# GOING BEYOND THE DYADIC PARADIGM: THE DYNAMICS OF SOCIAL PLAY AND BRAIN DEVELOPMENT

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## DEDICATION

For Theodore, Daisy, Emma, my parents, and for Sergio. You have all served as mentors, role models, and as inspiration throughout this entire journey. Without you, it would not have been possible. Thank you.

## ABSTRACT

Rough-and-tumble play (RTP) during the juvenile period is critical for developing social competency and some of its associated neural underpinnings in the medial prefrontal cortex (mPFC), yet little is known about how individual variability in play experiences affects development. This thesis investigates whether rats show preferences for specific play partners, what factors influence these choices, and how differences in RTP contribute to behavioral and neural outcomes. Using group play paradigms, I found that rats form partner preferences based on factors such as familiarity, play style, and strain; however, these preferences depend on social context. Indeed, among familiar group members, not all individuals are equally preferred, leading to unequal RTP experiences. The effects of juvenile variability in RTP was assessed by manipulating juvenile experiences and by tracking the life history of individuals reared in groups. Rats reared with a higher-playing strain engaged in less RTP and turn taking than those reared with same-strain partners and later exhibited social deficits and altered mPFC neuron morphology. Similarly, when reared in groups, individuals that naturally engaged in less role reversals during RTP showed impaired social competence and atypical mPFC development. These findings suggest that some rats fail to benefit from RTP. A possible reason for such failure is that early-life influences may impair rat sociability. To explore this, I used neonatal isolation as a model of early life adversity (ELA). ELA did not alter role reversals with familiar partners but reduced both RTP frequency and role reversals with unfamiliar individuals, suggesting that pre-juvenile experiences can indeed impair the mechanisms that maximize the benefits gained from juvenile RTP. Together, these results show that rats preferentially play with certain partners, and this can influence the value of experiences derived from RTP, which

combined with individual differences in play cooperation, can shape developmental outcomes.

## CONTRIBUTIONS OF AUTHORS

I am grateful to the many co-authors that have made this thesis possible. For each of the empirical chapters of this thesis, I have provided a CRediT statement along with a list of the authors.

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| | |
|---|---|
| ANOVA | Analysis of variance |
| Cg3 | Cingulate area 3 |
| CPP | Conditioned place preference |
| DFCTC | Discriminative fear conditioning to context |
| dLS | Dorsolateral septum |
| ELA | Early life adversity |
| EPM | Elevated plus maze |
| F344 | Fischer 344 |
| GLMM | Generalized linear mixed model |
| LE | Long Evans |
| mPFC | Medial prefrontal cortex |
| MWT | Morris water task |
| NMR | Nuclear magnetic resonance |
| OFC | Orbitofrontal cortex |
| PND | Post natal day |
| RTP | Rough-and-tumble play |
| sCPP | Social conditioned place preference |
| SD | Sprague Dawley |
| USV | Ultrasonic vocalizations |

# CHAPTER 1: GENERAL INTRODUCTION

> "*No behavioral concept has proved more ill-defined, elusive, controversial, and even unfashionable than play.*"
>
> — E. O. Wilson, *Sociobiology*, 1975

Often said to be 'easy to recognize, but difficult to describe' (Fagen, 1981), play behavior has been puzzling researchers for as long as it has been empirically studied. Despite play becoming an ever more 'hot topic,' our understanding of its form, function, ontogeny, and evolutionary pattern are still not well understood. To simplify the study of play, categories have been created, to facilitate its description and make phylogenetic comparisons. At present, play, at least in non-human animals, is most frequently subdivided into object play, locomotor play, and social play (Burghardt, 2005, 2011). Object play involves play with inanimate objects; though, in some cases, such as in the case of a killer whale (*Orcinus orca*) playing with, and ultimately killing, a gull, would also be considered object play, despite the object being animate at one point. Locomotor play is typically expressed as a solo behavior where animals engage in leaps, jumps, or rotations around a body axis in a seemingly uncoordinated manner. Finally, social play is play that is done with a partner, frequently involving behaviour derived from one or more behavior systems (e.g., sex, aggression) and is the focus of this thesis. While prevalent in a wide array of mammalian species from many orders (Burghardt, 2005), the species that has received the most attention in the study of social play is the humble laboratory rat (*Rattus norvegicus*).

