistical tests were considered significant for p values of ≤.05.

3.4 Experiment 1: Nicotine Behavioral Sensitization

The effect of prior play experience on the behavioral response to repeated nicotine exposure was tested. Behavioral sensitization is characterized as an increase in motor activity following multiple injections of a variety of psychostimulants, including nicotine (for a review, Robinson & Berridge, 1993). The working hypothesis for this experiment is that play experience during the juvenile period should attenuate the effects of exposure to nicotine.

3.4.1 Subjects and Experimental Groups

Seventy-two female Long-Evans hooded rats were used in this study. Of these, 24 were used as the experimental animals; while the remaining animals were used as housing partners (see Subjects and Experimental Groups under General Methods). All experimental rats and their partners in the play-condition were born in the vivarium at the University of Lethbridge, Canadian Centre for Behavioural Neuroscience, and were derived from the remaining 12 subjects that were originally obtained from Charles River Laboratories (St. Constant, Quebec, Canada). These 12 also served as the adult partner for the no-play group. Each group was housed in a 46 cm x 25 cm x 20 cm polyethylene tub with processed corn- cob as bedding, and maintained at a constant 21–23°C on a 12:12 light–dark cycle. Food and water were provided ad libitum. All animals were handled and cared for in accordance with the Canadian Council for

Animal Care (CCAC) regulations.

3.4.2 Drug Sensitization.

In order to test the behavioral sensitization to nicotine, overall locomotor activity was measured, as this serves as an appropriate index for behavioral sensitization (Wise & Bozarth, 1987). Subjects were habituated to the activity boxes (Accuscan monitoring system) for 30 min and then received their respective solutions (nicotine/saline). Immediately following nicotine injections, animals were returned to the activity boxes and activity was measured for 60 min. Following 10 days of injections, all animals were given a 2-week withdrawal period and were then all given a challenge-testing day. In the challenge day, every group received nicotine injections (.3 mg/kg), in order to test for the persistence of behavioral sensitization to nicotine. All locomotion activity was collected using the VersaMax^{TM} program and was converted using Versa-Dat^{TM} software (AccuScan Instruments, Inc., Columbus, OH).

3.5 Experiment 2: Nicotine Self-Administration

The working hypothesis of this experiment was that prior play experience would attenuate the voluntary consumption of nicotine, and that given the greater brain changes to nicotine injections in rats that had play experience as juveniles (Himmler et al., 2013b), the combination of both play and pre-treatment with nicotine should produce greater attenuation to voluntary ingestion of nicotine than play experience alone.

3.5.1 Subjects and Experimental Groups

Seventy-two female Long-Evans hooded rats were used in this study. Of these, 24 were used as the experimental animals; while the remaining animals were used as housing partners (see Subjects and Experimental Groups under General Methods). All

animals were obtained from Charles River (Wilmington, MA) and were housed at Arkansas State University. Sixty of these animals were juveniles, whereas the additional 12 were adults. Juvenile animals arrived, with dams, on postnatal day 8, while the adults arrived at age 32–35 days. All animals were given 14 days to recover from shipping and to habituate and adapt to the laboratory environment. Each group was housed in a 46 cm x 25 cm x 20 cm polyethylene tub with processed corncob as bedding, and maintained at a constant 21–23˚C on a 12:12 light–dark cycle. Food and water were provided ad libitum. All procedures used were approved by the University IACUC and were in accordance with guidelines presented by the Office of Laboratory Animal Welfare.

3.5.2 Oral Self-Administered Nicotine.

Following nicotine injections (see Nicotine Administration Section), all experimental animals were given a 2-week withdrawal period and were then individually placed in clear polycarbonate home cages equipped with a 5 bottle drinking arrangement (nicotine or water) for 10 consecutive days. All procedures used in the voluntary nicotine consumption phase of the study followed the general method used by Biondolillo, Pearce, Louder, and McMickle (2009). This arrangement consisted of one bottle of water and four bottles of nicotine. Two of the bottles containing nicotine had a low concentration (5 mg/mL) and two had a high concentration (8 mg/mL). In order to avoid bottle placement preferences for the rats, the bottle configuration was counter-balanced across cages, although the placement remained consistent within individual cages. All solutions were held in 50 mL centrifuge tubes fitted with rubber stoppers and drinking spouts.

Nicotine solutions were created by diluting a nicotine base (Sigma Aldrich) in tap water at concentrations of 5 and 8 mg/mL nicotine base/water. Nicotine solutions were mixed as needed and stored in amber bottles until distributed to animals daily. Drinking bottles were filled with their respective solutions or water daily, weighted, and returned to home cages. Approximately 23.5 hr later bottles were removed, weighed again and a difference score was calculated by subtracting second weight from the first. The difference score was used to measure intake from individual bottles during the previous 23-hr period. These procedures have been shown to yield a reliable method for measuring voluntary fluid intake (Biondolillo & Pearce, 2007; Biondolillo, Pearce, Louder, & McMickle, 2009; Stolerman & Kumar, 1972).

All test subjects were exposed to 10 consecutive days of self-administration of nicotine, although due to an error in bottle weight measurements, one full day (Day 6) was removed from the final analysis. Thus, the data for experiment 2 represent a total of 9 days of self-administration.

3.6 Results

3.6.1 Experiment 1

A 2 x 2 x 10 repeated measures ANOVA of motor activity following injection on each of the 10 days revealed a main effect of day [$F_{(9, 180)} = 24.189$, $p < .001$] and drug [$F_{(9, 180)} = 14.726$, $p < .001$], but not a main effect for play ($p > .05$). The main effect for drug was an increase of activity in the nicotine group (Fig. 3.1). The effect of nicotine was evident from the first day of injections with a 2 x 2 ANOVA for the first day of injections also showing a significant main effect for drug [$F_{(1, 20)} = 19.012$, $p < .001$], but not for play ($p > .05$). The main effect for drug was an increase

in activity in the nicotine group. Similarly, a 2 x 2 ANOVA for the challenge day of behavioral sensitization revealed a significant main effect for drug [F $(1, 20) = 34.805$, $p < .001$], but not for play ($p > .05$). The main effect for drug was an increase in activity in the nicotine group. In none of the above analyses were there significant interactions ($p > .05$).
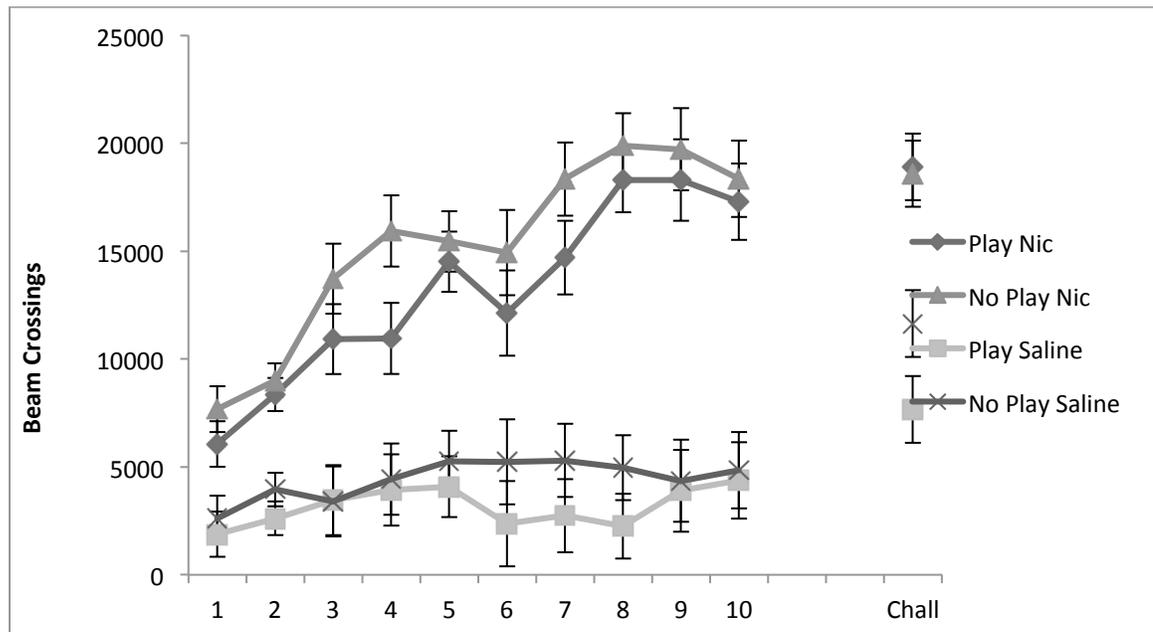
Figure 3.1

Mean (± SEM) locomotor activity recorded for 90 minutes following nicotine or saline injections for 10 days and the challenge day. Prior play experience had no effect on behavioral sensitization for any days.

3.6.2 Experiment 2

A 2 x 2 x 3 x 9 repeated measures ANOVA for the total amount of fluid consumed revealed a significant main effect of day [F $(8, 480) = 2.715$, $p = .006$] and for total fluid consumption [F $(16, 480) = 14.482$, $p < .001$], but not for drug pretreatment ($p > .05$) or for play ($p > .05$). There was a significant interaction

between day, play and total fluid consumption [$F_{(16, 480)} = 2.066$, $p = .009$]. Pairwise comparison revealed that the group without prior play experience consumed more water and less nicotine on Days 4 and 5 compared to the group with prior play experience (Fig. 3.2A). However, a problem with using absolute values for liquid consumption is that rats individually varied markedly in total fluid intake per day (lowest: .29 mL; highest: 24.46 mL). Therefore, the data were re-analyzed but this time the total proportion of liquid containing the two doses of nicotine was compared across groups.

A.   B.
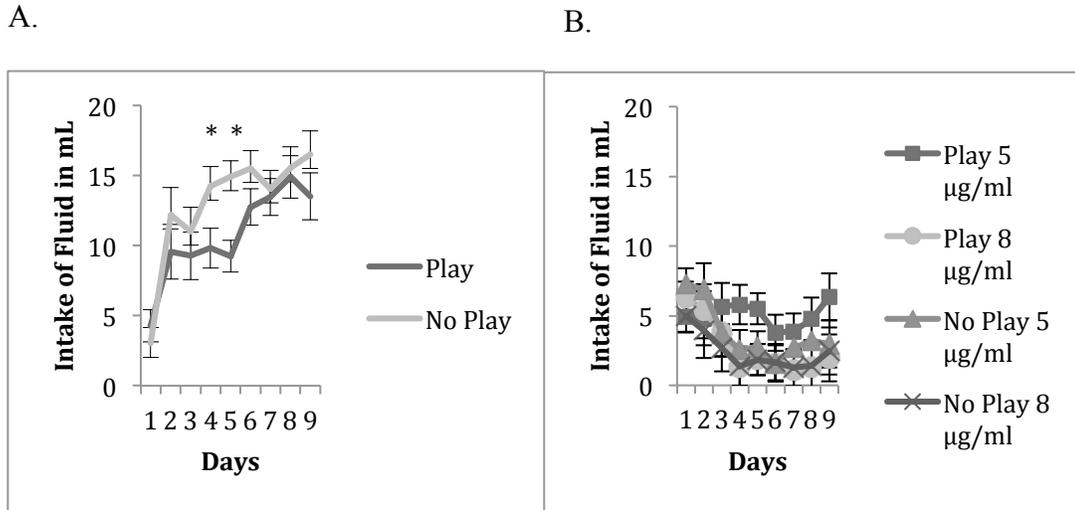
Figure 3.2
(a) Total fluid intake (mL) of water for rats with prior play experience and those without prior play experience. There was a difference in intake on Days 4 and 5, with rats receiving prior play consuming less water. Note that given that there was no difference between groups injected with nicotine and with saline, the data in this graph combines all rats pretreated with nicotine and saline into the play and non-play groups. (b) The total fluid intake of both the low (5 µg/ml) and high (8 µg/ml) dose of nicotine for rats with prior play experience and those without prior play experience. There were no differences between groups and the total intake of nicotine doses.

A 2 x 2 x 2 x 9 repeated measures ANOVA revealed a significant main effect of day [$F_{(8, 320)} = 19.580$, $p < .001$], but no main effect of drug pretreatment ($p > .05$), play ($p > .05$), or nicotine concentration ($p > .05$). There was a significant interaction between day, play and nicotine concentration [$F_{(8, 320)} = 3.552$, $p = .001$]. Pairwise comparison revealed that there was no difference between the animals with prior play experience and no prior play experience for the higher concentration of nicotine (8 mg/mL), but there were significant differences for consumption of the lower concentration (5 mg/mL) on some days. Animals with prior play experience drank less of the lower dose (5 mg/mL) on Day 1, but more on Days 4 and 5 ($p < .05$; Fig. 3.3A). On these same days, there was a significant preference for drinking the lower

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dose (5 mg/mL) compared to the higher dose (8 mg/mL) ($p < .05$). This implies that animals with prior play experience were drinking less of the lower dose of nicotine. To take into consideration the total amount of nicotine consumed rather than the fluid containing the nicotine the total mg of nicotine consumed was compared. A 2 x 9 repeated measures ANOVA showed a significant main effect of day [$F (8, 176) = 9.914$, $p < .001$], but no main effect for play ($p > .05$). Both groups decreased in total mg of nicotine consumed with a slight increase in the final days (Fig. 3.3b).

A.

B.
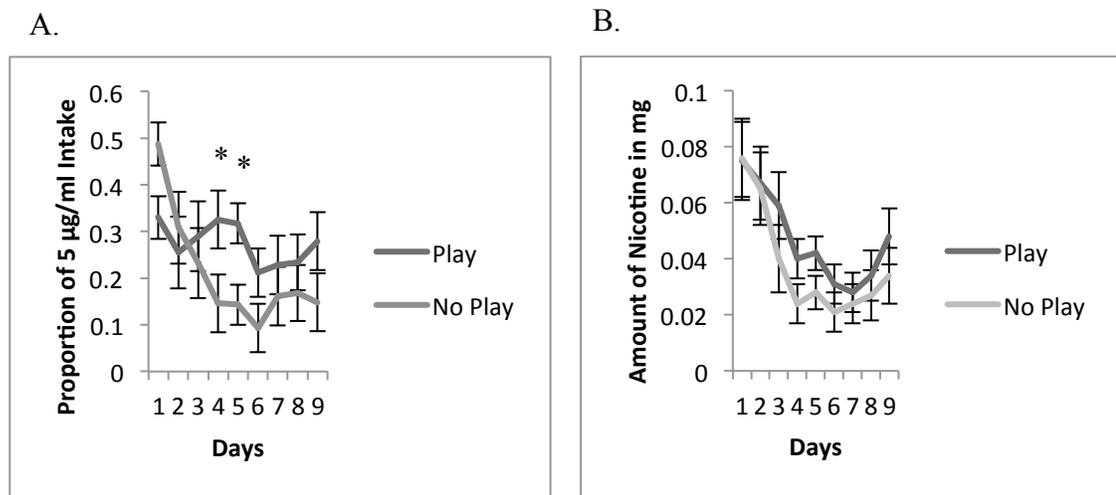
Figure 3.3
(a) The total proportion of the low dose of nicotine (5 µg/ml) consumed by groups with prior play experience or without prior play experience. The prior play group exhibited a decrease in consumption on day 1, but an increase on Days 4 & 5. (b) The amount of nicotine (mg) of nicotine consumed, combining the low and high concentration fluids, is shown for groups with and without prior play experience. There was no difference between groups.

3.7 Discussion

The present study investigated the effects of juvenile play experience on the response to nicotine exposure in adulthood. It was hypothesized that prior play experience would attenuate the effects of later nicotine exposure, both in behavioral

sensitization to the injections of the drug and also in the amount of nicotine that rats would consume voluntarily. Given the associated brain changes with combined prior play experience and nicotine exposure (Himmler et al., 2013b), it was further hypothesized that rats with play experience and pretreated with nicotine should show an enhanced attenuation of voluntary nicotine consumption. Neither of these hypotheses was supported. Prior play experience did not attenuate the motor effects induced by nicotine injections (Fig. 3.1), and prior play experience, either with or without pretreatment with nicotine, did not attenuate the oral consumption of nicotine (Figs. 3.2B and 3.3A,B). A possible reason for this lack of play-induced attenuation of response to nicotine is that juvenile play experience affects the development of brain areas that are not involved in the response to nicotine. This, however, seems unlikely given that both play and nicotine affect the development of the mPFC (Bell et al., 2010; Brown & Kolb, 2001; Himmler et al., 2013b). A more likely reason is that the play-induced changes in the mPFC are especially sensitive to social contexts given the marked social deficits in rats deprived of play as juveniles (e.g., van den Berg, Hol et al., 1999; Von Frijtag et al., 2002).

Nicotine given as a reward induce conditioned place preference (CPP) (e.g., Belluzzi, Lee, Oliff, & Leslie, 2004; Vastola, Douglas, Varlinska, & Spear, 2002) as does the opportunity to engage in social behavior (e.g., Calcagnetti & Schechter, 1992; Douglas, Varlinskaya, & Spear, 2004; van den Berg, Pijlman, Koning, Diergaarde, Van Ree, & Spruijt, 1999). Social reward combined with a low dose of nicotine produces a CPP in juvenile rats that is greater than either nicotine or social reward can produce independently (Thiel, Sanabria, & Neisewander, 2009). Moreover, nicotine can

affect the frequency of social play in juvenile rats (Thiel et al., 2009; Trezza, Baarendse, & Vanderschuren, 2009), suggesting that nicotine receptors affect the reward system that promotes play. Therefore, the effect of juvenile play behavior on later nicotine exposure may be revealed in social situations, not the non-social contexts used in the present study. Indeed, our animals were individually housed for the duration of the consumption of nicotine part of experiment 2, thus providing no opportunity for social influences.

The experience of juvenile play allows animals to exhibit improved regulation in response to both social and emotional situations (e.g., Arakawa, 2003; Von Frijtag et al., 2002). This may be, in part, due to the play-induced plasticity in the mPFC (Bell et al., 2010). The mPFC plays an important role in executive functioning, and if this area is damaged, animals exhibit deficits that resemble animals with play deprivation (Bell, McCaffrey, Forgie, Kolb, & Pellis, 2009). Recently, it has been shown that if animals with prior play experience are exposed to nicotine, there is a greater drug-related increase in the dendritic arbor of the mPFC (Himmler et al., 2013b). We interpret this as evidence that play makes the brain more responsive to later experiences (i.e., more plastic). Yet, the results of the present study show that play in the juvenile period does not affect behavioral sensitization and oral consumption of nicotine in adulthood. It is possible that the dose of nicotine used in this study may have been too large and therefore masked any changes due to prior play experience.

Studies using doses larger than the one administered in this study produce behavioral sensitization with fewer days of exposure, suggesting that sensitization is

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enhanced with higher doses (e.g., Li, DiFranza, Wellman, Kulkarni, & King, 2008; Reid, Ho, & Berger, 1996). The increased number of days required for behavioral sensitization at the dose used in our study (.3 mg/kg) may, nonetheless, have the same aggregate effect on brain changes and so the limited ability for prior play experience to attenuate the response to this drug. Smaller doses of nicotine (< .3 mg/kg) while less effective in producing sensitization, have been shown to induce conditioned place preference (see Le Foll & Goldberg, 2005), indicating that the drug is rewarding. Therefore, if rats with juvenile play experience were administered a smaller dose of nicotine, the possible attenuating effects of play experience may be more pronounced and prolonged and so detectable in the behavioral sensitization paradigm. The same applies to the voluntary intake of nicotine. Our results are consistent with past research, with rats generally preferring lower doses (Biondolillo et al., 2009). Doses lower than the lowest one used in the present study are also effective in inducing voluntary consumption (Biondolillo & Pearce, 2007; Flynn, Webster, & Ksir, 1989). Therefore, as for behavioral sensitization, it may be the case that by lowering the dose, the attenuation effect of juvenile play experience may become evident over a longer period of exposure to nicotine. Such an explanation, however, fails to account for why there was no initial attenuation of voluntary nicotine ingestion in the play experienced rats that were pretreated with saline, even if continued exposure to nicotine would end up swamping the play effect.

It may be the case that nicotine, compared to other psychostimulants, such as amphetamine (Muhammad et al., 2011), has a bigger effect on both brain and behavior. When compared to the effects seen in the nucleus accumbens following exposure to

amphetamine or cocaine, exposure to nicotine produces a 28% increase in overall dendritic length relative to 12% for cocaine or amphetamine (Brown & Kolb, 2001; Robinson & Kolb, 1999). The greater effect of nicotine on the brain compared to the other psychostimulants suggests that there may also be a larger change in the behavioral response to nicotine exposure. As a consequence, the use of the particular dose of nicotine in the present study may have resulted in producing a ceiling effect, reducing the capacity of play-induced brain changes to attenuate the behavioral response to this drug.

A surprising finding from the present study is that pretreatment with nicotine did not affect the oral consumption of nicotine. Given that nicotine drastically affects the plasticity of cells in numerous areas of the brain (Brown & Kolb, 2001) and repeated exposure results in the behavioral sensitization to the drug (e.g., Benwell & Balfour, 1992; Ksir, Hakan, Hall, & Kellar, 1985; present study), it appeared reasonable to assume that pretreatment with nicotine would influence the voluntary consumption of nicotine, whether there was a play effect or not. One possibility is that the nicotine solution was too bitter and thus the rats had an aversion in the consumption of nicotine. Indeed, our findings show that rats consumed more water than fluid containing nicotine on all days of testing (Fig. 3.2A,B). Therefore, it is possible that a pretreatment effect may become more evident if the oral solution was more dilute. Another possibility may be the age at which nicotine pretreatment is given. Whereas injections of nicotine during the periadolescence period (Days 34–43 lead to increased self-administration of nicotine in adulthood, injections during the postadolescence period (Days 60–69) do not (Adriani et al., 2003). Interestingly, the age at which the

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nicotine pretreatment is having an effect on later nicotine consumption is the same age at which play is having its effect on brain development (e.g., Baarendse et al., 2013; Bell et al., 2010; Himmler et al., 2013b; Markham et al., 2007), suggesting that the interaction of plasticity inducing experiences may be different when they co-occur in the juvenile period as opposed to when they occur before or after the juvenile period (Muhammad et al., 2011).

In sum, while prior play experience improves behavioral and physiological functioning in response to social contexts and emotion-inducing situations (e.g., Arakawa, 2003; Von Frijtag et al., 2002), as well as an enhancement of impulse control and decision making (Baarendse et al., 2013), our results suggest that prior play experience has little to no effect on the motor effects of nicotine exposure or the oral consumption of nicotine. However, this does not rule out the possibility of an interaction of play experience and nicotine treatment at different ages and in different behavioral contexts, especially social ones.

Chapter 4: The Role of the Medial Prefrontal Cortex in Regulating Interanimal

Coordination of Movements[†]

4.1 Introduction

For social species, including humans, interacting with conspecifics is a critical aspect of their day-to-day lives. A number of neural circuits are so critically involved in the organization of social behavior that this integrated circuitry has been labeled the "social brain" (e.g., Adolphs, 2009; Brothers, 1990). This circuitry includes, but is not limited to, the mesolimbic dopamine system, limbic structures, such as the amygdala, and areas of the frontal cortex. A major focus of research has been the involvement of the prefrontal cortex. The prefrontal cortex plays a large role in executive functions, such as monitoring behavior, attention, behavioral inhibition, planning, decision making and task switching (see Dalley, Cardinal, & Robbins, 2004, for a review), as well as impulse control (Baarendse, Counotte, O'Donnell, & Vanderschuren, 2013). The structure and function of the prefrontal cortex is influenced by a variety of social and nonsocial experiences during early development (Kolb, Mychasiuk, Muhammad, & Gibb, 2013), with one area, the medial prefrontal cortex (mPFC), being especially sensitive to peer–peer social interactions during the juvenile period (Baarendse et al., 2013; Bell, Pellis, & Kolb, 2010; Himmler, Pellis, & Kolb, 2013b).

The juvenile period is a critical time for the development of brain and behavior (Blakemore & Choudhury, 2006; Spear, 200), with the experience of social play with

peers being important for the development of behavioral flexibility and emotional regulation. For example, if rats are denied the opportunity to engage in playful interactions during the juvenile period, they either overreact or underreact to fearful or stressful contexts (Arakawa, 2003; da Silva, Ferreira, Carobrez Ade, & Morato, 1996; Lukkes, Mokin, Scholl, & Forster, 2009; Von Frijtag, Schot, van den Bos, & Spruijt, 2002) and exhibit weakened impulse control and decision making (Baarendse et al., 2013). These deficits are reflected in social interactions, with play-deprived rats failing to exhibit appropriate submissive behavior when confronted by a dominant male (van den Berg et al., 1999; Von Frijtag et al., 2002). Play-deprived rats also have difficulty in coordinating their movements with those of their social partners in both sexual and nonsexual contexts (Moore, 1985; Pellis, Field, & Whishaw, 1999).

At least some of these influences of play on social, cognitive, and emotional functioning may be mediated by changes in the mPFC (Baarendse et al., 2013). Indeed, in adult rats, the cells of their mPFC show evidence of extensive neural pruning as a result of the rats having had playful interactions in the juvenile period (Bell et al., 2010; Himmler et al., 2013b). Furthermore, if the mPFC is damaged in adulthood, the animals exhibit some of the same abnormal behavioral patterns as seen in adult rats that have been deprived of play as juveniles (Holson, 1986; Shah & Treit, 2003). For example, lesions to the mPFC affect the play behavior of rats (Bell, McCaffrey, Forgie, Kolb, & Pellis, 2009; Schneider & Koch, 2005). Even as adults, such rats initiate play and respond defensively to playful attacks at the same frequency as intact rats. However, although they are able to perform all defensive tactics, they mostly use simple tactics, such as evasion, during which the defender moves away from the attacker. More

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complex defensive tactics require multiple movements by the defender to occur in conjunction with movements made by the partner (Pellis & Pellis, 1987). Therefore, a hypothesis to account for this preference in using simpler tactics is that, in the absence of the mPFC, more sophisticated interanimal coordination is impaired (Bell et al., 2009). The present study tests this hypothesis.

A simple social task that critically depends on the performer coordinating its movements with that of a partner is robbing and dodging (Whishaw, 1988). In this paradigm, two rats are placed in a cylindrical test enclosure on a Plexiglas platform with a mirror placed at 45° to allow filming from below. Given that when one of the rats is given a piece of portable food, it will mostly defend it from the partner by producing lateral movements, this vantage point enables the movements of the two animals to be accurately measured in two dimensions (Field, Whishaw, & Pellis, 1996).