Like most mammals, rats begin to engage in social play during infancy, starting shortly before weaning, peaking in the juvenile period and then waning with the onset of sexual maturity (Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1990, 1997),

but continues well into adulthood, even though at a lower frequency (Pellis & Pellis, 1990; Thor & Holloway, 1984a). The social play of rats is characterized as rough-and-tumble play (RTP) or play fighting (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022). It involves (at least) two individuals attacking and defending the nape of the neck (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). If contacted, the recipient's nape is nuzzled with the snout of the attacker. That is, the goal of the attacking rat is to make contact with the nape of the defending rat. However, this competition for the nape of the neck is attenuated with cooperation, so that both partners have the opportunity to at least occasionally gain the advantage, thus creating a degree of reciprocity (Palagi, Cordoni, et al., 2016; Pellis et al., 2017, 2024). This produces an experience that is highly pleasurable and rewarding to the rats (Siviy, 2016; Vanderschuren, 2010). Indeed, rats engage in this behavior spontaneously (Pellis, Pellis, Ham, et al., 2022). While the main target of RTP in rats is the nape of the neck (B. T. Himmler, Pellis, & Pellis, 2013), there is a considerable degree of variation in how much RTP individuals initiate and in their preferred defensive tactics used to defend their napes (Achterberg et al., 2023; S. M. Himmler et al., 2016; Lampe et al., 2019; Lesscher et al., 2021; Pellis & Pellis, 1987; Poole & Fish, 1976). In terms of its developmental emergence, the attacking and defending of a particular body target, and the variation present across individuals in the quantity and style of play, the RTP of rats is similar to that of other mammals (Ham, Pellis, et al., 2024; Pellis et al., 2024), including humans (Blurton Jones, 1976).

In many mammals, RTP is a simulation of serious fighting, with the same targets being competed over in both types of contests (Aldis, 1975; Pellis, 1988), consequently, it can be difficult to distinguish them (Smith, 1997). In contrast, RTP in rats involves competing for nuzzling the partner's nape, whereas serious fighting involves biting the face

or the lower flanks and dorsum (Blanchard et al., 1977; Pellis & Pellis, 1987). Consequently, rats serve as a suitable model species for studying RTP as their social play can be unambiguously discerned from serious fighting (Pellis, Pellis, Ham, et al., 2022; Pellis et al., 2024). As such, rats have been intensely studied to characterize the underling neural mechanisms of play and the developmental consequences of engaging in RTP as juveniles. Recent comprehensive reviews (e.g., Achterberg & Vanderschuren, 2023; Pellis et al., 2023; Vanderschuren et al., 2016; Vanderschuren & Trezza, 2014; VanRyzin et al., 2020) clearly reveal the progress made over the past three decades. In brief, many of the mechanisms that generate and sustain play have been delineated (Achterberg et al., 2015, 2016, 2018, 2019; Achterberg & Vanderschuren, 2020; Dvorzhak et al., 2024; Reinhold et al., 2019), as have the functions in promoting the development of social (e.g., S. M. Himmler et al., 2016; Marquardt et al., 2023; Schneider et al., 2016; Stark & Pellis, 2020, 2021) and cognitive skills (e.g., Einon, 1980; Einon & Morgan, 1976, 1977). Studies from several laboratories have linked these socio-cognitive improvements to physiological and anatomical changes of the medial prefrontal cortex (mPFC) (Baarendse et al., 2013; Bell et al., 2010; Bijlsma et al., 2022, 2023; Ham, Szabo, et al., 2024; B. T. Himmler, Pellis, & Kolb, 2013; Stark et al., 2023). While the extent to which these findings generalize to other species, including humans, remains to be determined (Achterberg & Vanderschuren, 2023; Pellis, Pellis, Ham, et al., 2023), there are some promising clues that they may be, at least for animals with patterns of RTP that are as complex as those of rats. Comparative studies with primates have shown that the brain areas shown to be involved in RTP in rats (Siviy, 2016), are larger in species that engage in more RTP (Graham, 2011; Kerney et al., 2017; Lewis & Barton, 2006), suggesting a causal role of these brain areas in regulating play in a non-rodent clade of mammals. That the connection between RTP in the juvenile period

with refined socio-cognitive skills is not limited to rats is supported by comparable experimental findings of play's role in the development of the mPFC and social skills of Syrian golden hamsters (*Mesocricetus auratus*) (Burleson et al., 2016), and by quasi-experimental and correlational findings in human children (Diamond et al., 2007; Gibb et al., 2021; Nijhof et al., 2018).