Understanding how animals execute and regulate their movements during dodging is important to understand how this test paradigm can be used to evaluate the hypothesis that the mPFC contributes to the regulation of one's movements with those of a social partner. The initial studies on robbing and dodging (Whishaw, 1988; Whishaw & Tomie, 1987, 1988) indicated that just as in food hoarding, rats are more likely to dodge and dodge at a greater magnitude when protecting food of higher quality (Whishaw & Tomie, 1989). This suggests that rats seek to maximize their distance from real or potential social competitor (Whishaw, Gorny, & Dringenberg, 1991). If this were true, the rat would need to calculate the magnitude of the lateral movement prior to its execution using an algorithm that included salient environmental factors such as the food's qualities (Whishaw & Gorny, 1994) as well as features of the social partner (Pellis

et al., 2006). Once the appropriately sized dodge angle is calculated, further modification

would be irrelevant, making dodges relatively ballistic in their execution. However,

detailed analyses of dodging movements in relation to those of the robber reveal that this

is not what a rat does when it dodges. Rather, the defender moves so as to gain and

maintain a particular distance between itself and the robber (Bell & Pellis, 2011). The

quality of the food may influence how close the robber approaches before dodging is

triggered, but factors like food quality do not influence the magnitude of the dodge.

What determines the absolute magnitude of the dodge is the magnitude of the

movement toward the defender by the robber, leading to a significant positive

correlation between the amount of movement by the dodger and the amount of

movement by the robber. However, irrespective of how much the robber moves, there

is no correlation between robber movement and interanimal distance. That is, the

defender moves in a manner that compensates for the robber's movement, thus keeping

the interanimal distance constant (Bell & Pellis, 2011). Thus, during dodging, the lateral

movement is not executed in a ballistic manner, based on a precalculated value, but

rather, emerges from an interaction between the movements of the defender and the

robber. This finding, that during dodging the performer moves so as to gain and

maintain a constant interanimal distance with the robber, has been replicated in

independent samples of intact rats (Bell, 2014; Bell & Pellis, 2011). Not only rats, but

also crickets, organisms with a simpler nervous system, protect portable food using the

same organizing principle (Bell, Judge, Johnson, Cade, & Pellis, 2012). During dodging,

then, the defender has to make moment-to- moment calibrations of interanimal

distance and make compensatory movements to regain and maintain the protected

distance. For these reasons, dodging provides a good test paradigm within which to evaluate whether the mPFC has a role in coordinating the performer's movements with those of a partner. Importantly, previous studies have shown that rats with lesions of the mPFC can dodge when protecting food (Whishaw, 1988; Whishaw & Oddie, 1989), but using a unilateral damage paradigm, what they also found was that the rats were more likely to have their food stolen on the side contralateral to the lesion (Whishaw, 1988). This suggests that, while these rats dodged to protect their food, without input from the mPFC the dodging was less effective. If our hypothesis that the mPFC contributes to interanimal coordination (Bell et al., 2009) is correct, then one reason for this increased failure to protect their food may be because they are less able to coordinate their dodges with the movements of the robber on the side with the damaged mPFC. The present paper tests whether this is the case.

Just as how temperature is regulated by a thermostat, food- protecting dodges occur in the context of a cybernetic system, so it is important to understand what kind of evidence would reveal a reduction in interanimal coordination. Consider a thermostat that is regulating a room that has the preferred temperature set at 20 °C; however, if the actual temperature drops below 20 °C, this would trigger the turning on of the furnace, and warm air would be pumped into the room until the ambient temperature reaches 20 °C again, at which time, the furnace would switch off. However, if the thermostat can only detect temperature changes of 2:5 °C, there would be large fluctuations in the room's temperature, as the furnace would not turn on until the room temperature dropped to 15 °C and then would not switch off until the ambient temperature of the room was well over 20 °C. In contrast, if the thermostat can detect a

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1 °C change in room temperature, then only a small decrease from the preferred temperature would be needed for the furnace to turn on and off. Note that in both cases, the average room temperature would be 20 °C, what would differ would be the magnitude of the ranging behavior, that is, the moment-to-moment variability in room temperature. This is exactly what has been found for the regulation of body temperature in rats. When a rat's brainstem is disconnected from its cortex, its body temperature fluctuates to greater highs and lows than when the cortex is involved (Satinoff, 1978). Thus, it is not that in the absence of the cortex the rat is not capable of thermoregulation; what happens is that without its cortex, its ability to keep its body temperature under tight control is compromised. Similarly, following damage to the mPFC, we fully expect the rats to be able to dodge, but what is predicted to occur is that the close fit between the movements of the robber and the compensating movements of the rat with brain damage would be compromised. The measures scored are designed to detect such loss of coordination.

If damage to the mPFC diminishes interanimal coordination, then, in the food protection paradigm, rats with such lesions should have specific disturbances to their dodging behavior. First, as found in a previous study (Whishaw, 1988), rats with mPFC lesions should be less able to defend their food item than control rats. Second, their ability to gain and maintain their particular distance from a robber should be diminished. This should be reflected in two ways. (a) With very large or very rapid movements by a robber, the defender may fail to adequately compensate and so not be able to maintain the interanimal distance, leading to the interanimal distance being correlated with the robber's movements. (b) During a dodge, the moment-to-moment

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interanimal distance should be more variable in rats with lesions as they fail to compensate for the robber's movements adequately. Third, if the compensatory movements in the dodging of rats with mPFC damage are diminished, then the strength of the correlation between the movements by the defender and the robber should be increased. This last prediction appears counterintuitive, as the rats with brain damage are expected to show an improved correlation. However, it must be remembered that, in intact animals, the correlation is a product of the movements of both the robber, who is tracking the food, and the dodger, who is evading the tracking of the robber. If the dodger were less able to adjust its movements appropriately to those of the intact robber, the robber would be better able to track the dodger's movements, increasing the correlation between the movements of the two animals. So, a shorter or longer magnitude dodge by the defender would lead to a more precisely coordinated movement by the robber. Therefore, the predicted higher correlation in the lesion group would arise from the robber being better able to match its movements to those of the defender. But, in turn, the improved correlation would reflect a loss of performance by the rats with mPFC lesions.

Although these predicted changes in food protection would be consistent with the hypothesis that the mPFC contributes to inter- animal coordination, by themselves, they do not address how the mPFC plays this role. As rats with lesions of the mPFC play just as frequently as intact rats and are able to perform all the movements that intact rats can, it was hypothesized that their reduced use of the more complex defensive tactics is due to their greater difficulty in coordinating their movements with those of their partner (Bell et al., 2009). The implication of this hypothesis is that it

involves some higher-order cognitive integration, whereby the performer calibrates its position relative to its partner and then updates that calibration during the course of the dodge. However, studies have shown that rats with damage to the mPFC can suffer from sensory neglect (Johnson, Traver, Hoffman, Harrison, & Herman, 2013) and can have impaired fine motor skills (Kolb & Whishaw, 1983; Whishaw, Pellis, & Gorny, 1992). Therefore, the reduced ability to coordinate their movements with those of their partner in the robbing-and-dodging paradigm may simply reflect sensorimotor deficits, rather than deficits in higher-order cognitive function. Thus, in addition to the measurements taken during robbing and dodging to assess disturbances in interanimal coordination (described earlier), the animals were also assessed for sensory and motor deficits. Rats with impaired medium-to-long-range distance sensors (e.g., vision, vibrissae) begin to dodge when the robber touches the defender's fur or even when its snout is pressed against the defender. This sensory deficit is reflected in a reduced inter-snout distance between the robber and the defender at the moment dodging begins (Pellis et al., 1996). Similarly, rats with sensory neglect due to brain damage are inattentive to stimuli and need more intense stimulation than normally required to respond (Marshall, Turner, & Teitelbaum, 1971). Therefore, if rats with mPFC lesions begin their dodging at a shorter distance from the snout of the robber than do intact rats, this would provide evidence for sensory neglect in the rats with brain damage. To assess whether the rats with mPFC lesions had deficits in motor control, two approaches were used.

First, prior to the lesions, rats were trained in the robbing-and- dodging task until they all consistently dodged away from the robber and retained their food item

(see criteria detailed below). In this way, a decrease in interanimal coordination could not be because of the mPFC's role in the acquisition of new movements, but rather, due to the disruption of well-habituated movements. Also, during dodges, the total amount of movement and the velocity of the movements were calculated. It would be expected that motor deficits would result in smaller and slower movements. Second, following completion of the robbing-and-dodging task, these rats were tested on a task requiring fine motor skills of the forelimbs, which are known to be impaired following mPFC damage (Kolb & Whishaw, 1983; Whishaw et al., 1992). The motor task used was the sunflower seed-husking test, which provides a simple measure for motor disruption in the upper limbs and forepaws (Gomez, Santiago-Mejia, Ventura-Martinez, & Rodriguez, 2006; Whishaw, Sama, & Pellis, 1998). Briefly, once a rat receives a sunflower seed, it will grasp it with its forepaws and use its digits to manipulate the seed while ripping at the shell with its teeth. Initially, naïve rats will break the seed husk into multiple fragments, but after several trials, the rats will peel the shell of the seeds in two separate pieces. Thus, over trials, the average number of pieces of shell per seed decreases. This reduction in the number of seed pieces husked is associated with the development of a more stereotyped sequence of movements, making the husking procedure more economical in its execution (Whishaw et al., 1998). Therefore, the reduction in number of shell fragments can be interpreted as an increase is motor skill and so be used as a measure of motor performance (Gomez et al., 2006). Because it takes several trials over several days for a high level of performance to develop (Whishaw et al., 1998), by training and testing the rats after the lesion, both acquisition and production problems arising from the mPFC damage would be detected. Therefore, if damage to the mPFC

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produces persistent difficulty in husking seeds—a nonsocial task, then this would

provide evidence for a general motor deficit contributing to the deficits in the social

task of robbing and dodging.

4.2 Method

4.2.1 Subjects

Thirty-two male Long-Evans rats were used in this study. Of these, 16 (70 days

old) were used as experimental animals and 16 (80 days old) served as partners for the

robbing-and-dodging paradigm (see Robbing-and-Dodging Paradigm section). All

subjects were obtained from Charles River Laboratories (St. Constant, Quebec) and were

housed at the vivarium at the University of Lethbridge, Canadian Centre for

Behavioral Neuroscience. Upon arrival, the rats were given 3 days to habituate to the

new surroundings. The rats were then weighed and the experimental animals were

paired with an older partner, who was the robber in the robbing-and-dodging trials. Pairs

were housed together for four days prior to beginning the experiment. Each pair was

housed in a (46 cm x 25 cm x 20 cm) polyethylene tub with processed corn- cob as

bedding and maintained at a constant 21–23 °C on a 12:12 light–dark cycle. Food and

water were provided ad libitum. To ensure that the rats would protect their food in the

robbing-and- dodging experiment, they were then food deprived to 85–90% of their

original body weight (Whishaw, 1988). Rat weights were recorded daily. All animals

were handled and cared for in accordance with the Canadian Council for Animal Care

regulations.

4.2.2 Experimental Protocol

The pairs were trained on the robbing-and-dodging task prior to surgery. Trials were conducted daily and lasted for 15 days until all dodgers consistently defended their food. The experimental rat from each pair then underwent surgery. For pairs containing the experimental rats, the subject received bilateral lesions of the mPFC and for the pairs containing the control rats, the subject received sham lesions. Following 26 days of recovery and the reestablishment of food deprivation, the pairs were again trained on the robbing-and-dodging task for 11 consecutive days, with the final day used as the test day to measure the appropriate parameters of the dodge (see Robbing-and-Dodging Paradigm section). The day following the test day for dodging, the experimental and control rats were tested individually in the seed-husking task. The rats received daily trials for 9 days. At the completion of the behavioral testing, the experimental rats were sacrificed for histological analysis of the brain lesions.

Before proceeding, a brief justification is needed as for why we choose to use bilateral lesions rather than unilateral ones. For bilaterally organized brain systems, it is often useful to lesion one side and so use the intact side as the control condition. With such a matched sample, greater statistical power is achieved with fewer subjects. Indeed, some previous studies involving the robbing-and- dodging task used unilateral lesions (Whishaw, 1988), including the mPFC (Whishaw & Oddie, 1989). However, the unilateral strategy can have some logistical problems when trying to obtain high quality sequences of dodging for detailed analysis. For example, in using the unilateral strategy for studying the role of the orbital frontal cortex in regulating dodging, only a subset of the rats ended up providing sufficient dodges for statistical comparison of the

intact and damaged sides. The intact robber switched to attacking the contralateral side—the impaired side. But, then, the rat that was dodging learned to keep its damaged side pressed against the wall, thus preventing the robber from accessing the more vulnerable side (Pellis et al., 2006). Thus, for the present study, as we were uncertain as to the magnitude of the impairment produced by lesions of the mPFC, bilateral lesions were used, with the statistical comparison being between the rats with lesions and the sham-treated controls.

4.2.3 Surgery

All 16 experimental rats were anesthetized with isoflourane via a nose cone and were placed in a stereotaxic apparatus. Once the skull was uncovered, it was drilled with a 0.5-mm bit in order to keep the dura intact. For the rats receiving lesions, the neocortex was exposed by removing the skull with rongeurs from the bregmoidal junction anteriorly to the frontal bone suture and laterally ~2 mm from the midline on each side. The medial prefrontal cortex was then removed by aspiration with a glass pipette with the aid of a surgical microscope. The surgeries were intended to remove the anterior portion (in front of the bregma) of Zille's Cg1 and Cg3 as well as the infralimbic cortex. The incision was then sutured with 3–0 Vicryl and the rats were given subcutaneous injections of buprenorphine (0.04 mg/kg) and Metacam (2.0 mg/kg) to alleviate pain and inflammation, as well as saline (5 ml) for rehydration. Half of the sham animals ($n = 4$) were anesthetized, received a small incision on the base of the skull and were then sutured with 3–0 Vicryl. The other half of the sham group ($n = 4$) remained undisturbed. Following the surgeries, rats were placed individually into a standard polyethylene tub (46 cm x 25 cm x 20 cm) with Softzorb® bedding over a

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heating pad for recovery. Once recovered, the rats were reestablished in their home cage with their partners and were placed back on food restriction. One of the rats with a lesion of the mPFC had to be euthanized due to surgical complications, resulting in our experimental group consisting of seven pairs.

4.2.4 Robbing-and-Dodging Paradigm

The robbing-and-dodging apparatus consisted of cylindrical Plexiglas arena that was 40 cm in diameter and 45 cm high, sitting on a Plexiglas platform, with a 45° angle mirror placed below the arena in order to obtain a ventral view of the animals (Field et al., 1996; Pinel, Jones, & Whishaw, 1992). All trials were filmed using a DVD103 Sony Handycam.

Each pair of rats had a designated robber (the oldest of the pair), with their partner serving as the experimental subject. For each training day, rats were initially habituated to the arena for 5 min and were each given half an almond in order to promote motivation to obtain the almonds. Following habituation, the experimental subject from each pair was given almond halves until either 10 dodges were performed or the rat was given a maximum of five almond halves. All pairs underwent a training period of 15 days until they consistently produced 10 dodges with less than five almond halves. Following training, the experimental subject from each pair was then subjected to surgical procedures (see Surgery section). Following recovery, all pairs were retrained on the task for 11 days until the performance of the control group reached the previous asymptote in performance, and the final day of training was recorded as the test day. In order to measure retraining performance, the number of almond halves

used to reach the criterion of 10 dodges/trial was measured. Food access was then restored to the rats ad libitum.

In order to analyze robbing and dodging, all videos for each trial were played back and the first six dodges for each subject were selected by the experimenter. Each dodge was chosen in accordance with the criteria previously established by Bell and Pellis (2011) to ensure that the dodges used were not interrupted by the subject being pressed up against the enclosure wall and that they could be observed from beginning to end. Once all of the dodges were selected, they were analyzed frame-by-frame, using Vicon Motus digital tracking software (Vicon Motion Systems, Denver, CO). There were three locations on both the robber and dodger that were tagged and tracked throughout the entire clip: the tip of the snout, the midpoint on the nape of the neck, and the base of the tail. Using these points, the Vicon Motus system was used to calculate several parameters of the dodge: (a) interanimal distance, measured as the distance between the snout of the dodger and the snout of the robber (Bell & Pellis, 2011); (b) the amount of movement by each of the partners, measured by the total path length traveled (Bell et al., 2012); and (c) the maximum instantaneous velocity of movements performed by each of the partners, measured when the maximum was achieved in each trial (Bell & Pellis, 2011; Bell et al., 2012).

4.2.5 Seed-Husking Paradigm

Using the same experimental animals (seven lesion and eight control), all rats were habituated to the sunflower seeds by receiving five seeds in their home cage the day before testing in the seed-husking enclosure began.

All trials were conducted in a Plexiglas box (25 cm x 28 cm x 31 cm) one side constructed by wire mesh, a metal bar floor, and a Plexiglas lid. As the rats husked the sunflower seeds, the pieces fell through the metal rungs on the floor, which allowed for easy access by the experimenter to all of the pieces. For every trial, each rat received five sunflower seeds and the shell pieces of each seed were collected. All shell pieces were then wrapped in foil and labeled for later analysis. All rats were tested in these conditions for 9 days.

4.2.6 Histology and Anatomy

Following completion of all behavioral testing, subjects with lesions ($n = 7$) were deeply anesthetized with sodium pentobarbital and were then perfused with 0.9% saline and then 4% formalin. The brains were then subsequently harvested and immediately placed in 4% formalin. After 24 hours, the brains were then transferred to a solution that contained 4% formalin and 30% sucrose. Brains were then sliced on a cryostat into 40 μm coronal sections. Every fifth section was used and sections were placed onto 1% gel, 0.2% chromium aluminum-dipped glass slides. Sections were stained with cresyl violet.

4.2.7 Behavioral Analyses

The robbing-and-dodging experiment consisted of eight controls (sham-treated) and seven experimental animals, and they were trained for 11 consecutive days (with the final day being used as a test day) starting on the 26th day after surgery, with six trials per animal. The number of times a rat dropped a piece of food or had it successfully stolen was recorded daily for all trials. The values were summed for each rat and these were used to calculate group means (± standard deviations) for the control

rats and the rats with mPFC lesions. Based on previous findings (Whishaw, 1988), it was expected that the rats with mPFC lesions should suffer more losses. The actual performance when dodging was assessed was on the 11th of testing. On this day, for one of the control animals, only four viable dodges were available (i.e., dodges that met the criteria for selection—see Robbing-and-Dodging Paradigm section), and for another, there were only five. For three of the experimental animals, there were only five dodges available per animal.

The total movement (path length) of both the robber and dodger was calculated, as well as the distance between the snouts of the partners, the intersnout distance at the beginning of the dodge and the maximum velocity reached during a dodge by both partners. As detailed elsewhere (Bell & Pellis, 2011; Bell et al., 2012), from the digitized trials, the correlation between the movements of robbers and dodgers could be assessed, but given the critical importance of the predicted change in the capacity of the rats with mPFC lesions to maintain a constant interanimal distance (see Introduction), a second analytical procedure was used to test this deficit further. The rats with the mPFC lesions should be less able to maintain a constant interanimal distance, and this should lead to greater variability in interanimal distance during dodges. To measure this, the longest duration dodge by each rat was selected and the interanimal distance on each video frame was measured. These values were then used to calculate a mean and a standard deviation for each dodge, with greater magnitude oscillations away from the mean being reflected in larger standard deviations. To compare the variability between the experimental and the control rats, the coefficient of variation (CV: standard deviation/mean) was calculated as a measure of the variability in

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interanimal distance in each dodge. Then, the mean CV per group was calculated and compared, with the expectation being that the mean CV in the group containing the rats with the lesions of the mPFC should be greater than that of the control rats.

For the seed-husking experiment, the number of pieces of shell fragments for each seed was counted and group averages were calculated for each of the 9 consecutive days of testing.

4.3 Statistical Analyses

For the possible correlations between dodger and robber in the food protection task, Pearson's r was used to assess the strength of the relationship. For comparisons of single measures between the control and brain-damaged rats, one-tailed t tests were used for cases in which the direction of the difference was predicted, and two-tailed tests were used when direction was not predicted. Finally, an ANOVA was used to analyze the daily seed data for the two groups, using day as the repeated measure and group (lesion or sham) as the independent factor. The least significant difference post hoc test was used for pair-wise comparisons if further analysis of significant interactions was needed. Differences for all statistical tests were considered significant at $p < 0.05$.

4.4 Results

4.4.1 Anatomy of the Brain Lesions

As shown in Figure 4.1, the lesions removed the anterior portions of Zilles' Cg1, Cg3, IL, and Fr1. In all cases, there was sparing of the more posterior part of IL. In no case was there damage to the forelimb area or motor cortex. There was subtle degeneration in the lateral regions of the dorsomedial thalamic nucleus in about half of

the cases, although there was no obvious relationship between lesion extent and

dorsomedial thalamic nucleus degeneration.
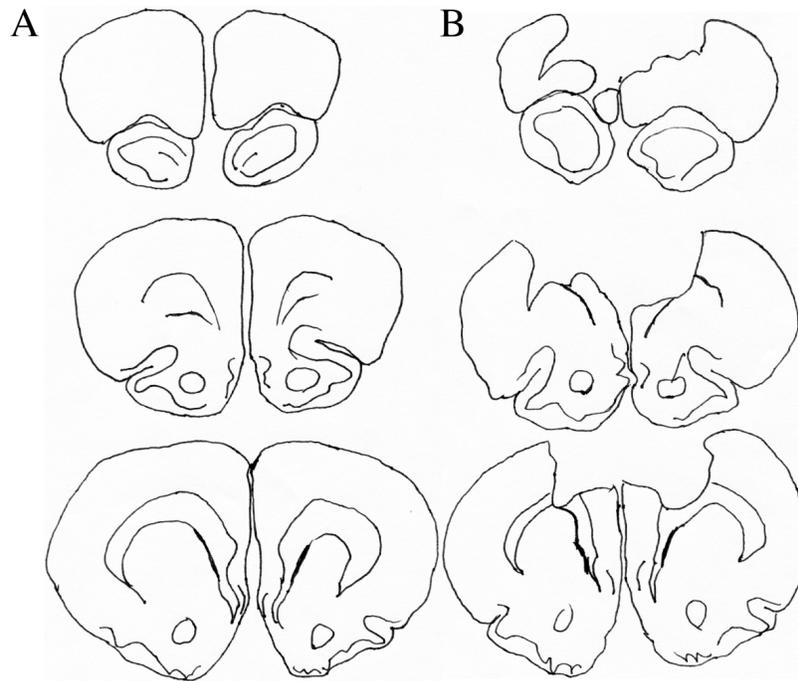
Figure 4.1
Representative coronal sections of the mPFC at three levels are shown for a non-brain damaged rat (A) and for a rat with an mPFC lesion (B).

4.4.2 Performance in the Food-Protection Task

The rats with lesions of the mPFC were just as likely to dodge when approached

by a robber, and as shown below, most of the parameters of the dodges were similar

across the experimental and control rats. What was of particular interest for the present

study was whether the dodges by the rats with mPFC lesions were as well coordinated

with the movements of the robber as were the dodges of the intact rats.

The 2 x 9 repeated-measures ANOVA for the number of almonds used to achieve the criterion of performing 10 dodges/ trial (see Robbing-and-Dodging Paradigm section) during the training phase did not reveal significant group, day or day-by- group interaction, effects ($p > .05$). These data suggest that there was no difference between groups in retraining performance with regard to how rapidly they achieved the presurgery levels of executing dodges. For both groups, it took between 3 and 3.5 almond halves to obtain the desired 10 dodges (see Figure 4.2).
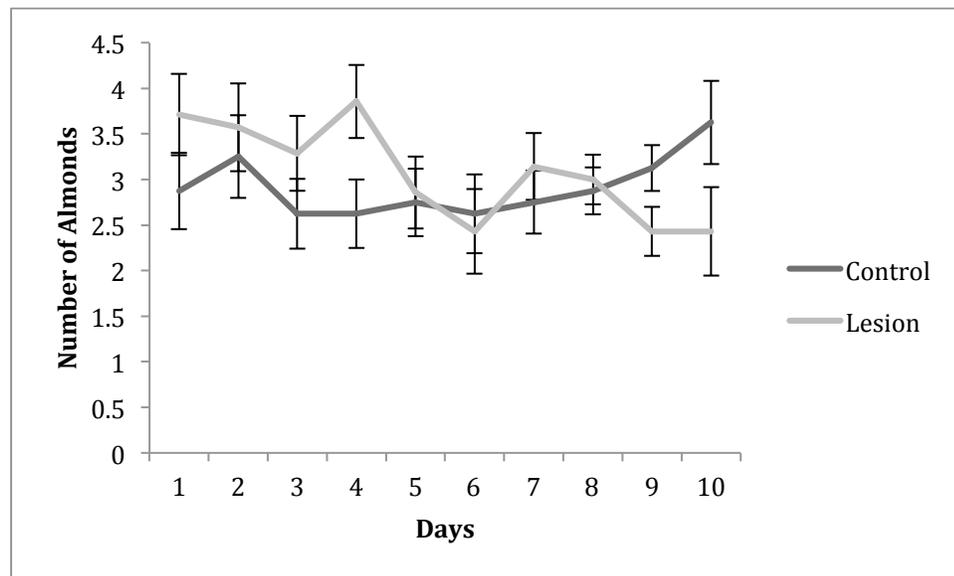
Figure 4.2
The mean number of almonds used to meet the criterion of 10 dodges/trial when protecting the food from a robber on post-lesion re-training days.

The control rats had fewer losses of food per trial (mean ± standard deviation: 0.375 ± 0.5) than did the rats with mPFC lesions (5.43 ± 6.1), with the difference being significant, $t(13) = -2.17$, $p < .05$. Also, whereas none of the losses in the controls were due to successful robbing by the partner, 85.7% of the

losses in the rats with lesions resulted from the food being stolen. Moreover, losses persisted over training days, with three of the rats with lesions suffering losses on the final day of testing. On the final test day, analysis of the dodges showed that there was no significant correlation between interanimal distance and robber movement for the control group, $r(43) = -0.036$, $p > .05$ (Figure 4.3A), but there was a significant negative correlation for the experimental group, $r(37) = -0.418$, $p < .01$ (Figure 4.3B). For dodger and robber movements, there was a significant correlation for both control, $r(43) = 0.869$, $p < .001$ (Figure 4.3C), and experimental groups, $r(37) = 0.980$, $p < .001$ (Figure 4.3D).
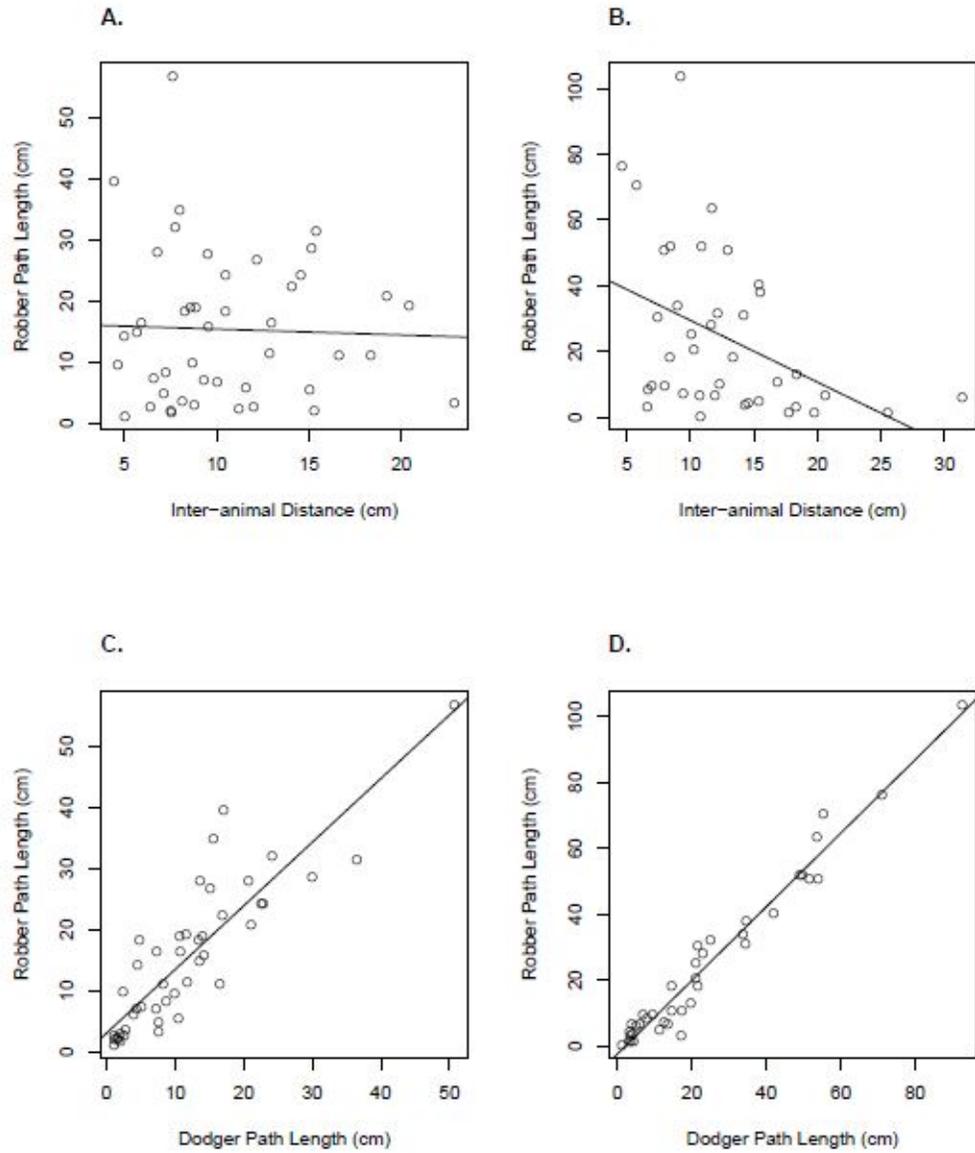
Figure 4.3
Scatterplots and lines of best fit are shown for the final inter-animal distance and the movement of the robber in the (A) control sham group and (B) the mPFC lesion group, and for the movements (path lengths) of the robbers and the dodgers in the (C) control sham group and (D) the mPFC lesion group.

Analysis of interanimal distance over the course of a single dodge revealed that this was more variable in the rats with mPFC lesions than control rats. The variability in the mean coefficient of variation (CV) was smaller in the control rats (CV (mean $\pm$ standard deviation): 0.389 ± 0.134) than the rats with mPFC lesions (0.571 ± 0.221), with the difference being significant, $t(13) = -1.91, p < .05$. The difference in the distance at the initiation of the dodging was not significant between control and experimental rats ($p > .05$), nor was the distance maintained during the dodge ($p > .05$). Robbers traveled further, $t(53.393) = 2.098, p < .05$, but not faster ($p > .05$) when approaching rats with mPFC lesions (see Table 1). In contrast, rats with mPFC lesions moved further, $t(52.018) = 3.232, p < .01$, and with greater velocity, $t(69.586) = 4.914, p < .001$, when dodging than did the control rats (see Table 4.1).

Table 4.1: Various parameters measured during dodges

Measures (mean $\pm$ SD[1])	Sham-treated controls	mPFC lesion rats
Start distance (cm)	11.601 ± 4.335	12.032 ± 4.335
Maintained distance (cm)[2]	10.526 ± 4.448	12.697 ± 5.516
Robber path length (cm)*[3]	15.303 ± 12.121	24.453 ± 24.794
Dodger path length (cm)*	11.815 ± 10.113	24.074 ± 21.732
Robber max velocity (cm/s)[3]	43.794 ± 13.356	44.001 ± 17.074
Dodger max velocity (cm/s)*	40.909 ± 18.899	65.159 ± 25.300

1. Sample standard deviation
2. As calculated in Bell & Pellis (2011)
3. As calculated in Bell et al. (2012)
*Significant group difference (p < 0.05) based on independent-samples t-tests

4.4.3 Performance in the Seed-Husking Task

The 2 x 9 repeated-measures ANOVA for the number of pieces into which the shells of sunflower seeds were shredded revealed significant main effect of day $F(8, 104) = 20.474, p < .001$ and a significant day-by-group interaction $F(8, 104) = 2.290, p = .027$, but not a significant group effect $(p > .05)$. Pairwise comparison revealed a significant group difference on Day 2 $(p = .004)$. However, because this test violated sphericity (as indicated by Mauchley's W), the values for the degrees of freedom were changed with the Greenhouse- Geisser correction. The corrected analyses continued to show an absence of a significant group effect $(p > .05)$ and a significant main effect of day, $F(3.915, 50.896) = 20.474, p < .001$. However, following this correction, there was no longer a significant day-by-group interaction $(p > .05;$ see Figure 4.4).
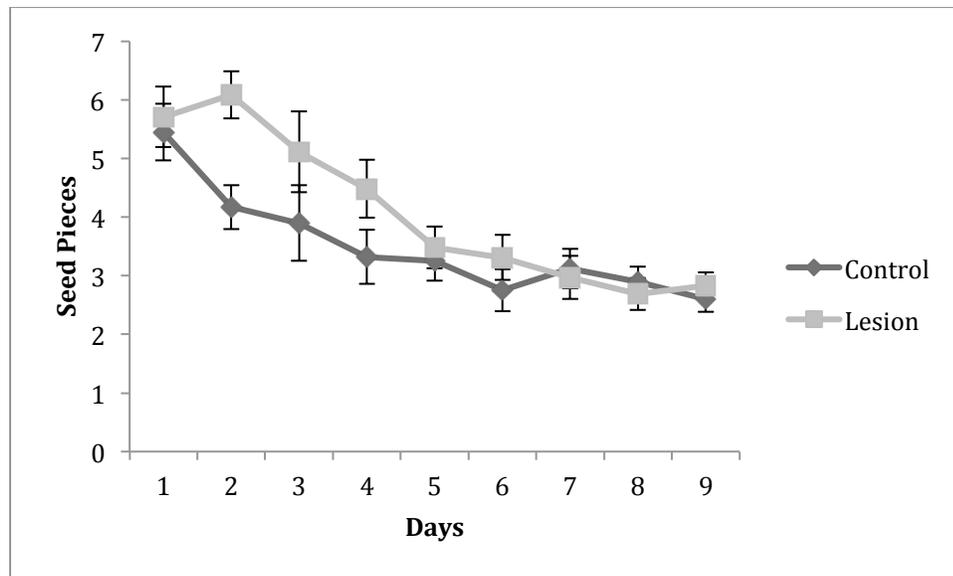
Figure 4.4
The mean number of seed pieces husked per day by sham and lesion groups.

4.5 Discussion

During the juvenile period, rats that do not actively engage in social interactions with their peers are impoverished in their ability to coordinate their movements with their social partners (Moore, 1985; Pellis et al., 1999) and fail to prune the dendritic arbor of the neurons of the medial prefrontal cortex (Bell et al., 2010; Himmler et al., 2013b). Because the rats with lesions of the mPFC preferentially use simpler defensive tactics during play fighting (Bell et al., 2009), the present study tested the hypothesis that the mPFC has a role in the coordination of movements with a partner. To test this hypothesis, the food protection task, in which one rat defends a portable food item from another rat, was used (Whishaw, 1988). Previous work has shown that, during food protection, one rat (the defender) dodges laterally away from its partner (the robber) and, while doing so, gains and maintains a particular interanimal distance. The maintenance of such a preferred distance requires the defender to compensate for the movement of the robber effectively (Bell & Pellis, 2011; Bell et al., 2012). If the mPFC is important for interanimal coordination, then it was predicted that rats with mPFC lesions should (a) be less successful in protecting their food from the robber, (b) display a diminished ability to gain and maintain a constant distance from the robber, and (c) have a strengthened correlation of their movements with those of the dodger. The results support these predictions.

The data show that rats with lesions of the mPFC are less able to protect their food with over 80% of their almond pieces that were lost being successfully taken by the robber. In contrast, robbers rarely succeed in stealing food from an intact rat (Field et al., 1996; Whishaw, 1988). In this study, the intact rats did not have a single food item

stolen. When dodging, the rats with mPFC lesions failed to maintain a constant distance from the robber. Rather, interanimal distance was correlated with the robbers' movements, unlike the case for the sham-treated control rats, and intact rats from other studies (Bell & Pellis, 2011). Moreover, interanimal distance varied more during dodges in the rats with mPFC lesions, suggesting a failure in them to compensate adequately for the robber's movements at a moment-to-moment level. Also, while the movements between the movements of the robbers and dodgers were positively correlated in both the control and experimental groups, the strength of the correlation was even greater in pairs of rats in which one had an mPFC lesion. The latter finding suggests that the intact robber is better able to exploit the failure of the brain-damaged rat to move adequately. Thus, all three predictions were supported by the findings, suggesting that the damage to the mPFC compromises the ability of those rats to coordinate their movements with a social partner.

Even though all the data from the robbing-and-dodging analysis converge on pointing to the main deficit caused by damage to the mPFC as being a diminution of a rat's ability to coordinate its movements with those of its partner, this paradigm has some limitations in characterizing how the mPFC may contribute to this ability. The main limitation arises from the short duration of dodges. As dodges can last as little as a fraction of a second, few episodes of movements are available in which the lag in response by one partner can be matched to the lag in the other and so provide the sample sizes needed for more sophisticated statistical analyses. A task requiring more prolonged sequences of coordinated movement would provide a richer data set with which to explore how and when the damage to the mPFC impaired interanimal

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coordination. One such task involves rats cooperating, rather than the competing as in the robbing-and-dodging task. In this cooperative task, the rats are rewarded for shuttling together across an open field, with the length traveled and paths taken readily shaped by adjusting the reward (Schuster, 2002). This means that long trains of coordinated movement ensue. If our findings are correct, then rats with lesions of the mPFC should be less able to coordinate interanimal movements, especially as the duration of the required coordination is increased. Small errors would be gradually compounded without the calibrations provided by the mPFC. To confirm our findings, the role the mPFC in interanimal coordination needs to be confirmed by such additional test paradigms.

4.5.1 The mPFC and Interanimal Coordination

The reduced ability of the rats with mPFC lesions to coordinate their movements with that of a social partner in the robbing-and- dodging paradigm could have arisen as a byproduct of sensory and/or motor deficits—these kinds of deficits have been previously reported following such damage (e.g., Johnson et al., 2013; Kolb & Whishaw, 1983; Whishaw et al., 1992). Data from the evaluation of the dodging suggest that this is not the case. The rats with mPFC lesions began to move away from their partner at the same interanimal distance as did the control rats and maintained the same average interanimal distance during dodges. These findings suggest that the rats with the mPFC lesions were able to detect social stimuli similarly to that of intact rats. That is, there is no evidence for the mPFC lesions to have produced sensory neglect in this task. In addition, rats with mPFC lesions not only performed defensive dodging movements, but also moved further and with a greater velocity than did the control rats,

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suggesting that the rats' ability to execute movements was not impaired. This conclusion was confirmed by the seed-husking test. Whereas it appears that there may be an initial attenuation of seed-husking performance in animals with lesions (see Figure 4), following a Greenhouse-Geisser correction for a violated Mauchley's W test, this transitory impairment was no longer significant. The results from the seed- husking test thus show that performance between the control and experimental group was the same, although the possible transitory attenuation in early performance may be worth exploring further. Therefore, neither gross movements (whole body turns—when dodging) nor fine movements (digit use—when husking seeds) revealed any motor impairments in the rats with mPFC lesions. Thus, the lack of interanimal coordination seen in the experimental group is likely not due to sensory or motor impairments. The present findings, then, indicate that the reduced ability of the rats with mPFC lesions to defend a food item from a robber is due not to their inability to execute movements in a timely and efficient manner, but because of an impairment in their ability to execute those movements in a way that is properly coordinated with those of their partners. As the robber moved toward the defender's snout, the defender moved laterally away, so yielding a strong positive correlation, just as with the control rats (see Figures 4.3C and D). As noted above, the stronger correlation in the experimental group likely arose from the intact robber being better able to counter the movements of the rats with mPFC lesions. The exaggerated amount of movement and its exaggerated velocity by the rats with mPFC lesions, likely reflects overcompensation for the robber's movements. Similarly, by tracking interanimal distance (snout-to-snout) over the course of the dodge, the rats with mPFC lesions deviated further from

the mean interanimal distance, again suggesting that the movements performed were not as effective in counteracting the movements of the robber. This led to a failure to compensate for all the robber's movements and so, an erosion of the ability to maintain a constant interanimal distance, producing a correlation between interanimal distance and robber's movements, unlike the case seen in intact rats (see Figure 4.3A and B; see also Bell & Pellis, 2011; Bell et al., 2012). That the resulting correlation was negative suggests that the excessive amount and velocity of the movement by the dodger was particularly ineffective for the smaller movements made by the robber. That is, the rats with mPFC lesions overcompensated, which became particularly evident when the robber made small movements. For these reasons, it appears that the primary deficit of the rats with mPFC lesions in a social context is not a reduced ability to execute movements, but a reduced ability to coordinate those movements with the movements of their partner. This supports the hypothesis that when engaged in more complex social interactions (i.e., involving more dimensions of movement and more varied actions by the partner), such as play fighting (Pellis & Pellis, 1987), the rats with mPFC lesions preferentially use maneuvers that require the least interanimal coordination (Bell et al., 2009). There are at least two ways in which the mPFC could contribute to such coordination of action. First, the mPFC may be important for higher-order functions, which in this case would involve monitoring one's own position with that of one's partner to calibrate the appropriate magnitude, speed, and direction of movement when the interanimal distance is disrupted by an encroachment of a robber. The mPFC is known to be involved in such higher-order functions (Ridderinkhof, Ullsperger, Crone, & Nieuwenhuis, 2004), so damage could have a direct impact on the calculations needed

to coordinate one's movements with those of one's partner effectively. Second, the mPFC has strong connections to limbic structures, such as the amygdala, and has an attenuating role on emotional reactions to fear-inducing stimuli and contexts (Etkin, Egner, & Kalisch, 2011). It is known that, when stressed or fearful, motor and cognitive performance is eroded (e.g., McEwen & Sapolsky, 1995; Metz, Jadavji, & Smith, 2005). Thus, the effects of the mPFC lesions on coordination would be indirect, as an excessive emotional response to the approach of a partner could impair performance. Indeed, the two may not be mutually exclusive, as different subcomponents of the mPFC are anatomically and functionally linked to sensorimotor and attention mechanisms as well as to cognitive and emotional systems (Euston, Gruber, & McNaughton, 2012). To tease apart these potential mechanisms, nonsocial, nonstressful tasks requiring calibration between different points in space would need to be tested to characterize the purely cognitive contribution. Similarly, a nonsocial task, performed in a stressful context, would need to be tested to characterize the contribution of emotional dampening on complex actions.

There are other, nonsocial functions of the mPFC that could have potentially caused the impairments in dodging of the experimental group. For example, the mPFC is involved in effort-based decision-making (Walton, Bannerman, & Rushworth, 2002) and with the reward system (Tzschentke, 2000). Given that all animals were food deprived prior to being tested on the two food-related tasks, the damage to the mPFC could have reduced the reward value of the food or altered their perception of the effort involved. However, our results suggest that this is likely not to be the case. During the robbing-and-dodging paradigm, both the rats with the mPFC lesions and the control

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rats defended their pieces of almonds, with the same number of almond pieces required to elicit the same number of dodges (see Figure 4.2). Moreover, both groups showed a high correlation between evasive movements of the dodger and the approach movements of the robber (see Figure 4.3), suggesting that the rats with the mPFC lesions were highly motivated to maintain possession of their food. Furthermore, there were no significant differences in the seed-husking skill task, again suggesting that the rats with the mPFC lesions were highly motivated to husk and eat the seeds (see Figure 4.4).

4.5.2 Back to the Role of Play

As noted earlier, social play interactions are important for the development of emotional, cognitive and social skills (Arakawa, 2003; Baarendse et al., 2013; da Silva et al., 1996; Lukkes et al., 2009a; van den Berg et al., 1999; Von Frijtag et al., 2002) and for the pruning of the dendritic arbor of the neurons of the mPFC (Bell et al., 2010; Himmler et al., 2013b). Given that the ability to coordinate movements with a social partner is compromised when rats are denied interacting with peers during the juvenile period (Moore, 1985; Pellis et al., 1999), and that rats with lesions of the mPFC have a reduced ability to coordinate their movements with a social partner effectively (present study), it is reasonable to hypothesize that at least some of the deficiencies seen in rats deprived of juvenile play experience arise from the failure to remodel the mPFC. If this is so, what needs to be determined is what changes in the mPFC are responsible for the particular deficits shown in different domains. For instance, the present study indicates that the loss of ability to coordinate actions with a partner does not arise from

sensorimotor deficits, but could conceivably arise from either reduced cognitive or emotional regulation.

Similarly, different facets of the deficits in cognitive, emotional and social skills following being reared in the absence of play with peers could arise from altered connections in different areas of the mPFC. This is reasonable given that different subareas of the mPFC are connected to different systems (Euston et al., 2012). More localized lesions of these systems need to be used to characterize the possible correspondence of play-deprived deficits with brain-damage-induced deficits. Conversely, play-experience-dependent changes to the neural circuitry of the various subsystems of the mPFC need to be identified and characterized.

Chapter 5: How Domestication Modulates Play Behavior: A Comparative Analysis

Between Wild Rats and a Laboratory Strain of Rattus norvegicus[‡]

5.1 Introduction

Play fighting is one of the most commonly studied forms of play (Pellis & Pellis, 1998b), and, for several decades, the species most commonly used for experimental studies of this play has been the laboratory rat (e.g., Bolles & Woods, 1964; Meaney & Stewart,1981; Panksepp, 1998; Panksepp, Siviy, & Normansell, 1984; Pellis, 2002a; Siviy, 1998; Thor & Holloway, 1984; Vanderschuren, Niesink, & van Ree, 1997). Even though the use of laboratory rats has enabled considerable progress in our understanding of the organization, development, and neural underpinnings of play (Pellis & Pellis, 2009), two problems have diminished the full value of this endeavor.

The first problem is that different laboratories often use different strains of rats, and some of the discordant findings across laboratories may be attributed to use of different strains. Laboratories using the same testing procedures and the same behavioral scoring schemes have clearly shown that different strains vary in the magnitude and robustness of sex differences in play, in the overall frequency of play, and in how they play when they do play (e.g., Reinhart, Pellis & McIntyre, 2004; Reinhart, McIntyre, Metz, & Pellis, 2006; Siviy, Baliko, & Bowers, 1997; Siviy, Crawford, Akopian, & Walsh, 2011; Siviy, Love, DeCicco, Giordano, & Seifert, 2003). The second problem is that, when comparing the play of rats with that of many other mammals, the studies

being compared are potentially confounding what is general to play and what may be a byproduct of domestication. The vast majority of the comparative literature on play fighting is derived from observations of free-living or captive wild animals (Bekoff & Byers, 1998; Burghardt, 2005; Fagen, 1981). Even when comparing across murid rodents, the subgroup of rodents to which rats belong, many captive or free-living nondomesticated species are compared to domesticated rats (Pellis & Iwaniuk, 1999; Pellis & Pellis, 1998b). That is, it cannot be taken for granted that the knowledge gained from a domesticated species can be readily generalized to nondomesticated species. The two problems may compound one another, because different strains may have been selected in such a manner as to emphasize different aspects of the wild-type phenotype (Stryjek, Modlinska, & Pisula, 2012).

There are at least three ways in which domestication can affect the expression of play. First, domestication involves the gaining of sexual maturity earlier, with an associated retention of juvenile- typical features (e.g., Coppinger & Coppinger, 2001; Morey, 1994; Trut, 1999). One consequence of this retention of juvenile features is that domestic animals tend to be more playful than their wild counterparts (Budiansky, 1999; Burghardt, 1984, 2005). Second, defensive aggression is greatly curtailed, leading to domesticated animals that are less hostile to both humans and conspecifics and, as a consequence, are more tolerant of intrusion into their personal space (e.g., Blanchard & Blanchard, 1994; Trut, 1999). Such tolerance reduces the risk of escalation of social encounters to serious aggression (Blanchard, Blanchard, Takahashi, & Kelley, 1977; Blanchard, Flannelly, & Blanchard, 1986; Takahashi & Blanchard, 1982). If the same is true for play, then domesticated rats should respond to

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a playful attack when the partner is closer, making body contact more likely. Moreover, when contact is made and playful wrestling ensures, there should be a reduced risk of the encounter escalating from playful to serious fighting. Third, domesticated animals become fatter, have less lean muscle, a weaker bone structure (Price, 1999; Richter, 1959) and, overall, are more reluctant to undertake complex behaviors than their wild counter- parts (e.g., Stryjek et al., 2012). A consequence on play may be that, unlike wild-type animals, domesticated animals are less likely to use the more energetically demanding and acrobatic tactics, and, if they do, given their larger body mass, would be less likely to use them effectively. Thus, as in the case of reduced interanimal distance, such changes in physical prowess could alter which tactics are used for playful attack and defense. In these various ways, the play fighting reported in domesticated strains of rats may differ from that present in the wild type.

One of the most intensively studied strains of domesticated rat with regard to play is the Long Evans hooded rat (LE) (Pellis & Pellis, 1998a, 2009). When mixed sex groups of LE rats are housed in seminatural conditions, they develop a social organization and use all the behavior patterns of both aggression and affiliation typically seen in wild rats (e.g., Adams & Boice, 1983; Blanchard, Flannelly, & Blanchard, 1988). Moreover, pigmented strains of domesticated rats, such as LE, have sensory capabilities (Prusky, Harker, Douglas, & Whishaw, 2002) and motor skills (Whishaw, Gorny, Foroud, & Kleim, 2003) that are superior to those of fully albino strains and are more comparable to wild rats. Therefore, in this study, we compared the play fighting of LE rats with wild-type rats. Both strains were housed in captivity and tested in a similar manner. Juvenile rats were tested between 30 and 35 days of age,

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which is within the peak play period reported across many strains by many laboratories using different testing paradigms (e.g., Bolles & Woods, 1964; Meaney & Stewart, 1981; Panksepp, 1981; Panksepp & Beatty, 1980; Pellis & Pellis, 1987, 1990, 1997; Takahashi & Lore, 1983; Taylor, 1980). Both males and females were used and both strains were compared in same sex, same strain pairs.

The Wild Warsaw Captive Pisula-Stryjek (WWCPS) strain served as our wild-type strain. This strain was derived in 2006 from genetic material obtained from five independent colonies of wild rats in Warsaw, Poland (Stryjek & Pisula, 2008). A major problem with using wild rats housed and bred in captivity is that unplanned selection for the most easily handled rats can quickly erode some features of wildness, such as the approach distance that triggers defensive aggression or flight (Blanchard et al., 1986). To offset this problem, the WWCPS rats are housed and handled in a manner that does not require that they be picked up by human hands for basic husbandry purposes (Stryjek, 2008, 2010). Moreover, to limit the effects of inadvertent selection further, testing was restricted to captive-born subjects from the second to fifth generations, with the colony being continually replenished with wild stock (Stryjek & Pisula, 2008). The animals that were wild caught were not used for testing because they remain highly stressed (e.g., Blanchard, Williams, Lee, & Blanchard, 1981; Blanchard et al., 1986).

Some researchers have suggested that play fighting is a form of immature serious fighting or quasi aggression (e.g., Hurst, Barnard, Hare, Wheeldon, & West, 1996; Silverman, 1978; Taylor, 1980). This is unlikely, because the targets attacked during play fighting and serious fighting differ. During play fighting, rats attack and defend the

nape of the neck, which is nuzzled with the snout if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). In contrast, during serious fighting, the rump and lower flanks are bitten (Blanchard et al., 1977; Pellis & Pellis, 1987). The play fighting of rats, therefore, appears to be like that of many other murid rodents, in that the targets attacked and defended are the same targets as those contacted during adult courtship (Pellis, 1993; Pellis & Pellis, 1998a). Even so, some murid rodents also attack and defend the rump during play fighting, as they do during serious fighting (Pellis & Pellis, 1989), and some nonmurid rodents attack both courtship and aggressive targets during play fighting (Pasztor, Smith, MacDonald, Michener, & Pellis, 2001; Pellis & Iwaniuk, 2004). In addition, when rats escalate from play to serious fighting, they switch from nuzzling the nape to biting the rump. That this switch reflects a change from play to aggression is supported by the presence of accompanying threat signals during rump attacks that are typically absent from play (Smith, Fantella, & Pellis, 1999; Takahashi & Lore, 1983). Because of the earlier onset of sexual maturity that occurs from domestication, it is possible that the courtship forms of play in domesticated rats have been retained while the role of play derived from aggression has diminished. Thus, wild-type rats, unlike domesticated rats, may combine both courtship and aggressive behavior in their play. The stronger presence of agonism in the play of wild rats would likely be reflected in the occurrence of both attacks to the rump and the nape during play fighting. In addition, the wild rats may also be more likely to escalate from play to aggression.