The work cited above, that has sought to answer a wide range of questions about RTP in rats, including its basic structure and behavioral patterns, has largely relied on testing rats in pairs. Dyadic testing has become the norm for experimental studies on play with rats, and for good reason (Pellis, Pellis, Burke, et al., 2022). First, testing animals in pairs limits experimental complications, such as the number of animals required for a given experiment. Second, by not having other rats blocking the view, it is easier to identify and score the behavior patterns performed when there are only two animals to follow. Indeed, for experienced observers, the scoring of some of the least ambiguous behavior patterns common to RTP in rats, can be done in real time without the aid of videorecording the trials which can greatly increase the sample sizes of experiments involving treatments with low effect sizes (e.g., Achterberg et al., 2023; Achterberg & Vanderschuren, 2020). However, play in a dyadic context does not represent the opportunities rats would encounter under more natural conditions, and so the niche within which RTP evolved in rats. Consequently, while practical, the dyadic test paradigm may mask some important factors that regulate RTP.

In the wild, rats live in colonies, some of which consist of hundreds of individuals (Schweinfurth, 2020). When litters from different females are born, they tend to arrive in close temporal proximity with one another (Calhoun, 1963), and since each litter consists of 6 to 12 individuals, juveniles living in colonies have access to multiple same-age play

partners, both siblings and non-siblings. Like children in playgrounds (Humphreys & Smith, 1987), young rats living in colonies would likely have the opportunity to choose with whom to play. For example, perhaps kids in the playground prefer to play with children they know over those from another class, who being less familiar, may be too scary. Likewise, rats may favor individuals that they are familiar with over novel individuals. Rats might also choose playmates with a compatible play style (the right degree of competitiveness and so roughness) and play frequency. Reflecting on my childhood, I remember kids who were too competitive and too rough for them to be my preferred play partners. Yet, these questions, and many more, remain unknown as rats are rarely studied in wild settings and, when studied in the laboratory, they are seldom tested in groups.

Since the natural ecology of rats involves living in large groups with a wide range of available play partners, a pattern that closely mirrors how humans interact with peers during childhood, it seems reasonable to try and replicate these naturalistic conditions when studying rat RTP in the laboratory. Doing so not only better reflects the real-world social environment of rats, but may also more accurately simulate human social dynamics, thereby enhancing the translational value of the research.

### 1.1 Addressing the gap

To address these overlooked areas of group dynamics, this thesis explores both play partner preferences in groups and the natural variation in the play experienced among members of groups over the juvenile period. The aim is to gain a deeper understanding of RTP in rats and to develop a more naturalistic approach to testing its developmental consequences.

### *1.1.1 Part I*

To do so, I first explore the dynamics of RTP in groups of rats in which testing involved placing more than two rats in the testing arena. The core issue being whether, when given the choice, do rats prefer some partners over others and, if so, what are the criteria they use to make such choices. To determine whether rats form RTP partner preferences, I first tested groups of four rats in which the familiarity of each partner was varied (Chapter 2). This design reflects a more naturalistic setting, where rats could choose to play with siblings (i.e., familiar individuals) or peers from neighboring litters (i.e., unfamiliar individuals). By varying familiarity, I introduced additional variation in social choice as novelty could plausibly drive preferences, either toward unfamiliar individuals or toward more familiar partners.

After establishing that rats can and do form play partner preferences (Chapter 2), I then designed experiments, involving groups of six members, to further explore the potential mechanisms underlying these preferences. In the first of these experiments, all individuals within the group were unfamiliar with one another (Chapter 3), and in the next all the potential partners were familiar with one another (Chapter 4).

In the former study (Chapter 3), by removing familiarity altogether, rats were forced to quickly decide with whom they wanted to play. They had only 20 minutes to interact with potential partners, leaving limited time and few cues to guide their choices. As such, partner selection was likely based on factors such as the playfulness and style of RTP of particular individuals, and/or their relative size and dominance.

The latter study (Chapter 4), which focuses on familiar partners, investigated whether preferences form among familiar individuals and whether these preferences persist throughout the juvenile period. Additionally, it explored how variability in partner preferences could lead to unequal play experiences, as some rats could be consistently preferred, while others avoided. Given RTP's known role in refining social skills (Pellis, Pellis, Ham, et al., 2022), such variation in play experience could have meaningful developmental consequences.