If domestication increases the motivation to play, then play should be more frequent in LE rats than WWCPS rats. There are three measures that, in different

ways, assess the frequency of play: (a) nape attack (Pellis, 1988; Pellis & Pellis, 1987), or nape contact (Siviy & Panksepp, 1987), assesses an individual's propensity to initiate play; (b) the probability of defending against a nape attack can vary across ages, sex, and strains (e.g., Pellis & Pellis, 1990; Reinhart et al., 2004, Reinhart et al., 2006), and assesses an individual's propensity to engage in play when attacked; and (c) during the juvenile period, rats from many domesticated strains frequently adopt a postural configuration during playful wrestling in which one rat stands over its supine partner (i.e., a "pin"; Panksepp, 1981). The pin assesses the playful propensity of pairs of rats. Contact with the nape and pinning, in particular, are among the most common behaviors used to assess the frequency of play in rats (e.g., Aguilar, Caramés, & Espinet, 2009; Calcagnetti & Schechter, 1992; Panksepp, 1981; Panksepp & Beatty, 1980; Thor & Holloway, 1983; Trezza & Vandershuren, 2008).

Detailed analysis of how pinning occurs shows that, in the majority of cases, the pin configuration arises when the recipient of a nape attack rolls over to fully supine (Pellis & Pellis, 1987, 1990, 1997). That is, in most cases, the pin configuration arises from the defensive action taken by the recipient of a nape attack (Pellis & Pellis, 1987). Therefore, a change in the frequency of pinning can arise from changes in the frequency with which the partner launches nape attacks and the likelihood that the recipient uses the rolling to supine tactic to defend the nape (Himmler, Pellis, & Pellis, 2013a). To assess changes in the frequency of pins, we scored the probability of use of the different defensive tactics.

Because most pins arise when the defender rolls over to supine and because this tactic is most likely to occur when the attacker has contact, or is near to contact, with the

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nape, strain differences in achieving such proximity could indirectly affect the frequency of pins. Furthermore, given the known differences between domesticated and wild animals, a difference in their regulation of interanimal distances could emerge in two ways. First, the wild rats could begin defending themselves at larger interanimal distances. Second, the wild rats could move away from an approaching partner more effectively (i.e., further and faster). These potential strain differences in sensorimotor capabilities were assessed.

5.2 Method

5.2.1 Subjects

A total of 84 rats were used for this study. Of these animals, 24 (12 males, 12 females) were LE hooded rats that were born in the vivarium at the Canadian Centre for Behavioral Neuroscience, University of Lethbridge. The mothers of these rats were obtained from Charles River Laboratories. All subjects were weaned at 22 days old and were then randomly paired with a sex- and age-matched (within 3 days) partner. Animals remained with the same partner for the entire experiment (i.e., until 35 days). The rats were kept in 46 X 25 X 20 cm polyethylene tubs, with processed corncob as bedding; they were maintained at a constant 21–23 °C on a 12:12-hr light-dark cycle. Food and water were provided ad libitum. All animals were handled and cared for in accordance with the Canadian Council for Animal Care regulations.

The other 60 rats (34 males, 26 females) were derived from wild-type stock (WWCPS strain) and were bred and housed at the vivarium at the Department of Psychology, Helena Chodkowska University of Management and Law, Warsaw, Poland. All subjects were weaned at 22 days old and were then randomly paired with a sex- and

age-matched (within 3 days) partner. Animals remained with the same partner for the entire experiment (i.e., until 35 days). All animals were housed in Eurostandard Type IV cages (61 X 43.5 X 21.5 cm), with dust-free softwood granules Tierwohl Super as bedding; they were given constant access to water and standard laboratory fodder. The day-night cycle was set at 12:12 hr, and the temperature was maintained at a constant 21–23 °C. All wild rats kept in the laboratory were housed, bred, and cared for in accordance with the Regulation of the Polish Minister of Agriculture and Rural Development of March 10, 2006, on laboratory animal care, and the experimental procedures were approved by the Fourth Local Ethics Commissions in Animal Experimentation, Warsaw, Poland.

5.2.2 Procedure

In all cases, play was tested between 30 and 35 days, which is within the peak period for playful interactions in rats (Thor & Holloway, 1984), and before the age at which, in males, dominance relationships begin to form (Pellis & Pellis, 1991). All animals were habituated to the play apparatus for 30 min each day for 3 days prior to testing. Each subject was isolated socially for 24 hr before the onset of testing to enhance playfulness (Niesink & van Ree, 1989; Panksepp & Beatty, 1980; Pellis & Pellis, 1990). Test sessions lasted for 10 min, which provided sufficient time to capture most aspects of play (Pellis & Pellis, 1990). Both habituation and testing sessions occurred in complete darkness, because it has been shown that social behaviors, including play, increase in frequency when in the dark versus low light or red light (e.g., Foroud & Pellis, 2002; Pellis & Pellis, 1987, 1990, 1997; Smith, Forgie, & Pellis, 1998). The pairs were tested twice, with a 1-day interval between trials. It should be

noted that, even though the WWCPS and LE rats were tested at two separate facilities, testing was conducted as similarly as possible. The sole difference was that, because WWCPS rats are more defensive, placement into and removal from, the testing cage was done with the experimenter wearing protective gloves rather than just latex gloves, as was the case for the LE rats.

5.2.3 Equipment

Play trials occurred in a 50 X 50 X 50 cm Plexiglas box, which was lined with approximately 1–2 cm of standard corncob bedding for LE rats and Tierwohl Super bedding for WWCPS. After each session for both LE and WWCPS rats, the boxes used were thoroughly cleaned with Virkon, and the bedding was replaced to clear the experimental box from any smells of previous rats.

The play trials were recorded from two different angles, obliquely from the front (at 45°) and directly from above (at 90°). In the LE strain, animals were filmed using a DVD103 Sony Handycam for the 45° angle shot and using an HDR-XR500V Sony Handycam for the 90° angle. In the case of WWCPS rats, a BCS 0804LE-A DVR system was used with an LC-471 camera filming from above and an LC-308D filming from 45°. All cameras used the night-shot option to film in the dark. Due to a camera malfunction, the second session for one male pair of the LE strain was not recorded at either the 45° or the 90° angles. In addition, due to file corruptions, the second session of the overhead view (at 90°) for three female LE strains was not used, as well as the top view for both sessions for two male pairs of WWCPS. The first set of WWCPS rat pairs was only filmed at the 45° orientation, whereas the second set (9 male

pairs, 7 female pairs) were filmed from both angles. The data for available trials were used for analyses.

5.2.4 Behavioral Analysis

For the scoring of play behavior, the 45° video orientation was used. Trials were inspected at full speed, in slow motion, and frame-by-frame. Because the WWCPS rats have a brown coat, and we wanted to avoid any undue stress that could arise from marking them, pair mates could not be individually recognized. In contrast, LE rats can be individually tracked because of their distinctive black and white pelage patterns (e.g., Pellis & Pellis, 1990). However, to be consistent in the scoring of the two strains, the behavior of each pair was recorded as a unit, summing the scores of attack and defense behavior of both pair mates.

Playful interactions begin with one partner approaching and attacking the nape of the other. The recipient of the attack can either respond to the attack or simply ignore it. If the recipient defends against the attack, the type of defense can be recorded (Himmler et al., 2013a). Therefore, the frequency of playful attacks (per 10 min), the probability of defense (percentage of all nape attacks that were defended), and the probability of each of type of defense tactic (percentage of each tactic used when defensive action was taken) were all recorded.

A *playful attack* was scored when one rat's nose was either in contact with its partner's nape or when one rat made a targeted movement toward the nape of the other. *Playful defenses* of the nape can take one of two major forms: (a) evasion, in which the defender moves its nape away from its attacker and does so by running, leaping, or swerving away and thus faces away from its partner, and (b) facing defense,

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in which the defender moves its nape away by turning to face its partner, thereby blocking access by situating its teeth between its partner and its own nape. Facing defense can also take one of three forms: (a) complete rotation, in which the defender rolls completely over onto its back, (b) partial rotation, in which the defender rotates its forequarters, but maintains contact with the ground with one or both of its hind feet, and (c) other, in which defensive actions involve rotations or other movements in other dimensions (e.g., rotating vertically in a horizontal plane; Pellis, Pellis, & McKenna, 1994; Pellis, Pellis, & Whishaw, 1992). The type of defensive tactic used was determined by the movements occurring in the first 2–3 video frames to ensure that what was recorded was the tactic first attempted by the defender rather than the outcome resulting from the continued attack movements by the partner (Himmler et al., 2013a). Irrespective of the defensive actions taken by the recipient of an attack, in domestic rats a common outcome is for one partner to end up on its back with the other standing over it (i.e., pin). Therefore, the frequency of pins during the 10-min play trials (Panksepp, 1981) was also scored.

For the scoring of sensorimotor differences between the strains, the 90° video orientation was used. Video clips were digitized and scored using the Vicon Motus motion capture software (e.g., Bell & Pellis, 2011; Pellis & Pellis, 1994; Sacrey, Alaverdashvili, & Whishaw, 2009). Clips used to measure interanimal distance were chosen only if an animal was attacked from the side and, at the onset of the attack, at least one rat-length away. The distance between the tip of the nose of the attacking animal and the middle of the nape of the defending animal was scored on the video frame in which the defender began to swerve laterally away from the approaching partner.

That is, to ensure consistency across individuals and strains, in all cases, the distance at the onset of defense was scored for the same configuration and type of defense (i.e., evasive defense and, specifically, a lateral swerve), because it is possible that different defensive tactics are employed at different interanimal distances and orientations (Bell, Johnson, Judge, Cade, & Pellis, 2012; Pellis et al., 1994). Clips used to measure the distance of the jump were chosen from the video at the point during which the performer leapt directly away from its attacker and involved a forward leap rather than an upward hop, and so was more likely a defensive action than a play solicitation action (Pellis & Pellis, 1983). Because the rat's head could turn to either side when landing, apparently to track the position of its partner, the spatial location of the nape was used to measure the distance of the jump rather than the tip of the rat's snout. The velocity of the jumps was also measured and, to be consistent across animals, the measurement of velocity was taken on the frame on which all four of the rat's feet lost contact with the ground.

Because scoring the subcomponents of play involves a greater degree of subjectivity (Himmler et al., 2013a) than the scoring the sensorimotor measures using the Peak Motus, it was important to verify that the scores derived were consistent. For the LE rats, many different experiments have been conducted using the current test paradigm and scoring scheme. The frequency of attack, probability of defense, and the probability of types of defense scored for the LE rats in this study were all within the ranges previously reported (e.g., Bell, McCaffrey, Forgie, Kolb, & Pellis, 2009; Kamitakahara, Monfils, Forgie, Kolb, & Pellis, 2007; Pellis & Pellis, 1990; Pellis, Pellis, & Whishaw, 1992; Smith et al., 1998). However, because this is the first time that this scoring scheme has been used for wild rats, we confirmed the reliability in the

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scores derived from WWCPS rats in two ways. First, the same experimenter (Himmler) rescored 6 pairs (3 male, 3 female) of the WWCPS rats 4 months after the original scoring. Comparison using Pearson's correlation of these two scores for each playful attack, probability of defense, total number of pins, evasive defense, and complete rotation defense showed a high level of intraobserver reliability (r range: .773-.992, with all comparisons being significant, $p < .05$). Second, another experimenter (Derksen), who was familiar with the scoring scheme, but who had never scored wild rats, also rescored a subset of 6 pairs (3 male, 3 female) of WWCPS rats previously scored by Himmler. Comparisons for all measures using Pearson's correlation showed that there was a high degree of interobserver reliability (r range: .402-.973, with four of five comparisons significant, $p < .05$). Thus, the scoring scheme could be applied to both strains of rats with a high degree of consistency. Nonetheless, given the greater consistency for intraobserver versus interobserver scoring for all the behavior categories used, all the data reported in the Results section were derived from the same experimenter (Himmler).

5.3 Statistical Analyses

The data were analyzed using a two-way analysis of variance (ANOVA), with strain (WWCPS or LE) and sex as independent factors; for pairwise comparisons, the least significant difference test was used for post hoc tests. Differences were considered significant for p values of $\leq .05$. For significant main effects of strain or sex, effect sizes were calculated using Cohen's d, with values of 0.8 or greater representing large effects. For graphical representation of the data, values are given for group means and 95% confidence intervals.

5.4 Results

5.4.1 Targets of Playful Attack

A 2 X 2 ANOVA for proportion of playful attacks directed at the nape showed no significant group (strain or sex) difference, $F(3, 38) = 0.075$, $p = .973$. Because there were no strain or sex differences, the data for each strain was summed across the sexes, showing that, for both strains, over 90% of attacks were directed at the nape (WWCPS rats $= 97.2 \pm 0.4$; LE rats $= 97.0 \pm 0.6$). When the rats, either WWCPS or LE, did gain access to the nape area, it was nuzzled with the snout and only occasionally nibbled or pulled, as has been previously shown for LE rats (Pellis & Pellis, 1998). Moreover, no instances of play escalating to serious fighting, as would be evidenced by a shift from nuzzling the nape to biting the rump, were present in either strain.

A.   B.
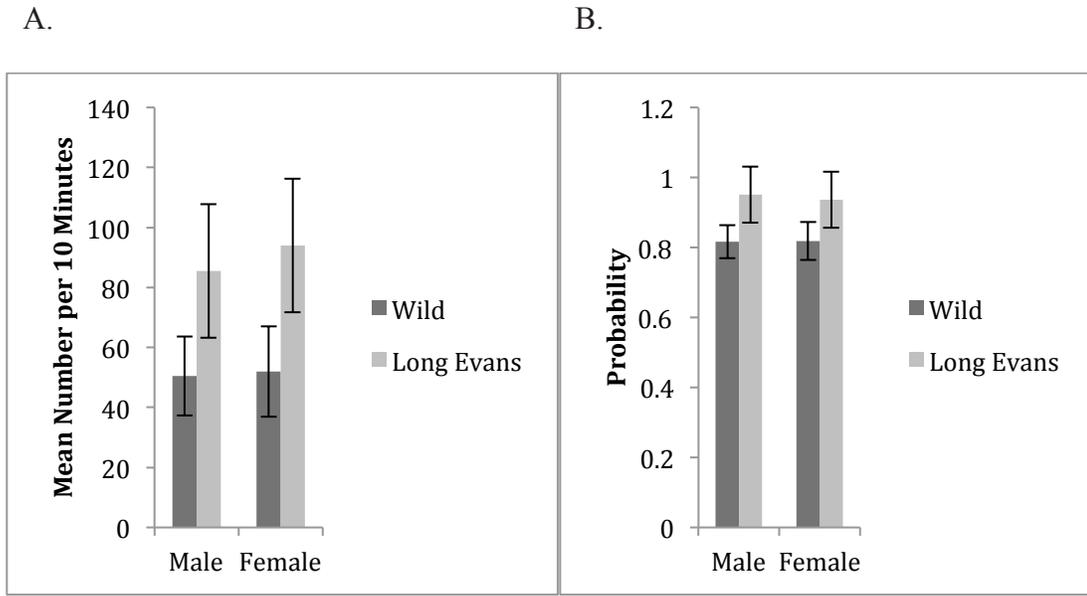
Figure 5.1
A. Number of playful attacks per 10 minutes. B. Probability of defending against a
playful attack. Unless otherwise specified the graphs are based on 30 pairs of WWCPS
rats (17 male, 13 female) and 12 pairs of LE rats (6 male, 6 female) and the error bars
represent 95% confidence intervals.

5.4.2 The Frequency of Play

A 2 X 2 ANOVA for the frequency of launching nape attacks revealed a

significant group difference, $F(3, 38) = 5.978$, $p = .002$, but no significant

interaction between strain and sex ($p > .05$). There was a main effect of strain,

$F(1,38) = 17.428$, $p < .001$, $d = 1.50$, but not for sex ($p > .05$), with LE rats

attacking more than WWCPS rats (see Figure 5.1a). There was a significant group

difference for the probability of defending against a nape attack, $F(3, 38) = 4.863$, p

$= .006$, but no significant interaction between strain and sex ($p < .05$). There was a

main effect for strain, $F(1, 38) = 14.408$, $p = .001$, $d = 1.37$, but not for sex ($p > 05$),

with LE rats being more likely than WWCPS rats to defend themselves (see Figure

5.1b).

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Pinning, which involves the configuration of one animal standing over a supine partner, was scored as its frequency of occurrence over the duration of the trial (Panksepp, 1981). There was a significant group difference for the occurrence of pins, $F(3, 38) = 34.101$, $p < .001$, but no significant interaction between strain and sex ($p > .05$). There was a main effect for strain, $F(1, 38) = 98.759$, $p < .001$, $d = 3.54$, but not for sex ($p > .05$), with LE rats being more likely than WWCPS rats to defend themselves in this manner (see Figure 5.2).
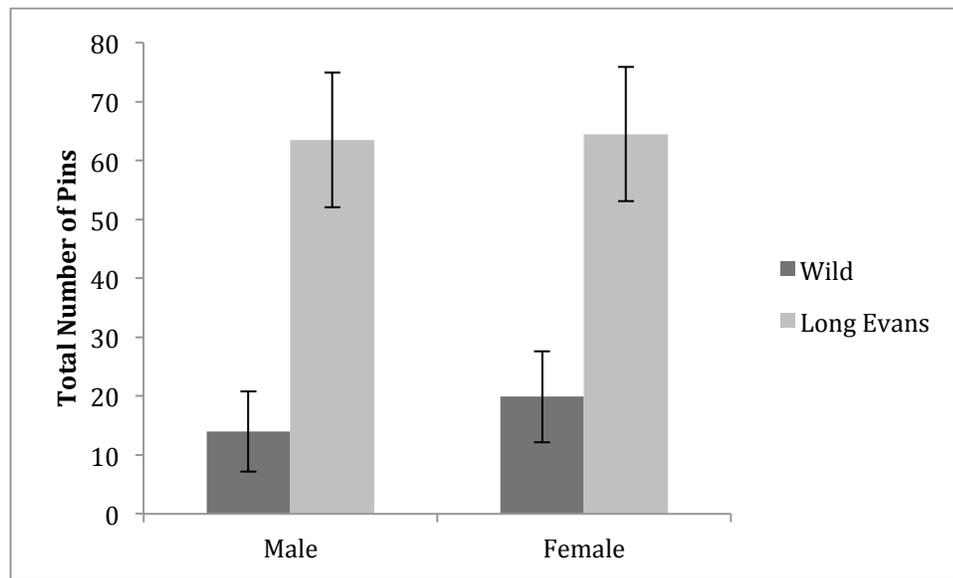
Figure 5.2

Total number of pins occurring per 10 minutes.

5.4.3 Tactics of Playful Defense

With regard to the types of defense against nape attacks, WWCPS rats were more likely than LE rats to use evasion (see Figure 5.3a). There was a significant group effect, $F(3, 38) = 8.158$, $p < .001$, but no significant interaction between strain and sex ($p > .05$). There was a significant main effect for strain, $F(1, 38) = 12.108$, $p <$

.001, $d = 1.18$, but not for sex ($p > .05$). For facing defense, use of partial rotation and

"other" was not readily distinguishable in all cases for WWCPS rats. Therefore, for

present purposes, these two types of defense were combined into "standing defense," and

this category was readily distinguishable from complete rotation, in which the defenders

rolled over to supine (see Method section). Complete rotation was calculated as a

proportion of all facing defenses, and this was compared across the strains and sexes.

There was a significant group difference for the probably of using complete rotations,

$F(3, 38) = 8.927$, $p < .001$, but there was no significant interaction between strain and

sex ($p > .05$). A main effect was shown for strain, $F(1, 38) = 22.436$, $p < .001$, $d =$

1.68, but not for sex ($p > .05$), with LE rats being more likely than WWCPS rats to

defend themselves in this manner (see Figure 5.3b). Given the significantly greater

proportion of the complete rotation in LE rats compared to WWCPS rats, the converse

was also true: There was a greater proportion of the standing defense in WWCPS

compared to LE rats (given the symmetry with the complete rotation, the latter is not

shown).

A.   B.
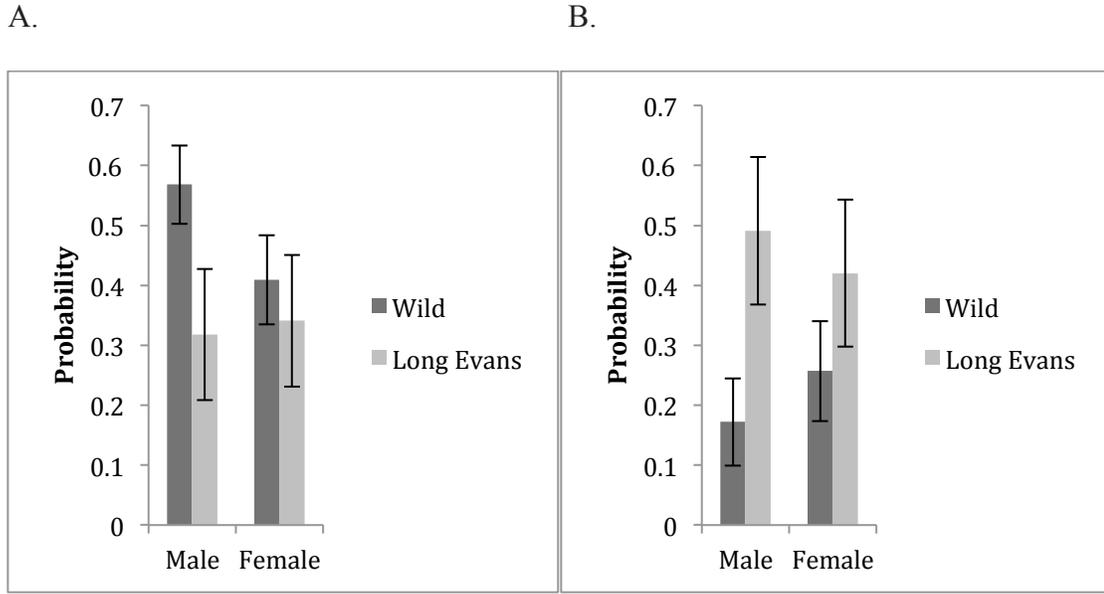
Figure 5.3
A. The probability of engaging in evasion when defending against a playful attack. B. The probability of engaging in the full rotation tactic, as a proportion of all facing defense, when defending against a playful attack.

5.4.4 Defense Distance and Acrobatic Capability

A subset of the pairs of rats provided instances that met the criteria needed for analyzing the interanimal distance when defense was initiated (WWCPS rats = 5 males, 7 females; LE rats = 5 males, 5 females). There was a significant group difference for interanimal distance, $F(3, 18) = 4.146$, $p = .021$, but there was no significant interaction between strain and sex ($p > .05$). There was a significant main effect for strain, $F(1, 18) = 11.407$, $p = .003$, $d = 1.63$, but not for sex ($p > .05$), with WWCPS rats beginning their defense at a larger distance away than did the LE rats (see Figure 5.4).
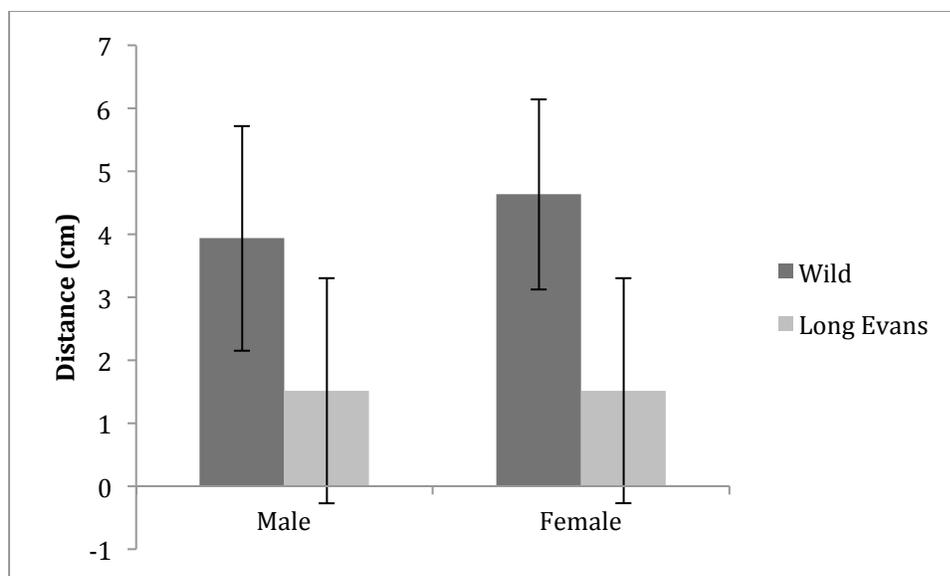
Figure 5.4
Distance (cm) between the nose of the attacker and nape of the defender on the first frame when evading an attack. See text for sample sizes.

A subset of the pairs of rats provided instances that met the criteria needed for analyzing jumps away from a partner (WWCPS rats = 6 males, 5 females; LE rats = 6 males, 5 females). There was a significant group difference for jumps, $F(3, 18)$ = 4.975, p = .011, but there was no significant interaction between strain and sex (p > .05). There was a significant main effect for strain, $F(1, 18)$ = 13.129, p = .002, d = 1.68, but not for sex (p > .05). Post hoc comparisons revealed that WWCPS males differed from LE males (p < .05). The WWCPS rats jumped further than the LE rats (see Figure 5.5a). For example, the maximum length of jumps recorded for WWCPS females (26 cm) was about one body length further than the maximum for LE females (18 cm). There was a significant group difference for velocities, $F(3, 18)$ = 4.076, p = .023, but there was no significant interaction between strain and sex (p > .05). There was a significant main effect for strain, $F(1, 18)$ = 11.783, p = .003, d = 1.63, but not

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for sex ($p > .05$), with WWCPS rats jumping with a greater velocity than their LE

counterparts (see Figure 5.5b). The maximum velocities recorded for WWCPS males

(200 cm/s) and WWCPS females (155 cm/s) were greater than those recorded for both

LE males (105 cm/s) LE females (98 cm/s).

A.   B.
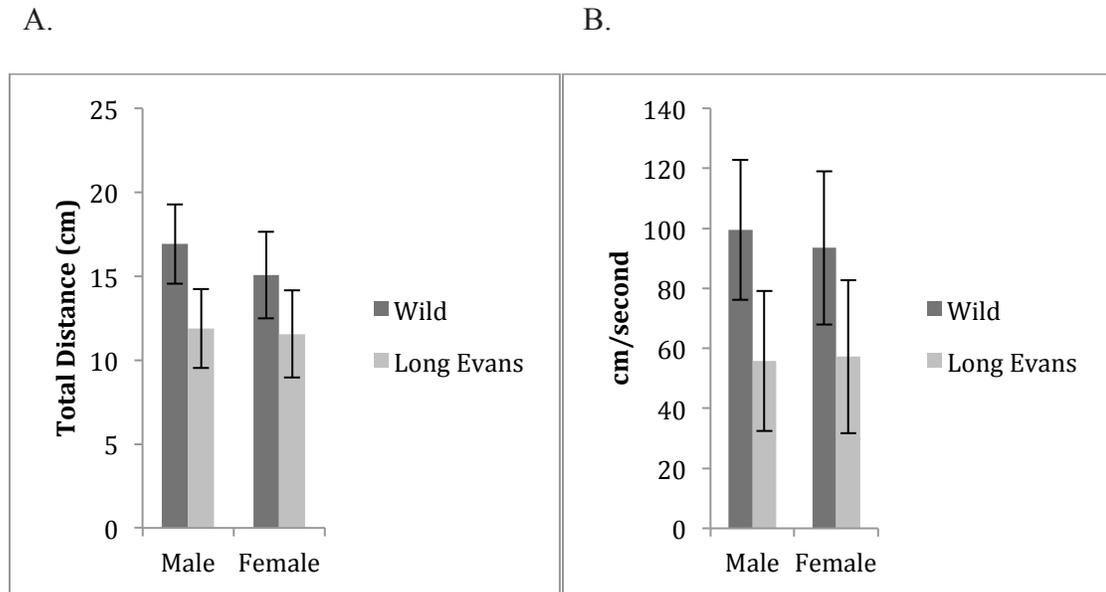
Figure 5.5
A. Distance (cm) jumped away from playful partners. B. Velocity of jumps. See text for
sample sizes.

5.5 Discussion

The main aim of this study was to determine whether domestication affects the

play behavior of rats. It was hypothesized that, if play were to be altered by

domestication, there were three possible routes by which this may have occurred. First,

play fighting in wild rats may contain a greater influence of aggression and aggressive

behavioral elements. This possibility was not supported, because, like their

domesticated counterparts, the wild rats attacked and defended the nape during play

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fighting, with biting attacks to the rump never being observed. Indeed, in neither strain did play fights escalate to serious fighting, suggesting a comparable propensity for play to remain playful. Second, it was hypothesized that domesticated rats, because of their more juvenile-typical features, should play more frequently than their wild counterparts. The differences in the frequency of launching nape attacks, the frequency of pins, and the probability of defending against attacks all support this prediction: the wild rats played less. Third, it was hypothesized that sensorimotor changes following domestication could influence the likelihood with which different playful tactics are used. There was clear evidence that wild rats initiated their defensive actions at a larger interanimal distance, and, with regard to acrobatic ability, the evidence was consistent with the view that wild rats have greater motor competence. Thus, even though the basic organization of play was the same across the wild and domestic forms, there were significant differences in the propensity to play and in the actions performed during play.

Before exploring the implications of these strain differences in play and sensorimotor capabilities in terms of domestication, it is important to consider two, potentially inadvertent, experimental confounds that could have produced at least some of these differences. First, even the minimum handling needed to place the rats into the testing enclosure could have been more stressful for the wild rats. Similarly, the 24-hr isolation prior to testing could have produced greater social stress in the wild rats. In turn, these two factors could have affected their play differentially, thus lowering the incidence of play in wild rats and making them less likely to engage in playful wrestling. There is evidence that there are strain differences in the stress response to

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social isolation (Ramos, Berton, Mormede, & Chaouloff, 1997). Although there are no specific data available for WWCPS rats, wild-type rats have been shown to be more resistant to social stress than the domesticated Wistar strain (Vidal, Buwalda, & Koolhaas, 2011). At the least, these competing data should caution one against making any premature conclusions about whether WWCPS or LE rats were more stressed by the present experimental protocol. We attempted to alleviate this possibility by ensuring that both types of rats were habituated to the testing enclosure, but the effects of possible differences in stress effects require experimental analysis. Nonetheless, observations from hundreds of play trials using LE rats conducted by one of the authors suggest that, when rats are stressed, their play is more severely depressed than was found with the WWCPS rats in the present study (S. M. Pellis, personal communication, September 3, 2012). Moreover, even when playing at a lower frequency than the WWCPS rats in the present study, LE rats still primarily use the rolling-to-supine tactic when engaging in facing defense (Foroud & Pellis, 2002; Pellis, Field, Smith, & Pellis, 1997). Therefore, it is likely that some of the differences in play between LE and WWCPS rats are robust strain differences in play rather than byproducts of strain differences in stress.

Second, even though tested in same sized enclosures, the two strains were housed, from weaning until test completion (i.e., a span of 13 days), in differently sized cages, with the wild rats having about 2.3 times more floor space (see Method section). Over the 2 weeks of housing and testing, this difference in housing enclosure could have provided the wild rats with extra opportunities to develop their sensorimotor capabilities. Although these sensorimotor differences are unlikely to account for the

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differences in the motivation to play (i.e., frequency of launching attacks and probability of defense to an attack), they are highly likely to influence the opportunity to express different defensive tactics. That is, these sensorimotor differences may not be a strain difference per se, but a difference that arose due to a small difference in rearing environment. There are, however, several pieces of evidence to suggest that this rearing effect is unlikely.

In the present study, the play fighting of LE rats is consistent with the frequency of playful attack and frequency of use of the various defensive tactics with previous studies of LE rats, irrespective of whether they were housed and tested in cages that were the same size as (e.g., Pellis et al., 1997; Smith et al., 1998; Smith, Field, Forgie, & Pellis, 1996) or larger than those used in the present study (e.g., Pellis & McKenna, 1992; Pellis & Pellis, 1987, 1990, 1991, 1992, 1997; Pellis, Pellis, & Kolb, 1992; Pellis, Pellis, & Whishaw, 1992). In all these studies, the typical distance at which defense began was 0–2 cm from the nape, as reported for LE rats in this study. This includes one study in which we used the same methodology (Peak Motus) to measure interanimal distance (Pellis et al., 1996). With regard to jumping behavior, one previous study explicitly analyzed developmental changes in hopping and jumping in LE rats. In that study, litters were reared and observed in a terrarium about the size of the housing cages used for WWCPS rats in the present study (Pellis & Pellis, 1983). Although jumping was not measured in the same way as it was in the present study, of the hundreds of jumps observed, no rat's leaps involved propelling their bodies 2–3 times their body lengths, a feat commonly observed in the wild rats. These studies have shown that, with respect to cage size, irrespective of rearing and testing conditions, the relative frequency of play, the

frequency of types of playful tactics used, and the overall sensorimotor capacities of LE rats all remain robustly consistent. Given the consistency for LE rats, it would seem unlikely that WWCPS rats would differ markedly in this regard.

Most critical to these considerations are the data on how the rats defended themselves. The wild rats were more likely to evade (swerve, run, or leap away) when attacked, and such defense could be facilitated by differences in sensorimotor capabilities. By beginning to move sooner and faster, the wild rats would likely succeed in their evasive maneuvers. Enhanced sensorimotor skills derived from being housed in larger home cages could, in this way, improve evasive defense. The same, however, cannot be argued for differences in facing defense, because this defense occurs when in close bodily contact and the animals remain in place rather than flee. Yet, when the rats defended against a nape attack using the facing defense, the WWCPS rats were less likely than the LE rats to roll over to supine (see Figure 5.3b), and were more likely to use a standing tactic. So, even if there were some rearing-induced changes in the sensorimotor abilities of WWCPS versus LE rats, these changes would not account for the strain differences in facing defense. Of course, to determine for certain which, if any, of the strain differences are sensitive to rearing experiences, LE and WWCPS rats housed in the same sized cages would have to be evaluated. Nonetheless, it would seem reasonable to conclude that, at least some of the differences, especially those involving the facing defense, between the play of LE and WWCPS rats are due to strain differences, and not because of the housing differences.

5.5.1 Play Fighting as a Species-Typical Simulation of Adult Sexual Behavior

Because wild rats, like domesticated ones (Pellis & Pellis, 1987; Siviy & Panksepp, 1987), attack and defend the nape, and nuzzle the nape if contacted (present findings), there is no evidence to suspect that domesticated rats have changed play fighting from a more aggressive to a less aggressive form. That is, wild rats, like domesticated rats, attack and defend a body target competed over during adult courtship encounters, as do a wide range of other murid rodents (Pellis, 1993; Pellis & Pellis, 1998). Therefore, the basic organization of play fighting found in domesticated rats can be reasonably compared with the play fighting of other species of rodents, even when, for many of those other species, the data are obtained from captive-born wild animals or from free-living wild animals (Pellis & Iwaniuk, 1999, 2004).

5.5.2 Differences in Styles of Play in Wild Versus Domesticated Rats

The main difference in the style of play fighting of the wild and domesticated rats was in the drastically reduced frequency of pinning in the former (see Figure 5.2). As already noted, in some strains of domesticated rats, the pinning configuration is a very frequent component of play fighting in the juvenile period (Panksepp, 1981; Panksepp & Beatty, 1980; Pellis & Pellis, 1990). For the LE hooded strain used in the present study, it has been shown that the majority of cases of pinning arise because, when attacked, the defender protects the nape by rolling over to supine (i.e., the complete rotation tactic; Pellis & Pellis, 1987). However, because using any particular tactic of defense is contingent on being attacked, a changed frequency of pinning may arise due to changes in the frequency of playful nape attacks launched at the subject or changes in the likelihood of using the complete rotation tactic (Himmler et al., 2013a; Pellis &

Pellis, 1987, 1990, 1997; Pellis, Pellis, & Whishaw, 1992). Moreover, other contextual factors, such as those arising from strain differences in sensorimotor capabilities, may also impinge on the opportunity to perform tactics that likely lead to a pin configuration. The data on the wild rats, compared to that on domesticated rats, indicate that a range of factors converge in accounting for the difference in the frequency of pins.

The wild rats were less likely to launch attacks (see Figure 5.1a), and, if attacked, less likely to initiate a defensive response (see Figure 5.1b), thus creating fewer opportunities for pins to occur. Furthermore, wild rats were more likely to evade an attacking partner (see Figure 5.3a), reducing the likelihood of close quarter wrestling that can lead to pins. In addition, the wild rats initiated their defensive actions at a longer interanimal distance (see Figure 5.4) and were able to move away further and faster (see Figure 5.5), again reducing the likelihood of creating a contact situation between partners that could lead to pins. Thus, in part, the low frequency of pins by the wild rats can be explained by a greatly reduced opportunity to perform actions that lead to pins. However, even when a partner closes the distance and actually contacts the nape, and does so from an orientation that enhances the utility of rolling to supine as a defensive tactic (i.e., the attacker approaches from the side, rather than from the rear or front), the wild rats differed. When standing their ground and adopting facing defense, that defense was much less likely to involve the use of the complete rotation tactic (i.e., the turn to supine; see Figure 5.3b). In such circumstances, they were more likely to use a defensive tactic that, while maintaining close body contact, involved the defender remaining in a standing posture.

Therefore, the reluctance of the wild rats to roll over to supine indicates a difference in preference and not just a reduced opportunity to do so. In LE rats, prejuvenile animals of both sexes (Pellis & Pellis, 1997) and sexually mature males (Pellis & Pellis, 1990) show a similar reluctance to roll to supine; instead they preferentially adopt defensive tactics that involve maintaining a standing posture (e.g., partial rotation). That these preferences reflect actual choices of action rather than opportunity is supported by the finding that damage to the cortex can change the juvenile preference for rolling to supine to one in which there is a greater likelihood for the standing defense, even though they do not exhibit any motor deficiencies and are attacked by intact partners that have not changed any facet of their attacking behavior (Foroud, Whishaw, & Pellis, 2004; Kamitakahara et al., 2007; Pellis, Pellis, & Whishaw, 1992). Together, these findings suggest that the reluctance of the wild rats to roll over to supine, even when the opportunity present itself, is partly accounted for by neural differences in the biases present for selecting particular defensive tactics. Therefore, the changes induced by domestication are threefold with regard to their effects on the pattern of play. First, there is an increased motivation to play, as is shown by an increased frequency of launching playful attacks and an increase in the opportunity for the close bodily contact afforded by pinning. Second, there are sensorimotor changes that affect the likelihood of coming into contact with a partner in a way that facilitates the occurrence of pinning. Third, there is an increased preference, when in the correct configuration, to use tactics that promote the occurrence of pinning.

The sensorimotor changes, while impinging on some aspects of play, are not in themselves to be explained as changes in the mechanisms regulating play. Rather, they

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are byproducts of domestication, ones that are common to many different lineages of animals that have undergone domestication. For example, wild mice have a greater maximal sprint speed than domesticated ones (Garland, Gleeson, Aronovitz, Richardson, & Dohm, 1995), wild fish swim at a greater speed than domesticated ones (Handelsman, Claireaux, & Nelson, 2010), and some wild species differ from their domesticated equivalents in muscle fiber types (Nimmo & Snow, 1983; Garland et al., 1995) and muscle strength (Barfred, 1971). In contrast, changes in the motivation to engage in play and changes in the selection of tactics used during play may have involved changes directly affecting the mechanisms regulating play. For example, the breeding of selected lines of rats with specific properties of neural function of the amygdala have led to differences in the motivation to engage in play (Reinhart et al., 2004; Reinhart et al., 2006). The neural and behavioral differences in these selected lines are likely the result of one selected line retaining more juvenile-like features than the other (Corcoran & Teskey, 2004). That is, selected lines of rats that retain characteristics more typical of juveniles as adults (e.g., impulsivity) tend to be more motivated to play. Thus, increased motivation to play among selected lines may be interpreted as a continuation of the observed increase in playful motivation of domesticated animals compared to their wild counterparts (Budiansky, 1999; Burghardt, 1984, 2005).

The lower likelihood of supine defense in many nondomesticated rodents (Pellis, Pellis, & Dewsbury, 1989) could suggest that this form of defense becomes more common after domestication. However, the preference for supine versus standing or evasive defense is not so clearly linked to domestication. For the two

lines of rats selected for differences in amygdala function, both types avoid supine defense, one by increased evasion and the other by increased standing defense (Reinhart et al., 2004; Reinhart et al., 2006). Therefore, it cannot be taken for granted that the change from a low likelihood of supine defense in wild rats to a higher likelihood of supine defense in LE rats (present study) is a necessary consequence of domestication. Indeed, preliminary data on the domesticated rats from the Sprague-Dawley strain have suggested that, these rats, like the wild rats, are reluctant to roll to supine even when given the opportunity to do so (Pellis et al., 1997). These findings suggest that different facets of the wild-type play phenotype may have been differentially modified by selective breeding, producing patterns specific to particular strains—a conclusion supported by existing comparisons across some strains (e.g., Siviy et al., 1997; Siviy et al., 2003, Siviy et al., 2011).

5.6 Conclusion

The present findings, comparing the play of wild rats with that of one commonly studied strain of domesticated rats, show that, even though the basic organization of play fighting is the same— the nape is still attacked and defended—some common occurrences in the domesticated rats, such as the pin configuration, are rare in the wild animals. That is, although the play fighting of domesticated rats is species-typical, there are some significant modifications in the frequency of use of particular behavior patterns from the wild type that occur as a consequence of domestication. Most strikingly, though, the present findings also raise the possibility that the differences observed across strains of domesticated rats (e.g., Reinhart et al., 2004, Reinhart et al., 2006; Siviy et al., 1997, Siviy et al., 2003, Siviy et al., 2011) may reflect selective

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differences in how different facets of play have been altered by domestication, with varying standards in housing and testing across different laboratory facilities potentially exaggerating the differences (Stryjek et al., 2012).

Such a possibility raises both a cautionary note and the prospect for novel avenues of research. On the cautionary side, the present findings reinforce the idea that mixing data from different strains of rats may confound coherent conclusions about the neural and behavioral mechanisms that regulate play. For example, rolling to supine is thought to provide the rewarding experiences in play because this behavior maximizes the physical contact between pairmates and such rewards are thought to be crucial to motivate play (e.g., Niesink & van Ree, 1989; Panksepp & Burgdorf, 1999). Rolling over to supine has also been thought to provide the critical experiences that promote the development of the prefrontal cortex (Pellis, Pellis, & Bell, 2010). That rats of different strains can sustain play even when, in some strains, such rolling over to supine is rare and close bodily contact infrequent, suggests that there must be other rewarding experiences that similarly promote play and influence brain development. On the positive side, if some aspects of play were transformed in the same way across all domesticated strains (e.g., increased motivation to play), while others were transformed in diverse ways (e.g., propensity to use supine defense), then this would point to different mechanisms regulating different aspects of play. Common mechanisms may be reflected in changes in systems that regulate the motivation to play (e.g., hypothalamus, amygdala), whereas idiosyncratic changes may involve specific changes at some levels of the motor system (e.g., motor cortex, striatum; e.g., Graham, 2011; Pellis & Iwaniuk, 2004; Pellis & Pellis, 2009). Selective cross-

breeding and modern genetic techniques could be used to reveal the particular molecular

controls that lead to neural changes specific to the different mechanisms involved in

producing and regulating play.

Chapter 6: Juvenile Play Experience has Different Roles in the Development of the Medial Prefrontal Cortex in Wild Rats and Domestic Rats

6.1 Introduction

Adult rats that have been deprived, as juveniles, of engaging in social play with peers exhibit social, emotional, and cognitive impairments. These include inappropriate reactions to stressful or fearful stimuli (e.g., Arakawa, 2003; da Silva et al., 1996, Lukkes, Mokin, Scholl, & Forster, 2007), an inability to follow social rules (van den Berg, Hol, Van Ree, Spruijt, Everts, & Koolhaas, 1999), overreacting to benign social contact (Einon & Potegal, 1991), and displaying higher levels of impulsivity and impaired decision making (Baarendse, Counotte, O'Donnell, & Vanderschuren, 2013). In part, many of these changes in behavior arise from the effects play experience has on the development of the medial prefrontal cortex (mPFC) (e.g., Baarendse et al., 2013). Juvenile play with peers leads to the reduction in the number of neurons in some areas of the prefrontal cortex (Markham, Morris, & Juraska, 2007), and most critically for the present study, such play leads to the pruning dendritic arbor and spine density of the neurons of the mPFC (Bell, Pellis, & Kolb, 2010; Himmler, Pellis, & Kolb, 2013b) and to increased sensitivity of these neurons to dopamine (Baarendse et al., 2013).

While these behavioral and anatomical results have been shown in several strains of laboratory rats, such as Long-Evans, Wistar and Lister-Hooded (e.g., Baarendse et al., 2013; Bell, et al. 2010; Himmler et al., 2013b, van den Berg et al., 1999), there is a potential confound in how to interpret these play-induced effects. Domestication affects a variety of traits including body composition (Lockard, 1968; Price, 1999; Richter, 1959) and behavior (Takahashi & Blanchard, 1982; Pisula, Turlejski, Stryjek, Nałecz-Tolak, Grabiec, & Djavadian, 2012). Therefore, it is possible that the effect of playful

experiences on the development of the mPFC may arise as a byproduct of domestication. If so, then wild rats should not exhibit this effect of play on brain development. Alternatively, it may be that play has this effect on the development of the mPFC irrespective of domestication. To test which of these two possibilities are correct, in the present study the same experimental paradigm previously used to reveal the effects of play experience on the development of the mPFC in Long-Evans rats (LE) (Bell et al., 2010; Himmler et al., 2013b) was used on wild rats.

The wild type used for this study is the Wild Warsaw Captive Pisula Stryjek (WWCPS) strain of *Rattus norvegicus*. This specific strain of wild rat was derived in 2006 from genetic material obtained from five different colonies of wild rats that were caught in Warsaw, Poland (Stryjek & Pisula, 2008). Since rats reared in captivity are susceptible to the effects of domestication, WWCPS rats are housed and handled in a way that reduces human contact (Stryjek, 2008). Further, only rats derived from the second-fifth generation are used and the colony is continually restocked from the wild (Stryjek & Pisula, 2008). Therefore, the rats are maintained in as wild a state as possible both with regard to taming and genetics. Importantly, for the tested relationship between play and brain development in this study, juvenile WWCPS rats engage in play and although such play is significantly less frequent than in domesticated rats, the variation in the form of the play falls within the range of variation seen across domesticated strains of rats (Himmler, Stryjek, Modlińska, Derksen, Pisula, & Pellis, 2013c; Himmler, Modlińska, Stryjek, Himmler, Pisula, & Pellis, 2014c). Based on previous studies using Golgi staining to evaluate dendritic length and branching and spine density (Bell et al., 2010; Himmler et al., 2013b), if play has the same effect on wild rats as it does on domesticated

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rats, then the length, branching and density of spines in the neurons of the mPFC should be reduced in the rats given juvenile play experience with peers compared to those that have not playfully interacted with peers. However, if this relationship between play and brain development only arises as a consequence of domestication, then there should not be any differences in the neurons of rats that have or have not played.

Given that we have consistently found comparable data on the complexity of pyramidal neurons in the mPFC of Long-Evans rats, and that the same investigators analyzed the brains of the WWCPS rats, we also availed ourselves of the opportunity to compare the complexity of the neurons in the domesticated and wild rats, irrespective of play experience. Given that domestication reduces brain size (Kruska, 1988; 2005), we anticipated that the neurons of the WWCPS rats might be more complex either in length and/or spine density than the Long-Evans rats. We were wrong.

6.2 Methods and Materials

6.2.1 Subjects

Twenty-four female (18 juveniles and 6 adults) Wild Warsaw Captive Pisula Stryjek (WWCPS) rats were used in this study. The juveniles were derived from 8 separate litters from the F3 generation. All rats were bred and housed at the vivarium at the Institute of Psychology, Polish Academy of Sciences, Warsaw, Poland.

All rats were housed in Tecniplast© Eurostandard Type IV cages (61cm×43.5cm×21.5cm) with dust-free softwood granules Tierwohl Super© as bedding and with constant access to water and standard laboratory fodder (Labofeed H, WP Morawski, Kcynia, Poland). The day/night cycle was set at 12/12h, with the temperature being maintained at a constant 21-23ºC. All rats kept in the laboratory were housed, bred

and cared for in accordance with the Regulation of the Polish Minister of Agriculture and Rural Development of 10 March 2006 on laboratory animal care, and the experimental procedures were approved by the 2nd Local Ethics Commission in Animal Experimentation, Warsaw, Poland.

6.2.2 Experimental Groups

On postnatal day 21, the 12 experimental animals were weaned from their mothers and randomly selected for placement in one of two rearing conditions. In one, the young rat was housed with an age-matched partner (play group) and in the other; the young rat was housed with an adult female (no-play group). They remained in these two pairing conditions until after sexual maturity. Housing with an adult female provides the juvenile rat with a variety of social experiences (e.g., huddling and grooming) but little, if any, opportunity to play since adult females typically do not engage in play with juveniles (Einon, Morgan, & Kibbler, 1978). This contrasting pattern of rearing has been repeatedly used to demonstrate that play with peers in the juvenile period affects the development of the mPFC (Bell et al., 2010; Himmler et al., 2013b).

6.2.3 Histology

On postnatal days 74-75, subjects were deeply anesthetized using 3ml of ketamine (Bioketan) (100mg/1ml) and 2ml of medetomidine hydrochloride (Domitor) (1mg/1ml) in NaCl and were then perfused with 9% saline and their brains harvested. All brains were prepared using the modified Golgi-Cox procedure (Gibb & Kolb, 1998). Following collection, brains were placed in Golgi-Cox solution for 14 days and were then placed in 30% sucrose solution for seven days. The brains were then cut into 200 micron (μm) sections using a vibrating microtome and placed on 2% gelatin-dipped glass slides. Slides

were placed into a darkened and airtight container for three days and were then stained, cover slipped and left to dry for approximately 2 weeks.

6.2.4 Anatomy

In order to quantify the neuronal morphology of the mPFC, cells were traced onto paper using a *camera lucida* at a magnification of 250x. A total of three to five cells were selected from each hemisphere, with the mean score of each measure being used for analysis. Cells were only selected if they met two criterions: (1) the cell was fully impregnated with stain, and (2) the cell was not overlapping other cells.

6.2.5 Quantification

Layer III pyramidal neurons were traced from Zilles (Zilles, 1985) area Cg3 (mPFC) and both apical and basilar dendrites were drawn. Three methods of analysis were used to obtain information of dendritic morphology: Sholl analysis, branch order analysis and spine density analysis (Gibb & Kolb, 1998). Sholl analysis was used to determine the total dendritic length by overlaying a transparency of concentric circles onto the drawing of the neuron and counting the number of dendrites that crossed each circle (16 circles). In order to estimate the complexity of branching of each dendrite, branch order analysis was used (Coleman & Riesen, 1968), in which complexity is calculated by counting the number of bifurcations on each specific dendrite. In order to measure spine density, third-order branches or higher terminal branches were selected and then traced in high magnification (1000x). To calculate spine density, the total number of spines was divided by the length of the traced branch.

6.3 Statistical Analysis

6.3.1 Effects of Play on Dendritic Organization

The data between hemispheres were compared and analyzed using two-tailed paired t-tests. As there were no significant differences between hemispheres for any of the measurements, the subsequent analyses combined data from both hemispheres. The combined data were analyzed using one-tailed independent t-tests for both the apical and basilar dendritic fields with rearing condition (play, no play) as the independent factor. Significance was considered for p values of < 0.05.

Given that we have replicated the finding that juvenile experience with play has the effect of pruning the dendrites of the mPFC neurons using five different cohorts of Long-Evans rats over a span of seven years (e.g., Bell et a., 2010; Himmler et al., 2013b), we are confident of this relationship and of the overall magnitude of the effects. Thus, to facilitate seeing whether play has comparable effects on wild rats, the data from the LE cohort most closely matching the age and experimental condition of the current study are shown as well (derived from Bell et al., 2010; Himmler et al., 2013b).

6.3.2 Sholl Analysis

There were no significant differences in dendritic length for either the apical

(t(22) = 0.741, p = 0.23) or the basilar (t(22) = -0.284, p = 0.39) fields (Fig 6.1).
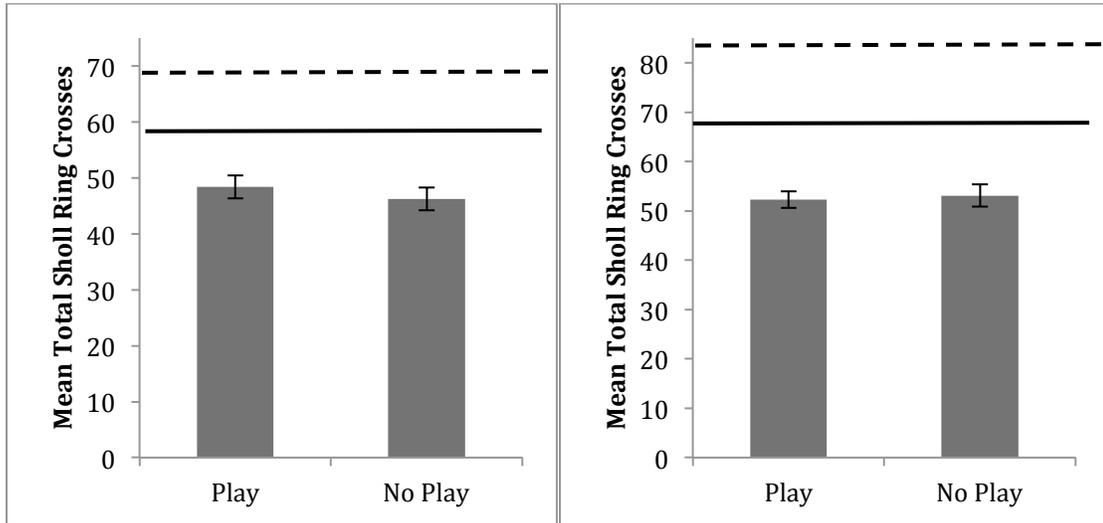
Figure 6.1

Mean dendritic length between groups for the apical (a) and basilar (b) dendritic fields are shown. For comparison, in this and subsequent figures, the average values for the effects of play experience (horizontal solid line) or the absence of play experience (horizontal dashed line) on the mPFC of LE rats from previous studies are shown.

6.3.3 Branch Order

There were no significant differences for the number of branches in either the apical ($t(22) = -0.105$, $p = 0.46$) or basilar ($t(22) = 0.586$, $p = 0.28$) fields (Fig 6.2).
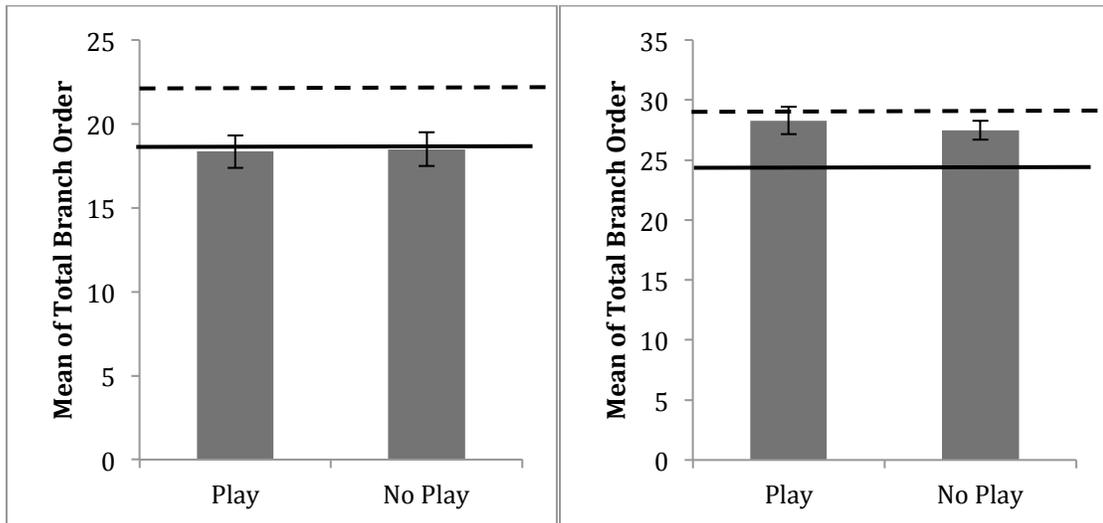
Figure 6.2:
Mean dendritic branching between groups for the apical (a) and basilar (b) dendritic fields are shown.

6.3.4 Spine Density

There were no significant differences for either the apical (t(22) = 0.291, p = 0.06)

or basilar (t(22) = -0.293, p = 0.39) fields (Fig 6.3)
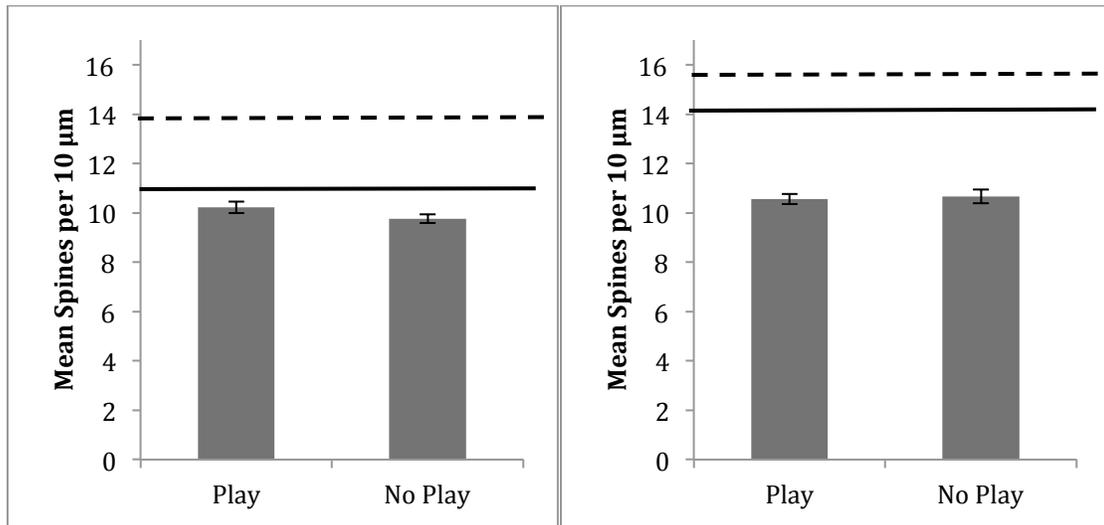
Figure 6.3

Mean spine density between groups for the apical (a) and basilar (b) dendritic fields are
shown.

6.3.5 Effects of Strain on Dendritic Organization

It is clear from Figures 6.1-6.3, 1) that the neurons of the WWCPS rats are

simpler than those of the Long-Evans rats with restricted play experience; and, 2) that

play in the Long-Evans rats reduces the dendritic profile to that of the WWCPS rats.

6.4 Discussion

If the effect of play experience on the development of the mPFC is not a

byproduct of domestication, then it was predicted that the same pattern of pruning of the

mPFC neurons reported for domesticated rats (Bell et al., 2010; Himmler et al., 2013b)

should also be found in wild rats. Our data do not support this prediction; the dendritic

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arbor and spine density were the same whether the rats were reared with playful peers or non-playful adults. Therefore, it seems that the linkage between juvenile play and the development of the mPFC has arisen due to the effects of domestication.

However, given that juvenile experience with play changes dopamine sensitivity of mPFC neurons (Baarendse et al., 2014), it is possible that the neurons of the wild rats were affected, but at a physiological level, not detectable by the method of analysis used in the present study (Gibb & Kolb, 1998). Even so, domesticated strains of rats have been shown to have both anatomical (Bell et al., 2010; Himmler et al., 2013b) and physiological (Baarendse et al., 2014) changes in the mPFC resulting from juvenile experience with playful peers. Therefore, the present findings on the anatomy of the mPFC would suggest that, at the very least, the effect of play in the wild rats is attenuated compared to that present in domesticated rats. The question remains as to why the development of the mPFC in wild rats is not as sensitive to the influence of experiencing play. It may be that the changes in brain and behavior arising from domestication are involved in play gaining this ability to modify the mPFC.

The domestication process is often accompanied by a reduction in brain size (Kruska, 1988) and changes in behavior that likely reflect modifications of the neural circuits involved, as is suggested by variations across domesticated strains (e.g., Himmler et al, 2013c; Himmler et al., 2014c; Coppinger, Glendinning, Torop, Mathay, Sutherland, & Smith, 1987; Siviy, Love, DeCicco, Giordano, & Seifert, 2003; Siviy, Crawford, Akopian, & Walsh, 2011). For example, wild animals are less tolerant to intrusion of interpersonal space and display greater levels of defensive aggression (Blanchard & Blanchard, 1994; Trut, 1999). Therefore, it is possible that neural and behavioral changes

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following domestication have created a context in which play experience is able to influence the development of the mPFC. Supporting this possibility is the finding that play-induced pruning of the neurons of the mPFC has also been reported in Syrian golden hamsters (Burleson, Pederson, Seddighi, & Cooper, 2014), another domesticated species of rodents. Taken together, these findings suggest that it is under the conditions of domestication that play has been co-opted to influence the development of the mPFC. However, these findings raise an important question: why are play-induced improvements in social skills, cognitive skills and emotional regulation important for domesticated rats (Arakawa, 2003; da Silva et al., 1996; Lukkes et al., 2009; van den Berg et al., 1999; Einon & Potegal, 1991; Baarendse et al., 2013), but if the anatomical data are to be believed, are not so for wild rats?

A potential clue emerges from the actual values of the anatomical measures from this study and those previously published on domesticated rats (Bell et al., 2010; Himmler et al., 2013b) (see Figures 6.1-3). The data show that wild rats, regardless of play experience, have similar values for all dendritic measurements as have domesticated rats with play-induced pruning of the mPFC.

Once weaned, mothers largely ignore their offspring (Cramer, Thiels, & Alberts, 1990) and young rats need to be able to fend for themselves: to interact with a variety of other rats in the colony, to find food and to protect themselves from potential predators. However, in captivity, domesticated rats live in an environment in which food, water and shelter are all provided and predation is absent. For practical purposes, such as cost and maintenance, the usual housing for laboratory rats has been to maintain them singly, from weaning. Even given recent changes in providing social enrichment, the number of social

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partners is limited [Boggiano, Cavigelli, Dorsey, Kelley, Ragan, & Chandler-Laney, 2008; Brown & Grunber, 1995; Gonder & Laber, 2007). This is very different to the dozens or hundreds of potential partners in naturally occurring colonies (Calhoun, 1962). Given the stability and lack of life-threatening challenges that accompany domesticated living, the need for behavioral flexibility is relaxed. In contrast, not only do wild rats have to deal with these challenges, but they also have to deal with them from weaning once the protection of the mother is withdrawn. That is, wild rats cannot wait until the end of the juvenile period to accrue the behavioral flexibility benefits derived from play behavior as domesticated rats do (Pellis, Pellis, & Himmler, 2014). Therefore, the pruning of the mPFC neurons (Bell et al., 2010; Himmler et al., 2013b) that is associated with improved executive function (Baarendse et al., 2013) needs to occur regardless of play experience in wild rats and achieve functional improvements in behavioral performance shortly after weaning.

If this is correct, then the maturation of the mPFC in wild rats should be accelerated relative to that reported in domesticated rats. In rats, play behavior peaks between 30-40 days (Meaney & Stewart, 1981; Panksepp, 1981), just shortly after weaning (Cramer, Thiels, & Alberts, 1990; Thiels, Alberts, & Cramer, 1990), and the full benefits of play are not fully in place until about 60 days (e.g., Arakawa, 2002; 2003; Lukkes et al., 2009) when the prefrontal cortex achieves its adult-typical level of maturity (Kolb, 1990). Therefore, if achieving an mPFC that is functionally more adult-like earlier in life is necessary in wild rats, then there should be evidence of pruning of the neurons at the onset of the peak play period. Similarly, if this is so, then testing wild rats that have not had playful experiences as juveniles on tasks evaluating executive function should

produce results akin to those of domesticated rats that have had play experience and better than those of domesticated rats without play experience (Arakawa, 2003; da Silva et al., 1996; Lukkes et al., 2009; van den Berg et al., 1999; Einon & Potegal, 1991; Baarendse et al., 2013). But why is it that domesticated rats have co-opted play to influence the development of the mPFC and executive function?

As has already been noted above, domesticated rats do not have to deal with the challenges of daily life. As such, reproductive success depends on traits deemed important to humans, not to the ability to deal with naturally occurring threats and challenges (e.g., Trut, 1999). Indeed, until relatively recently, rats were housed in isolation from weaning and so faced few social challenges. Moreover, the common husbandry practice for breeding in which rats are placed in relatively small enclosures, diminishes the need for sophisticated male-female interactions in order for the animals to mate successfully. In large, naturalistic enclosures, in which multiple potential mates are present, the inter-animal coordination and communication needed for successful mating can be very complex (McClintock, 1984; McClintock & Adler, 1978). Therefore, not only have rearing and breeding practices relaxed the need for rats to maximize their executive functions, but they have also likely expanded the time during which the prefrontal cortex matures. We suggest that this relaxation has created the conditions by which the experience of play can influence the development of the mPFC. Expanding the time period over which neural maturation takes place can increase the opportunity for experiences to influence those developing systems (Hogan, 2001). Moreover, under captive conditions, only some situations confronted by adults require an mPFC that is functioning at its peak capacity, thus leading to greater opportunity for experience-

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dependent modification. In this context, then, play has become an optional means by which, under the right conditions, it can influence the development of the mPFC and improve executive function.

6.5 Conclusions

The theory that we are proposing is that, in wild rats, executive functioning is so important that it is not left up to chance, but in domesticated rats, the window of opportunity to facilitate the growth of these executive functions is extended and can thus be influenced by playful experiences. This would suggest that play may have evolved in domestic rats as an avenue through which they are able to gain behavioral flexibility that they otherwise would not get from their natural environment. Thus, the dendritic organization in the domestic rats is more complex until the environment in which they find themselves actively prunes the neurons.

Chapter 7: The Development of Juvenile-Typical Patterns of Play Fighting in Juvenile Rats does not Depend on Peer-Peer Play Experience in the Peri-Weaning Period[§]

7.1 Introduction

Play fighting in rats typically involves the attack and defense of the nape, which if contacted is nuzzled with the snout (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). To protect the nape, the defender either evades, by fleeing or swerving away, or turns to face to block the attacker. When turning to face the attacker, the defender can either rotate onto its back (supine defense) or use a variety of tactics that involve remaining standing on one or both of its hind paws (standing defense) to ward off its partner (Pellis & Pellis, 1987). Playful attack begins to emerge at around 15-17 days of age (Baenninger, 1967; Bolles & Woods, 1964; Thiels, Alberts, & Cramer, 1990) and the tactics of playful defense do not attain their juvenile-typical pattern until 28-30 days (Pellis & Pellis, 1997). Moreover, play reaches its peak frequency between 30-40 days of age (Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1990; Thor & Holloway, 1984).

Comparison of play behavior in juveniles from wild rats and four strains of domesticated rats showed that play fighting in all these rats involved the attack and defense of the nape, and all use the same repertoire of defense tactics to defend the nape (Himmler et al., 2013c; Himmler et al., 2014c). However, strains differ in their

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Chapter 7: The Development of Juvenile-Typical Patterns of Play Fighting in Juvenile Rats does not Depend on Peer-Peer Play Experience in the Peri-Weaning Period[§]

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frequency of use of these different defensive tactics, with the largest difference being between Sprague-Dawley (SD) and Long-Evans (LE) rats. SD rats tend to use evasive tactics more frequently than facing defense, while LE rats use facing defense more frequently than evasive tactics. Moreover, when using facing defense, LE rats rotate to supine more often than SD rats. This strain-typical preference is maintained irrespective of whether attacked by same-strain or opposite-strain partners (Himmler, Lewis, & Pellis, 2014b).

The study on the effects of the strain of the attacker revealed an unexpected result: rats housed in mixed strain groups converged in their use of defensive tactics to ones that were intermediate between the two strains, and these altered preferences in defense remained the same irrespective of the strain of the attacker. That is, as little as seven days of exposure to partners from different strains in the week proceeding weaning is sufficient to change strain-typical preferences in use of defensive tactics. Given that the development of play fighting from the week preceding to the week proceeding weaning is piecemeal (Bolles & Woods, 1964; Pellis & Pellis, 1997), the findings from the cross-housing experiment (Himmler et al., 2014b) suggest that the practice that is gained from playing in an immature form prior to the juvenile period may be necessary for the consolidation of the pattern of play that is typical of juveniles.

In order to test this hypothesis, two experiments were conducted that manipulated the experience of peer-peer play in the peri-weaning period. For the first experiment, rats were socially isolated for the same seven-day time period (24-30 days) that was effective in changing strain-typical preferences in defense

due to housing with another strain (Himmler et al., 2014b). Given that LE rats showed a marked change in patterns of playful defense when reared in mixed strain groups than did the SD rats (Himmler et al., 2014b), for this experiment, LE rats were used. If peer-peer playful experiences are needed in order to develop strain-typical patterns of playful defense in the juvenile period, then social isolates should exhibit strain-atypical playful defense as juveniles.

If the play following isolation is atypical, this may not, however, be due to the lack of peer play interactions, as complete social isolation produces various abnormalities in the development of emotional regulation, as well as in cognitive and social skills (e.g., Baarendse, Counotte, O'Donnell, & Vanderschuren, 2013; Byrd & Briner, 1999; da Silva, Ferreira, Carobrez, & Morato, 1996; Hall, 1998; Lukkes, Mokin, Sholl, & Forster, 2009, Von Frijtag, Schot, van den Bos, & Spruijt, 2002), with some impairments evident when isolation is limited to the first week proceeding weaning (Arakawa, 2002; 2003; 2007a, b). Therefore, if the rats in the post-weaning social isolation experiment were to show atypical patterns of play as juveniles, this could be due to an indirect effect of isolation on emotional, cognitive, and social development, and not necessarily due to the lack of practice of play fighting with peers.

In contrast, if the pattern of play is juvenile-typical following post-weaning social isolation, this may not in itself show that peer-peer play with littermates is not necessary. In the Himmler et al. (2014b) experiment, what was shown was that living and playing with a strain of rat that plays differently in the week proceeding weaning can change the manner in which an individual plays. It may be the case

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that play with same-strain peers in the week preceding weaning, when play first begins to emerge (Bolles & Woods, 1964; Pellis & Pellis, 1997), provides the critical social experience for the development of juvenile-typical play, with the pattern of play only subject to change later if the post-weaning experiences are in conflict with those that occurred prior to weaning. That is, to capture the critical role of peer-peer interactions in the maturation of juvenile-typical play, depriving infants of such experiences over a wider swathe of the peri-weaning period may be needed.

Therefore, the second experiment was designed to control for these two confounding factors. First, the infant was housed with an adult female, which eliminates the effects of complete social isolation and provides it with a variety of social experiences (e.g., grooming, huddling), but little-to-no experience of play, and certainly no play with a peer (Einon, Morgan, & Kibbler, 1978). Second, infants were denied the opportunity for peer-peer play over the whole peri-weaning period (15-28 days) during which play fighting matures (Pellis & Pellis, 1997). Therefore, in Experiment 2, individual pups were reared with only their mothers as social companions and the play of these pups as juveniles was compared to the play of juveniles that had been reared with both a mother and siblings. However, given that weaning rats and cats early can affect the frequency of their play in the juvenile period (e.g., Bateson & Young, 1981; Brunelli, Shindledecker, & Hofer, 1989; Guyot, Bennett, & Cross, 1980; Janus, 1987; Ikemoto & Panksepp, 1992; Shimozuru et al., 2007), an additional control group was used. Pups were reared over the peri-weaning period with peers alone, in the absence of their mother.

With domestication, animals are reared for many generations in environments free of stressors, such as predation and food shortages and live in an atypical social organization; this may have reduced the critical importance of some early developmental experiences in shaping later juvenile behavior (Bateson & Martin, 2000). Thus, while for Experiment 1, the males from a domesticated strain were used for direct comparison to the results from Himmler et al. (2014b), for Experiment 2, wild rats born and raised in the laboratory (see Method) were used, diminishing the potentially confounding effects of domestication. Note also, that in the first experiment, only males were used, but in the second, both sexes were used. The reason that males were used in Experiment 1 was to parallel the study by Himmler et al. (2014b), but while some studies reveal little or no difference in the play fighting of males and females (e.g., Himmler et al., 2013c; Himmler et al., 2014c; Panksepp, 1981), others have revealed both quantitative and qualitative differences (e.g., Meaney & Stewart, 1981; Pellis, 2002b). Therefore, in Experiment 2, both males and females were used to increase the likelihood of detecting peer-influences on the development of play fighting.

While the present study is primarily focused on the development of the tactics of defense, the potential effects of peer-peer play experience on the development of these tactics could arise indirectly due to experience-induced effects on the quality of playful attack. Even though from the very outset, pre-weaning rats focus their playful attacks on their peers' napes and this does not appear to change with age under normal rearing conditions (Pellis & Pellis, 1997), it is possible that the tight focus on the nape is maintained by experience with attacking the nape. That is, in the

absence of peer-peer play in the peri-weaning period, the targeting of the nape may degrade and this could indirectly affect the pattern of playful defense that emerges in the juvenile period. Therefore, in addition to scoring the tactics used for defense in the play fighting of juveniles, measures of the accuracy of playful attacks were also scored. Finally, while play-deprived rats may begin by playing in a typical manner, their lack of experience may erode their ability to modulate their actions in a way that enables play to remain playful (Pellis, Pellis, & Reinhart, 2010). Therefore, measures that evaluate the ability for play facilitating actions to be deployed by the rats (e.g., see Kisko, Himmler, Himmler, Euston, & Pellis, 2015) were also scored.

7.2 Method

7.2.1 Subjects

A total of 151 rats were used in these studies. Of these, 24 Long-Evans (LE) male rats were used for Experiment 1. These rats were obtained from Charles River Laboratories (St. Constant, Quebec) at around 23 days of age and housed at the Canadian Centre for Behavioral Neuroscience. All animals were housed in their respective conditions at 24 days of age. The rats were maintained at a constant 21-23°C on a 12:12-hour light-dark cycle and were kept in 46cm x 25cm x 20cm polyethylene tubs, with processed corncob bedding. Food and water were provided ad libitum. All animals were handled and cared for in accordance with the Canadian Council for Animal Care regulations.

The remaining 127 rats were derived from a wild-type stock (WWCPS – Warsaw Wild Captive Pisula Stryjek) and were bred and housed at the vivarium at the Department of Psychology, Helena Chodkowska University of

Management and Law, Warsaw, Poland (Stryjek & Pisula, 2008), and were handled in a way that minimizes human contact (Stryjek, 2008, Stryjek, 2010; Stryjek & Modlińska, 2013).

All WWCPS rats were housed in Tecniplast© Eurostandard Type IV cages (61cm×43.5cm×21.5cm) with dust-free softwood granules Tierwohl Super© as bedding. Food (Labofeed H, WP Morawski, Kcynia, Poland) and water were provided ad libitum. The day/night cycle was set at 12/12h, and the temperature was maintained at constant 21-23ºC. All rats kept in the laboratory were housed, bred and cared for in accordance with the Regulation of the Polish Minister of Agriculture and Rural Development of 10 March 2006 on laboratory animal care, and the experimental procedures were approved by the 4th Local Ethics Commissions in Animal Experimentation, Warsaw, Poland.

7.2.2 Apparatus

All play trials were in a 50cm×50cm×50cm Plexiglas box, with the floor having a 1-2cm layer of Softzorb® bedding for LE rats and Tierwohl Super© bedding for WWCPS rats. Based on previously established protocols, following each trial, the box was thoroughly cleaned with Virkon© and the bedding replaced in order to ensure that the experimental box was free of smells from the rats previously tested. Even though this may introduce some novelty to the testing enclosure that could affect playfulness (e.g., Vanderschuren, Niesink, Spruijt, & Van Ree, 1995), the pre-test habituation appears sufficient to ensure that the effects of strain and experimental treatment can be detected (Himmler et al., 2013c; Himmler et al., 2014c; Kisko et al., 2015). Play trials were recorded with a

DVD103 Sony Handycam for the LE rats and a LC-308D camera for the WWCPS rats. Both cameras were equipped with the night-shot option and were placed so that video recordings were recorded from an oblique (45$^{\text{o}}$) angle.

7.2.3 Procedure

In all groups, play was tested between 31-35 days, which is within the peak period for playful interactions for rats (Thor & Holloway, 1984) and before the age at which, in males, dominance relationships begin to form (Takahashi & Lore, 1983; Pellis & Pellis, 1991). All rats were tested for their play in a standard paradigm (Himmler, Pellis, & Pellis, 2013a). They were habituated to the test enclosure for 30 minutes per day, for three consecutive days, prior to testing. Following habituation, each subject was socially isolated for 24 hours prior to testing, as brief periods of social isolation increase playfulness, and then tested for 10 minutes. Both habituation and testing sessions were conducted in complete darkness, as play increases in frequency when in the dark as compared to normal light levels, low light or red light. Placement into, and removal from, the testing cage, was done with the experimenter wearing protective gloves.

7.2.4 Behavioral Analysis

Playful interactions were first inspected at full speed, then in slow motion and frame-by-frame. Whereas Long-Evans rats can be easily identified from pair mates due to black and white pelage patterns, all WWCPS rats have a brown coat, and thus pair mates could not be readily tracked as individuals. Therefore, the play behaviors of both LE and WWCPS were scored and summed for pairs as we have done previously (Himmler et al., 2013c).

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Playful interactions begin when one partner approaches and attacks their partner's nape. The recipient of the attack can then either respond to the attack or simply ignore it. If the recipient defends against the attack, the type of defense can be recorded (Himmler et al., 2013a). Therefore, the frequency of playful attacks per trial, the probability of defense (percentage of all nape attacks that were defended) and the probability of each type of defense tactic (percentage of each tactic used when defensive action was taken) were all recorded.

7.2.5 Playful Attack

A playful attack was scored when one rat's nose was either in contact with its partner's nape, or when one rat made a targeted movement towards the nape of the other, but a defensive movement by the recipient precluded actual contact. If the recipient initiates a defensive action before the attacker reaches the nape, the point of contact on the defender's body was also scored, thus enabling the relative frequency of nape directed play fights versus non-nape directed play fights to be evaluated (Himmler et al., 2013c; Himmler et al., 2014c). The total frequency of attacks per pair per the 10 min trials was scored.

To assess the quality of the execution of playful attacks, three aspects of how rats move during an attack were measured: *aim*, *vigor* and *maintenance*. The first two were measured at the onset of the attack and the third was measured in the cases in which the defender lay on its back to protect its nape (i.e., pin, see below). All three aspects of the execution of attacks were scored on a three-point scale (0, 1 or 2).

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For *aim*, if the attacker failed to make contact with the nape (i.e., over or undershoot the target), that attack was given a score of "0," whereas if the attacker had clearly targeted and made contact with the nape, that attack was given a score of "2." Attacks that were intermediate between these two were given a score of "1." For *vigor*, if the attacker had walked over or simply moved its snout towards the nape of the other animal, a score of "0" was given for the attack. However, if the attacker had pounced or made swift movements towards the nape of the other animal, the attack was given a score of "2." Attacks that were intermediate between the two were given a score of "1." For *maintenance*, a "0" was given if the attacker either walked over to the supine defender or held the defender down with its forepaws, but in neither case made any movements of the snout toward the nape. A score of "2" was given if the attacker continued to target or maintained snout contact with the nape of the supine defender. Attacks that were intermediate between these two were given a score of "1."

For *aim* and *vigor*, a total of 10 playful attacks per pair were used and for maintenance, eight per pair were used. As these represented only a subset of the total attacks that occurred in the 10 min trials, to ensure that all pairs were sampled similarly, the minute in which the peak frequency of attacks was identified for each pair. The first eight or ten cases, depending on the measure, occurring during this peak period, were used. For *maintenance*, two male pairs and one female pair of WWCPS rats did not meet the minimum of eight supine configurations and so were not included in this analysis.

7.2.6 Playful Defense

Attacks to the nape can be defended using two major types of tactics: The first tactic is evasion, in which the defender moves its nape away from its attacker and does so by running, leaping or swerving away and thus faces away from its partner. The second tactic is facing defense, in which the defender moves its nape away by turning to face its partner, so blocking access by opposing its teeth between its partner and its own nape. Facing defense can also take one of three forms: (i) complete rotation, in which the defender rolls completely over onto its back, (ii) partial rotation, in which the defender rotates its forequarters, but maintains contact with the ground with one or both of its hind feet, and (iii) other, in which defensive actions involve rotations or other movements in other dimensions (e.g., rotating vertically in a horizontal plane). The type of defensive tactic used was determined by the movements occurring in the first two to three video frames, to ensure that what was recorded was the tactic first attempted by the defender (Himmler et al., 2013a). Based on the total frequency of attacks and defenses scored per pair, the probability that an attack led to a defensive maneuver and the probability of which defensive maneuvers were used were calculated. Given that previous studies have shown that the biggest strain differences are in the use of evasion and complete rotation (Himmler et al., 2013c; Himmler et al., 2014b, c), for simplicity, unless other tactics emerged as significantly different, only data on these two tactics will be presented graphically.

7.2.7 Outcomes of Play Fights

Playful interactions can last for a few seconds, and irrespective of the initial defensive tactic used, can lead to a number of different outcomes. For example, the playful interaction can end with one partner on its back, with the other standing on top, in what has been called a 'pin' configuration (Panksepp, 1981), or the partners may end up standing on their hind legs facing and holding one another (rearing) (Poole & Fish, 1975; Silverman, 1978). Which outcomes arise can provide insight into the motivational organization of the behavior. Some studies have shown that, an increase in rearing, especially if coupled with boxing (i.e., hitting one another with the forepaws), has been associated with increased aggression (e.g., Hurst, Barnard, Hare, Wheeldon, & West, 1996; Reinhart, Pellis, & McIntyre, 2004; Taylor, 1980). In contrast, increases in pinning have been interpreted as an increased motivation for playful contact (e.g., Panksepp, Siviy, & Normansell, 1984; Pellis & McKenna, 1995; Varlinskaya, Spear, & Spear, 1999). Therefore, to assess whether the motivational substrate was altered by the different rearing conditions, rearing, with and without boxing, as well as pinning were scored.

Rearing was scored when both partners were standing on their hind legs facing each other. Once in the rearing position, boxing was scored if one, or both rats, slapped the other on the face (Grant & Mackintosh, 1963). Irrespective of the duration of the rearing position, each bout was scored as a single event. Pinning was scored if the rats ended in a position with one partner on its back and the other standing on top (Panksepp, 1981). The frequency of rearing and pinning was scored as the absolute frequency per pair per trial.

During play fighting, rats may also launch counterattacks after successfully defending their nape from their partner (Pellis & Pellis, 1990). Successful counterattacks to the nape lead to role reversals, in which the original attacker is put on the defensive (Pellis, Pellis, & Foroud, 2005). For play to remain playful, interactions need to be reciprocal with the frequency of reversals providing a measure of the reciprocity (Pellis et al., 2010b). Therefore, changes in the frequency of role reversals can provide insight into altered social competence (Kisko et al., 2015). A sequence of attack and defense that led to the original attacker becoming the defender was recorded as a role reversal. For each pair, the percentage of attack-defense sequences that led to a role reversal was calculated and these were used to calculate group means.

7.2.7 Experiment 1

A total of 24 rats were used. Twelve were singly housed at 24 days of age and were not exposed to a social partner until testing began between 31-33 days. The other 12 animals were housed in pairs at 24 days of age for the duration of the experiment. Given that, by necessity, the socially isolated animals were tested with unfamiliar partners, the individual subjects from the pair-housed condition were also tested with unfamiliar partners by using rats from different dyads. In this way, any group differences would be due to rearing effects, not the identity of the play partner.

7.2.8 Experiment 2

Of the 127 wild rats used in this study, 21 were adult females used for breeding. Sixteen of the adult females provided the young for the mother-only

and sibling-only groups, and also the rearing companions for the mother only group. The 16 adult females gave birth to a total of 73 pups (36 male and 37 female), with 16 of the pups (8 male and 8 female) being used for the mother-only group, 24 pups (12 male and 12 female) being used as the experimental animals for the sibling-only group, and the remaining 33 pups (16 male and 17 female) being used as the partners for the sibling-only groups. The other 5 adult females gave birth to 33 pups (16 male and 17 female) and these were used for the control group containing both siblings and the mother. Of the 24 pups born to these 5 females, 12 males and 12 females were used to form the control groups, with the remaining 9 pups (4 male and 5 female) being used for other experimental purposes (see below for a more full description of the rearing conditions used for Experiment 2).

The WWCPS rats used in this study were of the F3 generation. With further generations of breeding within a laboratory context, there is the increased risk that the domestication process would begin to change various aspects of behavior (Barnett & Stoddart, 1969; Blanchard, Flannelly & Blanchard, 1986). At the same time, wild captured rats or their offspring were not included in the experiment, as there is no possibility of assessing, let alone controlling for, the conditions in which such animals had developed. Also, for the wild caught animals, the drastic change in environmental conditions may have had a profound effect on their levels of stress, and consequently, on their behavior during tests, as well as on their ability to raise offspring. Therefore, using the F3 generation allows us to control the conditions for the rearing environment

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experienced by the WWCPS rats, while reducing the potential early effects of domestication (Himmler et al., 2013c; Stryjek, Modlińska, Turlejski, & Pisula, 2013).

7.2.8.1 Rearing Conditions

Single pregnant females were placed in separate, standard cages (Tecniplast© Eurostandard Type IV) with food and water provided ad libitum. After birth, the health of females and their litters was monitored. All rats were kept under identical conditions until day 15, when the pups were randomly divided into one of three groups (siblings-only, mother-only, and sibling-and-mother). All rats remained in these experimental conditions until day 27, when they were randomly paired with a sex and rearing condition matched partner. After pairing, all animals remained with the same partner for the remainder of the experiment (i.e., until they were 35 days of age).

7.2.8.1.1 Siblings-only (SO)

A total of 57 (28 male and 29 female) pups from 10 litters were used in this condition. On day 15, pups were taken from their mothers and placed in incubators (Happy Chick II mini) (67cm×41cm×32cm), in groups of 6-8 siblings of the same sex. The incubator was equipped with a thermostatically controlled red light, heat lamp, which ensured a constant ambient temperature of 35°C. All pups were fed standard fodder ad libitum. To make the standard food accessible to the pups, it was mashed and soaked with substitute milk (Bebilon Comfort 1, Nutricia, Poland). The feed was replaced twice a day until the rats were able to consume unmashed standard fodder. In order to ensure the pups were receiving

sufficient nutrition, two steps were taken. First, for the first two days of separation from the mother (day 15 and 16), the pups were also fed milk by a pipette two times daily. Second, the feed was weighed at each inspection to monitor the amount of food ingested by the pups and the pups were weighed daily to ensure that they were gaining weight. The rats remained in the incubator until 27 days old, at which time 12 male and 12 female rats were randomly selected as the experimental animals and placed in same-condition, same-sex pairs. These pairs (6 male and 6 female) served as the experimental pairs for this condition.

7.2.8.1.2 Mother-only (MO)

The litters of 16 mothers were reduced to a single pup (8 male and 8 female). The single pups remained with the mother and did not receive any peer-peer interactions until day 27, at which time the rats were randomly placed in same-condition, same-sex pairs. These pairs (4 male and 4 female) served as the experimental pairs for this condition.

7.2.8.1.3 Control-Mother and Siblings (CO)

A total of 33 (16 male and 17 female) pups from 5 litters were used for this condition. Pups were reared with both mother and groups of 4-8 siblings until day 27, at which time 12 male and 12 female rats were randomly selected as the experimental animals and placed in same-condition, same-sex pairs. These pairs (6 male and 6 female) served as the experimental pairs for this condition.

7.3 Statistical Analyses

The data for Experiment 1 were analyzed using two-tailed independent sample t-tests. For Experiment 2, the data were analyzed using a two-way

analysis of variance (ANOVA), with sex and group (mother-only, sibling-only, mother and sibling) as independent variables. For pairwise comparisons, the least significant difference test was used for post hoc tests. For multiple comparisons, the Bonferroni correction was used when needed. Because the measures for the aim, vigor and maintenance were ordinal (i.e., scores of 0, 1 or 2), a non-parametric test, the Kruskal-Wallis non-parametric one-way analysis of variance, was used, and for pairwise comparisons, Mann-Whitney U tests were used. Differences were considered significant for p values ≤ 0.05. For graphical representation of interval data, values are given for group means and standard deviations, and ordinal data are given as group medians and ranges.

Inter-observer reliability for the same scorers was previously evaluated for the standardized measurements of playful attack and defense (Himmler et al., 2013c; Himmler et al., 2014c). However, because the measurements of aim, vigor and maintenance were new, these were evaluated for inter-observer reliability. For each of these measurements, 12 examples (two for each condition for each sex for the WWCPS rats), previously scored by one observer (B. T. H.), were re-scored by another observer (S. M. H.). Pearson's correlation revealed a high degree of inter-rater reliability (*aim*: $r = 0.834$; *vigor*: $r = 0.946$; *maintenance*: $r = 0.908$). All correlations were significant ($p < 0.05$).

7.4 Results

7.4.1 Experiment 1

7.4.1.1 Playful Attack

There were no significant differences between groups for the proportion of

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playful interactions that began with the defense of the nape rather than contact on other areas of the body ($p > 0.05$). All groups attacked the nape in over 90% of cases. The frequency of launching nape attacks was significantly different, $t(10) = 5.736$, $p = 0.0001$, with socially isolated rats attacking more often (Figure 7.1a). With regard to the execution of attacks, there were no significant differences between groups for *aim* ($p > 0.05$), *vigor* ($p > 0.05$), or for *maintenance* ($p > 0.05$) (Table 7.1).

7.4.1.2 Playful Defense

There was no significant difference for the probability of defense ($p > 0.05$) (Figure 7.1b). Both groups defended their napes in $\geq$ 90% of cases. Also, there were no significant group differences for the probability of using either of the defensive tactics: evasion ($p > 0.05$) or complete rotation ($p > 0.05$) (Table 7.1).

A.   B.
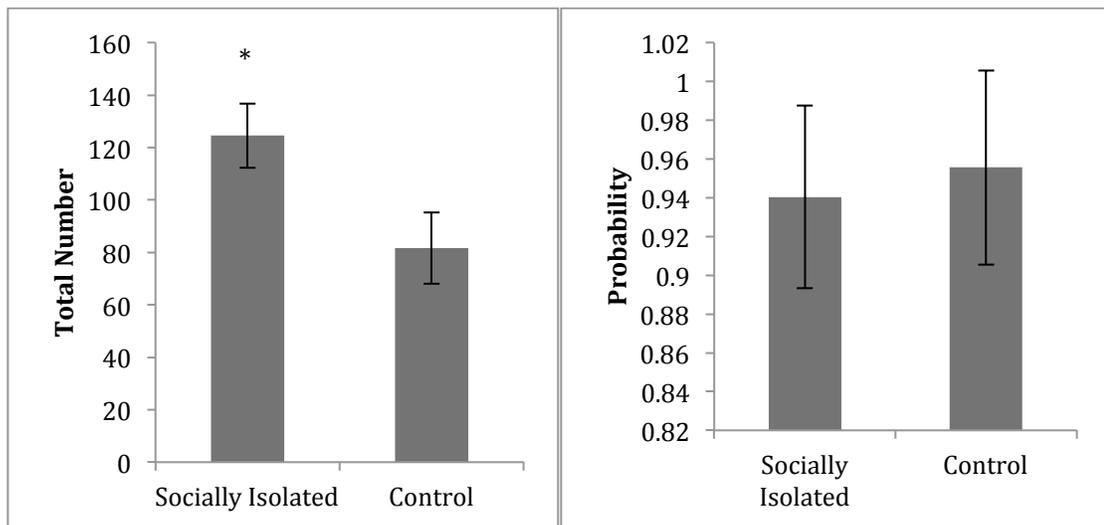
Figure 7.1
(A) The total number of attacks per 10 minutes and (B) the probability of defending against a playful attack for LE rats.

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7.4.1.3 Outcomes of Play Fighting

The probability of defense involving rearing revealed a significant difference between groups with the control group rearing more than isolates, $t(10) = -2.781$, $p = 0.019$ (Table 7.1), but the probability that rearing led to boxing did not differ significantly between groups ($p > 0.05$). There was no significant difference between groups for pinning ($p > 0.05$) or in the probability of role reversals ($p > 0.05$) (Table 7.1).

Table 7.1: The three aspects of play measured for LE rats from experiment are shown.

	Attack[a]			Defense[b]		Outcome[b]		
	Aim	Vigor	Maintenance	Evasion	CR	Rearing	Pinning	Role Reversals
LE-Paired	1.85 (1.6-2.0)	1.6 (1.4-1.8)	1.88 (1-2)	0.31 ± 0.02	0.43± 0.08	0.06 ± 0.01	0.69 ± 0.12	0.28 ± 0.08
LE-Socially Isolated	1.6 (1.3-2.0)	1.6 (1.3-1.8)	1.75 (1-2)	0.30 ± 0.05	0.42 ± 0.03	0.04 ± 0.01	0.62 ± 0.07	0.31 ± 0.06
p	ns	ns	ns	ns	ns	< 0.05	ns	ns

Note. [a]The scores for these measures are shown as medians and ranges (as shown in parentheses), and the statistical comparisons were done using the Mann-Whitney U. [b]The scores for these measures are shown as mean ± SD, and the statistical comparisons were done using independent t-tests

7.4.2 Experiment 2

7.4.2.1 Playful Attack

A 2 x 3 ANOVA for the proportion of playful interactions that began with the attack of the nape rather than contact on other areas of the body failed to reveal any significant difference for sex ($p > 0.05$), group ($p > 0.05$), or interaction

between sex and group ($p > 0.05$). All groups attacked the nape in over 90% of cases.

A 2 x 3 ANOVA for the total number of playful attacks did not show a significant sex difference ($p > 0.05$), but did reveal a significant main effect for group, $F(2, 26) = 4.632$, $p = 0.019$. Pair wise comparisons revealed that rats in the SO group launched more playful attacks than those in the CO group ($p < 0.05$), but neither group differed from the MO group (Figure 7.2a). Even though there was no significant interaction for sex and group ($p > 0.05$), inspection of Figure 7.2a indicates that most of the increase in the frequency of play by the SO group was likely due to the females.

A.   B.
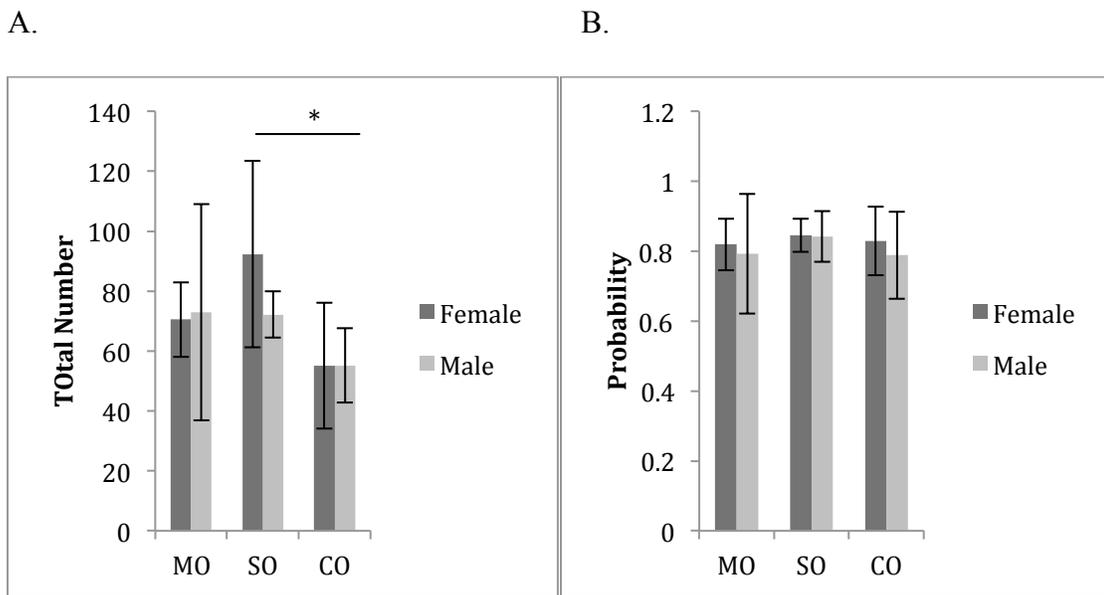
Figure 7.2
(A) The total number of attacks per 10 minutes and (B) the probability of defending against a playful attack for WWCPS rats.

With regard to the execution of attacks, a Kruskal-Wallis test revealed a significant difference for *aim*, H(2) = 12.228, $p = 0.002$, with the CO group scoring

significantly higher than the SO and MO groups ($p < 0.05$), but the MO and SO did not differ significantly from each other ($p > 0.05$). There was no significant main effect for sex ($p > 0.05$). For *vigor*, a Kruskal-Wallis test revealed a significant difference for group, $H(2) = 7.605$, $p = 0.022$ with the MO and SO groups scoring higher than the CO group ($p < 0.05$), but the MO and SO did not differ significantly from each other ($p > 0.05$). There was no significant main effect for sex ($p > 0.05$). For *maintenance*, there were no significant main effects of group or sex ($p > 0.05$) (Figure 7.3).
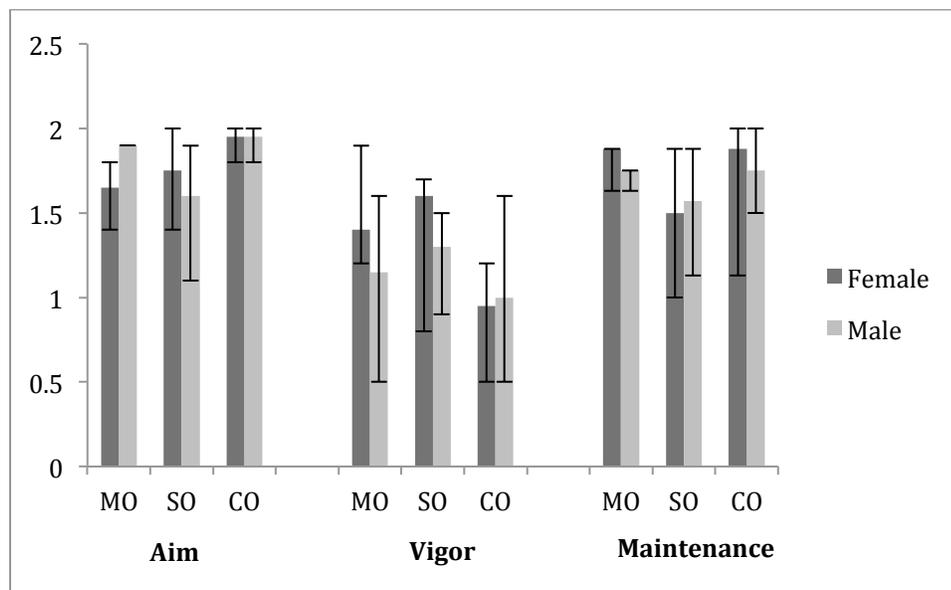
Figure 7.3
The median scores (and ranges) for the *aim*, *vigor*, and *maintenance* of playful attacks to the nape in all groups of WWCPS rats.

7.4.2.2 Playful Defense

A 2 x 3 ANOVA for the probability of defending against a nape attack revealed no significant main effects or a significant interaction ($p > 0.05$). All three

groups defended their napes in $\geq$ 90% of cases. A 2 x 3 ANOVA for the probability of using evasive playful defense did not reveal significant group effect ($p > 0.05$), but did reveal a significant main effect of sex, $F(1, 26) = 8.474$, $p = 0.007$, with males doing more than females ($p < 0.05$). For facing defenses, there were no significant main effects or interactions for   the probability of using complete rotation ($p > 0.05$). However, there was a significant main effect for the probability of using other defenses for sex, $F(1, 26) = 9.619$, $p = 0.005$, with females using this defensive tactic more often ($p < 0.05$). There was no significant main effect for group ($p > 0.05$), nor a significant interaction between sex and group ($p > 0.05$) (Figure 7.4).

A.

B.

C.
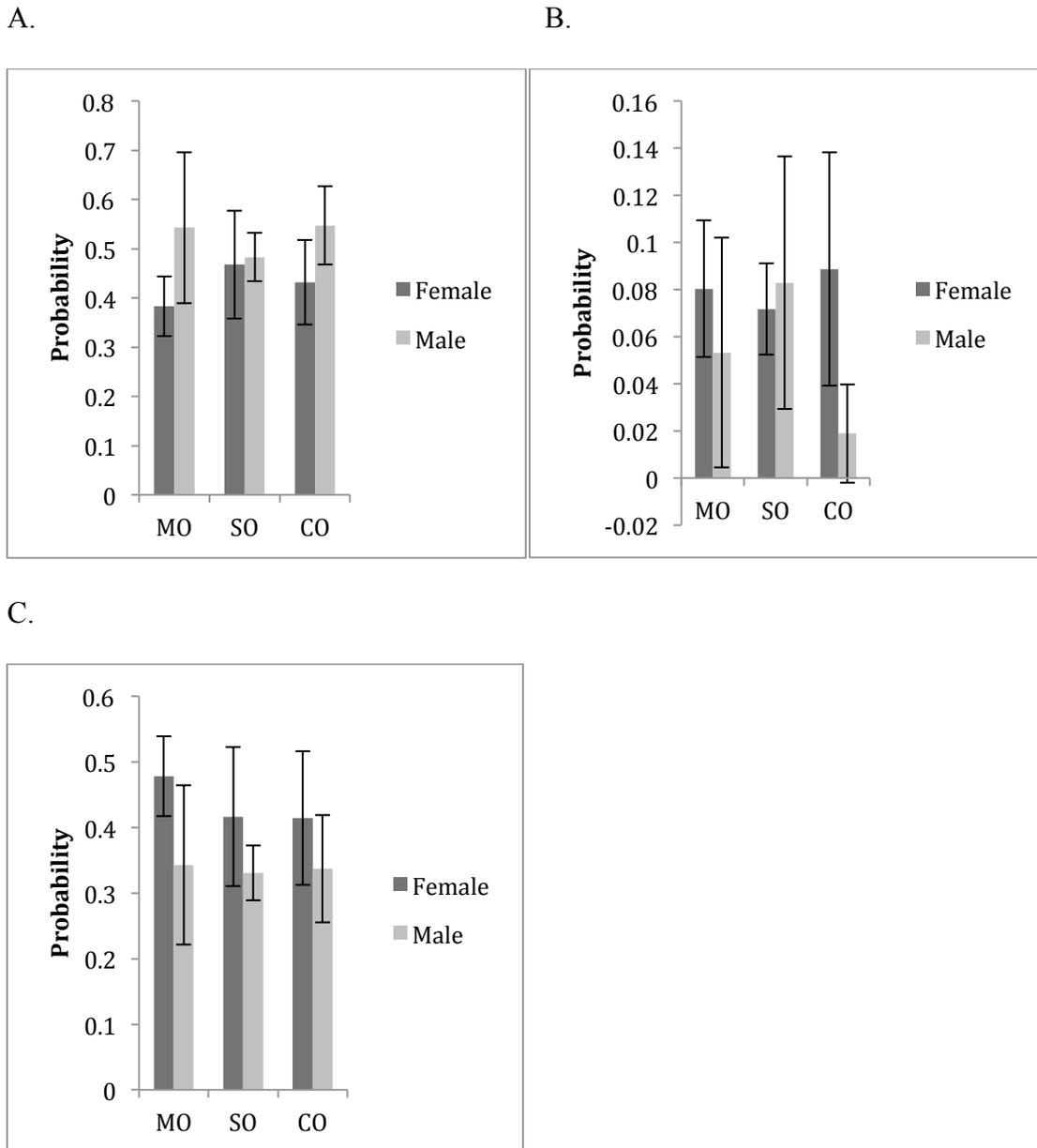
Figure 7.4

The probability of using evasive tactics (A) the complete rotation tactic (B) and the 'other' tactic (C) in response to a playful attack for all groups of WWCPS rats.

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7.4.2.3 Outcomes of Play Fighting

A 2 x 3 ANOVA for the probability of defense involving rearing revealed a significant main effect for group, $F(2, 26) = 4.027$, $p = 0.030$, with the MO group having more rearing than the SO group ($p < 0.05$), but neither group differed from the CO group (Figure 7.5). The probability that rearing led to boxing did not differ significantly among groups or between the sexes ($p > 0.05$), but there was a significant interaction, $F(2, 26) = 4.928$, $p = 0.015$. Pair wise comparison revealed that the females in the SO group were more likely to engage in boxing than both the MO and CO groups ($p < 0.05$) (Mean $\pm$ SD: CO: Males = 0.37 ± 0.11; Females = 0.24 ± 0.11; MO: Males = 0.29 ± 0.14; Females = 0.18 ± 0.14; SO: Males = 0.07 ± 0.11; Females = 0.57 ± 0.11). A 2 x 3 ANOVA of pinning revealed no significant differences among the groups or between the sexes ($p > 0.05$) (Figure 7.5).
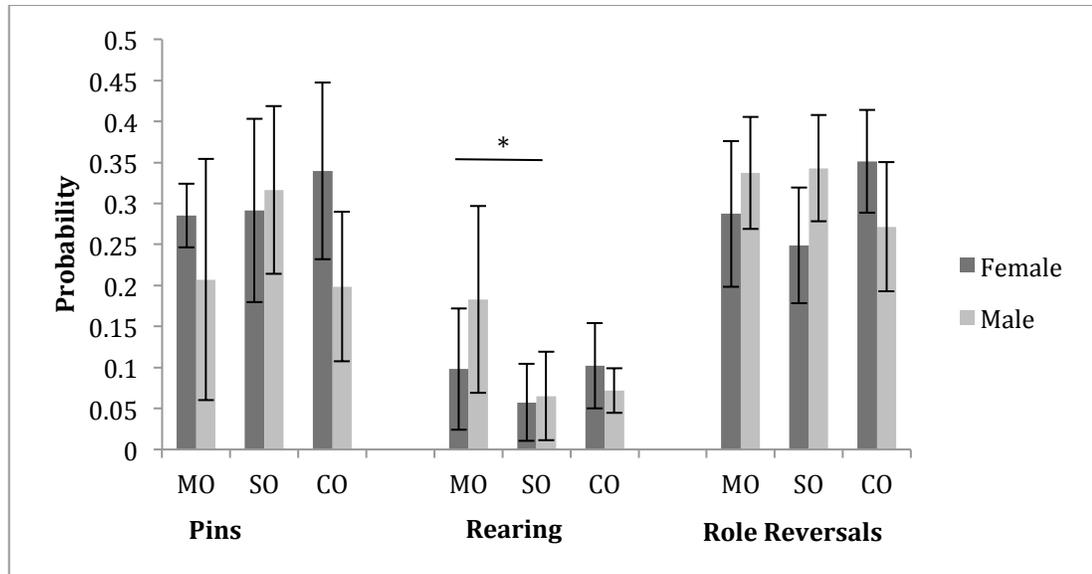
Figure 7.5
The probability of defensive maneuvers resulting in a *pin*, a *rear*, or a *role reversal* for all groups of WWCPS rats.

For the probability of role reversals, there were no significant main effects for sex or group ($p > 0.05$). However, there was a significant interaction, $F(2, 26) = 4.665$, $p = 0.019$). Pair wise comparisons revealed role reversals were more common in females in the SO group compared to females in the MO and CO groups ($p < 0.05$), although, within the SO group, they were more common in males as compared to females ($p < 0.05$) (Figure 7.5).

7.5 Discussion

The present study was designed to test the hypothesis derived from the findings by Himmler et al. (2014b) that peer-peer experience during the peri-weaning period is necessary for the development of juvenile-typical patterns of playful defense. In order to determine if peer experiences are needed, two experiments were conducted. The first experiment investigated the play of juvenile LE rats that were

socially isolated for the week following weaning (i.e., matching the age at which the Himmler et al. (2014b) results were found) and the second experiment investigated the play of juvenile WWCPS rats that were reared as singletons with only the mother during the entire peri-weaning period. In both experiments, lack of play experiences with peers in the peri-weaning period did not affect the development of strain-typical preferences in the tactics of defense used during play in the juvenile period or for the consolidation of the nape as the target of playful attack. That is, peri-weaning play with peers is not necessary for the development of juvenile-typical patterns of attack and defense in play fighting, although the aim of nape attacks were less accurate and the vigor of their execution was increased in the experimental subjects from Experiment 2. However, given that these changes were present in both the MO group, which did not experience peer-peer play, and the SO group which did, it is likely that these may have resulted from underlying changes in excitability or motivation, rather than in the ability to play in the typical manner (see below).

A change that occurred in both experiments was in the motivation to play, as measured by the frequency of nape attacks (Panksepp, 1981; Pellis & McKenna, 1995; Thor & Holloway, 1984). There was a large increase in the frequency of nape attacks for the LE rats in the socially isolated group, which is consistent with other studies showing that rats that have been isolated for an extended period of time tend to play at a higher frequency (Byrd & Briner, 1999; Ikemoto & Panksepp, 1992; Panksepp & Beatty, 1980; Varlinskaya et al., 1999). An increase, albeit a smaller one, was also seen in the SO WWCPS rats. There may be two separate

mechanisms involved in these two cases of increased of playfulness.

Following a period of social deprivation, rats will increase their initiation of playful contact with the nape, but this is not associated with a comparable increase in the frequency of social investigation (Panksepp, 1981; Panksepp & Beatty, 1980). This suggests that being deprived of peers is not simply producing an increase in the motivation for social contact, but a specific increase in the motivation to engage peers in play. This is supported by other studies, which show that simply suppressing play without social isolation also produces a rebound in the frequency of play when the opportunity arises. For example, Baldwin and Baldwin (1976) showed that the frequency of social play in squirrel monkeys decreases when food is scarce, as more time is required to find food. However, once food is made readily available, the frequency of play increases to above normal baseline levels. These studies suggest that the motivation for play can be manipulated independently of other forms of social motivation. Such a selective increase in the motivation to play may account for the findings on the socially isolated LE rats from Experiment 1.

In the case of the SO WWCPS rats from Experiment 2, the increased play of the juveniles is consistent with findings from other studies showing that early weaning, involving separation from the mother, tends to lead to increased playfulness (e.g., Brunelli et al., 1989; Janus, 1987). A possible avenue for this effect is that maternal contact involving licking, grooming and huddling, that is effective in altering juvenile play when received in the first two weeks after birth (e.g., Arnold & Siviy, 2002; Birke & Sadler, 1987; Karkow & Lucion, 2013; Moore & Power, 1992; Parent & Meaney, 2008; Veenema & Neumann, 2008), may continue to have some influence

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in the week preceding weaning. Whether this is the case has yet to be established, as is whether the change in playfulness arises from a specific influence on the motivation to play or from some more generic factors, such as changes in stress regulation (e.g., Caldji et al., 1998; Francis & Meaney, 1999), that may indirectly influence playfulness along with all social behavior.

That there were such potential stress-induced changes due to early rearing influences is the finding that, in the SO group, there was a significant increase in rearing. This suggests that, in the absence of the mother, there may be reduced regulatory control and an increase in aggression (see also Diamantopoulou et al., 2012). That both MO and SO reared subjects appeared more excitable (i.e., more vigorous nape attacks) and less accurate in their nape contacts, suggests that, in late infancy, both the presence of the mother and of siblings may contribute to the maturation of regulatory mechanisms that affect social behavior. That the changes in playfulness arising from atypical social environments in the peri-weaning period (Experiment 2) and from social isolation (Experiment 1) involve different mechanisms, is supported by the data on rearing in Experiment 1, in which the social isolates engaged in significantly less rearing. The reduced rearing typically results in more time engaged in contact promoting wrestling (Pellis & Pellis, 1987), suggesting that the increased frequency of launching playful attacks by the isolates is, indeed, a reflection of an increased motivation to engage in play.

7.5.1 The Prejuvenile Development of Playful Attack and Defense

During the peri-weaning period, play is still developing, not becoming fully juvenile-typical until between 28-30 days of age (Pellis & Pellis, 1997). This

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continuing development suggests that the brain mechanisms involved in the regulation of play are also still maturing. The altered patterns of play induced over this period by the experience of play with peers of a different strain (Himmler et al., 2014b) further suggests that these brain mechanisms are sensitive to alteration by social play experiences. For these reasons, it is surprising that rats with no peer-peer play during the peri-weaning period still developed the juvenile-typical patterns of attack and defense.

The normal development of behavior patterns without prior experience with their performance has been categorized as prefunctional (Hogan, 2001). This label does not mean that no experience is necessary for the development of the behavior only that functional feedback from the performance of earlier forms of that behavior is not necessary. For example, dust bathing in fowl involves a sequence of movements, starting with the fowl pecking and scratching at the ground, dropping and spreading its wings, one at a time, rolling over to one side and then the other, and then finally standing and shaking its body. Dust bathing gradually matures in the young. Young fowl will add elements of the dust bathing sequence to their unfolding repertoire, in the same order in which the complete sequence is performed. However, neither functional feedback from the incomplete versions of the dust bathing nor exposure to dust is necessary for the development of the complete dust bathing sequence (Vestergaard, Hogan, & Kruijt, 1990).

No deficits were found in the juvenile patterns of playful defense in rats that had been deprived of play with peers in either the post-weaning period alone or the whole peri-weaning period, extending from the beginning third week to the end of

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the fourth week after birth. The absence of practicing defensive tactics when attacked (the isolates in Experiment 1 and the MO rats in experiment 2) or of receiving slightly deviant attacks (the SO rats in Experiment 2) did not affect the achievement of juvenile-typical patterns of playful defense. That is, like the dust bathing in fowl, the development of juvenile-typical play in rats appears to be prefunctional in that it does not require experience from its performance during the peri-weaning period to develop into its mature form.

7.5.2 Why is the Development of Juvenile-Typical Play Fighting So Robust?

Play and other social interactions during the juvenile period have been found to provide important experiences for developing and refining a variety of social, emotional and cognitive skills by modifying the brain mechanisms that regulate them (e.g., Arakawa, 2002, 2003, 2007a,b; Baarendse et al., 2013; Bell, Pellis, & Kolb, 2010; Delville, David, Taravosh-Lahn, & Wommack, 2003; Einon & Morgan, 1977; Einon et al., 1978; Hall, 1998; Himmler, Pellis, & Kolb, 2013b; Siviy, 2010; van den Berg, et al., 1999; Vanderschuren & Trezza, 2014; Von Frijtag et al., 2002). We hypothesize that, because play in the juvenile period is so critical in the development of these skills, its maturation is highly robust. That is, irrespective of small differences in experiences due to litter sizes, the sex-composition of those litters and the involvement of the mother, the form of the play expressed in the juvenile period converges onto the same pattern.

The increase in rearing and boxing, especially in the SO females, may suggest that they have become more aggressive; however, the finding that rats from all groups were able to maintain playful interactions as playful (i.e., all had similar

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levels of role reversals and pinning), suggests that this may not be the case. Therefore, even in the absence of earlier play experience with peers, juvenile rats must still be capable of using play signals or other cues which enable them to communicate these interactions as being playful (e.g., Bekoff, 1995; Himmler, Kisko, Euston, Kolb, & Pellis, 2014a; Kipper & Todt, 2002; Palagi, 2008; Pellis & Pellis, 1983) and must have the neural mechanisms in place to ensure that the interactions remain reciprocal (Pellis, Pellis, & Bell, 2010a).

7.6 Conclusions

While social experiences with peers and the mother in the peri-weaning can influence the development of some aspects of play (e.g., level of motivation), the data from the present study converge in showing that playing with peers in the pre-juvenile period is not necessary to develop juvenile-typical patterns of attack and defense. These findings apparently contradict those of Himmler et al. (2014b), which showed that housing with members of a different strain in the week following weaning alters the juvenile-typical pattern of playful defense. A possible resolution to these seemingly conflicting findings may be as follows. Under the normal range of variability in rearing experiences (e.g., different size of litter, sex ratio), playful attack and defense matures to its typical form. Moreover, as shown in the present paper, this maturation can proceed to its typical end-point without the need for functional feedback from playing with peers.

Thus, juvenile-typical play does not require to be reinforced by particular feedback from playing to emerge. However, encountering feedback that is discordant, as is provided by playing with a member of a strain with a marked difference in

preference of particular defensive tactics, can reset the trajectory of development. Interestingly, once reset, the form of the play remains resilient and unchanging even when encountering rats of different strains with differing preferences in playful defense (Himmler et al., 2014b). Such resiliency suggests that the resetting involves changes to neural mechanisms that regulate play. This model suggests that there are bounds of experience within which juvenile-typical play develops unchanged, but that there are experiences that can alter that development. The question then becomes whether such pattern-altering experiences are within the naturally occurring range of variation likely experienced by some rats under natural conditions.

Chapter 8: General Discussion

Play behavior occurs relatively sporadically in the Animal Kingdom, leading to the conclusion that the conditions making play possible rarely arise (Burghardt, 2005). Play is a far more common feature of mammals (Burghardt, 2005; Fagen, 1981), with at least some mammals relying on play for several important functions (Pellegrini, 2009; Pellis & Pellis, 2009). Insights into the mechanisms by which play arises and the functions it serves have been greatly enhanced over the past 40 years by studies of laboratory rats (Panksepp et al., 1984; Pellis & Pellis, 1998; Siviy & Panksepp, 2011; Thor & Holloway, 1984; Vanderschuren, Niesink, & van Ree, 1997). Rats are useful for the study of play for several reasons. First, laboratory rats are relatively cheap to use in the laboratory. Second, compared to other rodent species so far studied, rats exhibit a complex pattern of play very similar to that seen in primates, so data derived from rats may also apply to humans. Third, the brain and many aspects of its development are well understood in rats, and as its organization and its cyto-architecture are similar to that of humans, therefore, understanding the neural underpinnings of play in rats has the potential to translate into understanding how play influences the human brain.

Whereas much progress has been made in our understanding of how the brain produces play and, in turn, how play influences the brain, there are a number of outstanding issues that remain. For example, why do some animals play and others do not, and in those animals that do play, why do some species play more and why do others play in more complex ways than others? The aim of this thesis was to explore some of these outstanding issues. Whereas a number of interesting insights have emerged from my thesis research, there are two that I consider to be particular useful for future research

on this topic. 1) The play and non-play induced remodeling of the prefrontal cortex (PFC) has varying degrees of longevity, with the play-induced change of the medial prefrontal cortex (mPFC) remaining relatively stable into adulthood, whereas the partner diversity-induced change to the orbital frontal cortex (OFC) erodes in adulthood. These differential age-related changes point to important functional differences in these sub-regions of the PFC. 2) Even though wild rats play in a similar manner to domesticated rats, the play-induced changes to the mPFC are not seen in wild rats. This suggests that complex patterns of play fighting have evolved independently of their role in the development of the PFC, with changes in ecological conditions creating the opportunity for play to be co-opted for this function.

8.1 Social experiences in the juvenile period and the development of the PFC

Whereas the number of social partners, regardless of whether they provide playful experiences or not, induces the proliferation of the dendritic arbor of neurons in the OFC, the experience of social play induces the pruning of the dendritic arbor of the neurons in the mPFC (Bell et al., 2009; Himmler et al., 2013b). Other studies have shown that, in adulthood, both the mPFC and OFC undergoes age-related pruning, so that, with increasing age, the neurons of the PFC become simpler in dendritic organization (Kolb et al., 2012; 2014; Koss et al., 2013; Markham, et al., 2007; Milstein et al., 2013). In chapter 2, it was shown that the partner-induced proliferation of the neurons in the OFC arising from juvenile social experiences was present at 60 days of age, but not at 100 days, which is consistent with the natural age-related pruning reported in other studies. However, the neurons of the mPFC do not show any further pruning in adulthood. The finding regarding the mPFC is thus not consistent with previous studies that have shown pruning

of the mPFC in rats in adulthood (Koss et al., 2013; Markham et al., 2007, Milstein et al., 2013). The differences in the functions of the OFC and the mPFC may account for these results.

It has been suggested that juvenile play provides the context within which animals refine various social, emotional and cognitive skills (Pellis, Pellis & Bell, 2010; Pellis, Pellis, & Himmler, 2014), with at least some of these arising from play-induced remodeling of the mPFC (Vanderschuren & Trezza, 2014). It is likely that these skills are refined during the juvenile period, as this is the time period in which the brain and behavior are most malleable (Spear, 2000). Consistent with this possibility is that this is also the age at which play is most frequent (Panksepp, 1981; Thor & Holloway, 1984), and has the characteristics that produce the kinds of experiences that appear to be critical in remodeling the PFC (Foroud & Pellis, 2003; Pellis & Pellis, 1997; Pellis, Pellis & Foroud, 2005). A clue as to why the remodeling of the mPFC in the juvenile period is resistant to change in adulthood may be found in the types of skills that are improved by play.

One such skill was characterized in chapter 4, in which it was shown that the mPFC is important in enabling rats to coordinate their movements with those of a partner, an ability found to be decreased in rats that are deprived of social interactions during the juvenile period (Pellis, Field & Whishaw, 1999). This skill is essential to navigate through complex social interactions successfully, such as mating. Both rats and rhesus monkeys that have been denied the opportunity for peer-peer interactions in the juvenile period have deficiencies in coordinating their inter-animal movements during sexual encounters in adulthood (e.g., Mason, 1960; Moore, 1985). The mechanism hypothesized

to be driving the development of this skill, and others, is the pruning of the mPFC (Pellis, Pellis, & Bell, 2010; Pellis, Pellis, & Himmler, 2014), which is associated with modified function (Baarendse et al., 2013). Skills such as being able to coordinate one's movements effectively with that of one's partner are important at all ages. Therefore, a possible explanation for the finding of an absence of any additional age-related pruning of the mPFC between 60-100 days in this thesis is that these animals reached a level of remodeling that was sufficient for the maintenance of the requisite skills. That is, because as adults they continue to use these skills, the level of pruning attained during the juvenile period is maintained. If that is so, then holding rats in social isolation as adults, so that they do not use these social skills, may create an environment in which the level of juvenile-induced remodeling is lost.

In the case of the OFC, the increased complexity of the dendritic arbor arises from the experience of interacting with multiple partners during the juvenile period (Bell et al., 2010). Given that rats come into contact with many new partners over the course of their life (e.g., Barnett, 1975; Calhoun, 1962), I hypothesize that once familiarity is gained with existing social partners, the OFC undergoes pruning, thus allowing the OFC to be responsive to new social partners. That is, over the course of a rat's life, the OFC undergoes cycles of proliferation and pruning as old partners becomes familiar and new ones are encountered. If this is true, then adult rats should show an increase in the dendritic arbor of OFC neurons when exposed to novel social partners. Some preliminary data support this possibility. If adult rats are housed in pairs every second day for 14 days, they displayed an increase in the complexity of the dendritic arbor of the OFC compared to control rats (Hamilton, Silasi, Pellis, & Kolb, 2003).

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8.2 The wild side of the PFC

One of the potential problems in using rats to understand play behavior and its neural underpinnings is that the laboratory research has been conducted on domesticated rats (Pellis & Pellis, 2009; Vanderschuren & Trezza, 2014). Given that domestication has been shown to influence a variety of behavioral, physiological and neural mechanisms (e.g., Albiach-Serrano, Brauer, Cacchione, Zickert, & Amici, 2012; Castle, 1947; Coppinger & Coppinger, 2001; Lockard, 1968; Pisula, Turlejski, Stryjek, Nałęcz-Tolak, Grabiec, & Djavadian, 2012), it is possible that domestication has changed the way in which rats play, and consequently in the way that play influences the development of the PFC. Whereas the results of chapter 5 showed that the basic organization of juvenile-typical play is similar in both wild and domesticated rats, the results from chapter 6 revealed that the play-induced pruning seen in domesticated rats was not seen in wild rats. These results suggest that, even though wild and domestic rats play in a similar manner, in wild rats, play does not influence the development of the PFC. Rather, regardless of whether they had played or not, wild rats had a level of complexity in the neurons of the mPFC that was like that of the domestic rats that had played as juveniles. That is, wild rats, regardless of whether they played or not, appear to have a pruned mPFC. This finding thus questions the importance of the experience of play in the development of social, emotional and cognitive skills.

Aside from the influences of selective breeding, one of the main differences between wild and domesticated rats is in the environment in which they live following weaning. For domesticated rats, the environment created by humans provides very little in terms of environmental challenges, as fresh bedding, water and food are provided.

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However, in the wild, once weaned, rats have to fend for themselves in an environment in which resources are not always abundant and there is greater risk to survival, especially from predators, suggesting that juvenile wild rats need to have a level of skill not required by their domesticated counterparts. Also, in free-living conditions, not all environments accommodate play. Several studies of free-living animals have shown that when conditions are not favorable, the occurrence of play is reduced, or even abolished (e.g., Barrett, Dunbar, & Dunbar, 1992; Baldwin & Baldwin, 1974; Pellis, 1981; Stone, 2008). That is, under poor environmental conditions, play may not be available as a means of refining skills. Therefore, it would be beneficial for wild rats to have the neural mechanisms that are associated with the skills necessary for survival to be refined to a suitable functional level by the juvenile period. This may account for the presence of a level of pruning in the mPFC of wild rats that is comparable to that of domestic rats that have had play in the juvenile period. Thus, it would be expected that wild rats, regardless of prior play experience, would display similar executive functioning skills in adulthood as domesticated rats that have had play experience as juveniles.

These findings in wild rats raise the importance of understanding the function of play in wild animals. Most of the effort in understanding the benefits of play has been on its delayed functions (Baldwin, 1986; Fagen, 1981; Martin & Caro, 1985). That is, what does play in the juvenile period buy you in adulthood? As mentioned previously, play is important for the development of social, emotional, and cognitive skills in rats (e.g., Von Frijtag et al., 2002; Arakawa 2002, 2003; da Silva et al., 1996; Baarendse et al., 2013). In free-living animals, several cases of such long term benefits have been shown. For example, in marmots, juvenile social play leads to an improved capacity to gain

dominance in adulthood (Blumstein, Chung, & Smith, 2013). Similarly, the experience of juvenile social play leads to improved reproductive success in female ground squirrels (Nunes, 2014). Given these findings, it is even more surprising that juvenile social play in wild rats has not shown changes to the mPFC, as shown in laboratory rats. Another finding in wild animals provides a possible avenue by which this seeming inconsistency can be understood.

It has long been known that play does not occur when animals are stressed (Fagen, 1981). For example, an outbreak of intra-troop aggression in rhesus monkeys leads to the youngsters slinking away and not playing for some period of time (Symons, 1978). Similarly, detection of a predator, even its odor, leads to prolonged periods of suppression of play in rats (Siviy, Harrison, & McGregor, 1996). In fact, stress is so effective in reducing play, that one of the commonly used criteria used to define a behavior as being playful is that it occurs in stress-free situations (Burghardt, 2005). However, there are a number of studies that suggest that, under some conditions, play may be used as a way to reduce or attenuate stress (e.g., von Frijtag et al., 2002; Norscia & Palagi, 2011; Palagi, Cordoni, & Borgognini Tarli, 2004). For example, in captive bonobos, the frequency of play is increased directly before feeding, which is thought to help ease the tension of competition, which often accompanies feeding (Palagi, 2007). Among marmosets, the severity of the physiological stress response following a stressor is reduced if the animals engage in play (Mustoe et al., 2014). Importantly, this stress-reducing effect of playing occurs in both juveniles and adults (Mustoe et al., 2014; Norscia & Palagi, 2011). Therefore, not only does playing in the juvenile period have a delayed benefit - that of rendering adults to be less sensitive to stressful situations

(Lukkes, Summers, Scholl, Renner, & Forster, 2009; Mustoe et al., 2014) - but it also has an immediate benefit at all ages, that of using play to attenuate stress (see above).

These findings on the role of play in attenuating stress may at first seem contradictory to the findings that stress reduces play (e.g., Siviy et al., 2008; Symons, 1978), but they may not be. A potentially virulent stressor such as the presence of a predator may be antithetical to play, but more moderate stressors such as encountering unfamiliar conspecifics (e.g., Antonacci, Norscia & Palagi, 2010; von Frijtag et al., 2002) may stimulate play, which in turn attenuates the severity of the stress response. That is, play as a stress attenuation tactic works for dealing with mild to moderate stressors, not severe ones. Such a capacity to regulate stress was suggested to account for the increased survival of wild brown bear cubs that played more in their first year of life (Fagen & Fagen, 2004). Given that play can be used to regulate emotions both in juveniles and adults, it is possible that one of the original functions of play was for its ability to attenuate the stress response.

Some species use play in adulthood as a means of social assessment and manipulation (Brueggman, 1978; Palagi, 2011; Pellis & Iwaniuk, 1999, 2000). For example, rats live in highly complex social colonies that consist of dominant males, subordinate males, and females. In such a system, rats, particularly subordinate adult males, have to adapt their social interactions according to the identity of the partner with whom they are interacting. That is, subordinate rats will behave differently when interacting with a dominant male as compared to interacting with another subordinate male or a female (Pellis, Pellis & McKenna, 1993; Pellis et al., 2006). As such, adult subordinate males use play as a method for navigating their relationships within the

colony (Pellis, 2002a). This ability to adapt their social behaviors based upon partner status suggests a higher order of cognitive control.

If rats are decorticated, they display similar frequencies of play (Pellis, Pellis, & Whishaw, 1992; Panksepp, Normansell, Cox, & Siviy, 1994) as intact rats and use similar defensive tactics. However, there are variations in the organization of their play, suggesting that the cortex appears to be involved in how the animals play depending on the context. For instance, damage to the OFC reduces the ability to modulate social interactions with different partners (Pellis et al., 2006), whereas damage to the mPFC reduces the ability to coordinate complex movements with social partners (Bell et al., 2009; Chapter 4). This suggests that although the expression of play can unfold normally with only subcortical mechanisms in place, input from the cortex allows the animals to play with the play - that is, express a higher level of cognitive control over the actions during playful interactions. This latter capacity allows for play to be used in a more strategic manner, enabling animals to manipulate social partners to their advantage (Palagi et al., 2015; Pellis & Pellis, 2011).

However, not all playful species, even closely related ones, use play in this way (Ciani et al., 2012; Pellis, 2002a). For example, as juveniles, both Japanese and Tonkean macaques display the same pattern of play-targets and tactics, but Tonkean macaques are more likely to use play in adulthood to manipulate relationships (Ciani et al., 2012). This would suggest that juvenile Tonkean macaques are modifying their play in a way that enhances these skills, which likely depends on prefrontal control - as suggested by the PFC lesions studies in rats. And indeed, the pattern of play in juvenile Tonkean macaques involves more reciprocity and the use of tactics that create more unpredictable events

during the play (Reinhart et al., 2010). This is what would be predicted if these experiences are assisting in developing these skills. Consistent with this role of play in shaping cognitive skills, are the findings in rodents, that whereas many species play, only rats have been shown to have cognitive deficits if they are denied the opportunity to play as juveniles (Einon et al., 1981). From an evolutionary perspective, this suggests that the primary, immediate function of play is to regulate emotional responses, which then leads to a secondary, delayed function of refining the mechanisms that control emotional responses. A tertiary function is to co-opt play for another immediate function - that of social assessment and manipulation in older animals - by changes to higher levels of cognitive control (i.e., via the PFC) which deploy the ability to manipulate others, strategically, in social situations. In turn, this use of play creates the opportunity for a quaternary, delayed function to evolve, that of modifying juvenile play so that it helps train these higher-level control mechanisms, and so be more skilled at navigating social relationships as adults.

Given that both wild and domesticated rats show a robust pattern of development of juvenile typical play (chapter 7), but the wild rats do not show any play-induced changes of the mPFC (chapter 6), play in wild and domesticated rats could be present for emotional regulation. However, it may be the case that the environment to which domestic rats have adapted (i.e., no threat of predation, freely available food and water, but critically, a variable social environment), a context has been created in which play has been co-opted to affect the development of the mPFC. That is, environmental changes have allowed play to take on a role in refining different prefrontal functions in domesticated rats. Therefore, this thesis provides a window through which we can

investigate the mechanisms by which play can be co-opted for use in novel functions. The evolutionary changes in the play-brain relationship evident in rats may thus provide insights into how evolutionary changes in play across species may arise.

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