While preferences were found in the previous Chapters (2-4), the mechanisms by which rats select partners remained elusive, although each of the three studies provided some valuable clues. One hypothesis is that rats select partners whose play styles complement their own. This is because there is a considerable degree of individual variation in both play frequency and play style (Achterberg et al., 2023; Lampe et al., 2019; Lesscher et al., 2021; Pellis & Pellis, 1987; Poole & Fish, 1976). Therefore, I predicted that when given a choice, rats would prefer complementary play partners. To test this prediction, I tested rats from different strains with known differences in play styles together. I used Long Evans (LE) rats as the focal subjects in groups composed of same-strain and mixed-strain partners, allowing the rats to choose which strain to engage with during RTP (Chapter 5). The strains selected, Sprague Dawley (SD) and Fischer 344 (F344), were based on well-established strain-typical differences in RTP frequency and style. For example, SD rats, an albino strain, engage in more RTP than LE rats and exhibit more evasive defensive tactics than facing defenses (S. M. Himmler, Modlinska, et al., 2014; Orsucci et al., 2024). Conversely, the F344 rat, another albino strain, is relatively low playing compared to LE and SD rats (Siviy et al., 1997, 2003; Stark et al., 2021), and like SD rats, F344 rats prefer

to evade rather than face their attacker (Siviy et al., 2023). By selecting strains with extreme differences in play style, this potential mechanism of partner selection is isolated and tested.

In addition to testing RTP in groups of four, I also assessed RTP behavior in mixed-strain dyads to determine whether LE rats adjust their RTP behavior when the choice is removed. Finally, I used a socially conditioned place preference (sCPP) paradigm (Achterberg et al., 2012, 2014; Trezza et al., 2009, 2011), conditioning LE rats to two boxes. In one box they were exposed to a same strain LE partner and the other to a F344. If partner compatibility influences preferences, I predicted that LE rats should prefer the box associated with the same-strain partner.

Together, Part I of this thesis explores whether rats have partner preferences during RTP and, if so, what factors may influence these preferences. A consistent finding is that rats show individual differences in social behavior, and within group contexts, not all individuals are equally preferred. This suggests that play experience during the juvenile period is not uniform, and thus not all rats may benefit from RTP to the same extent. To better understand how RTP contributes to development, and what factors shape these outcomes, Part II investigates aspects of behavioral and brain development.

### *1.1.2 Part II*

This thesis builds on the insights gained from studying group play by examining the consequences of juvenile RTP experiences. In addition, it focusses on how rats interact in their home cage environment and how those interactions impact development, as well as what early life factors might contribute to differences in behavior and brain development. This section includes analysis of juvenile and adult RTP behavior, as well as mPFC brain

anatomy, to explore whether natural variation in RTP experienced during the juvenile period has lasting consequences.

Although there are many ways to reduce or completely deprive juvenile rats of RTP experiences (e.g., Bell et al., 2009; Einon & Morgan, 1977; B. T. Himmler, Pellis, & Kolb, 2013; Marquardt et al., 2023; Stark et al., 2023), not all methodologies are equal (Pellis, Pellis, Ham, et al., 2023). One of the earliest approaches involved complete social isolation during the juvenile period (Einon & Morgan, 1977). While this method successfully eliminates RTP, it also deprives rats of all forms of social interaction, making it difficult to attribute developmental outcomes specifically to the absence of RTP. As an alternative, rats can be reared with adults, who naturally engage in less RTP than juveniles (Bell et al., 2010; B. T. Himmler, Pellis, & Kolb, 2013; Pellis et al., 2017). This approach reduces juvenile RTP while preserving other forms of social interaction. However, it is only effective with pairs of females, as adult males are aggressive with young males (A. R. Burke et al., 2010, 2013), and males reared with adult females often attempt to copulate with them shortly after reaching sexual maturity (Pellis & Pellis, 1990). In both cases, as with social isolation, developmental outcomes cannot be clearly attributed to reduced play, since aggressive or sexual experiences may also contribute. To mitigate these limitations, a clever method developed by the Schnieder group involves rearing two same-aged peers together, ensuring typical social interactions, however, one member of the pair is from a low-playing strain, reducing the amount of RTP play experienced by the other, more playful partner (Schneider, Bindila, et al., 2016; Schneider et al., 2014; Schneider, Pätz, et al., 2016).

The Pellis lab has adopted this technique by rearing LE rats, a relatively high playing strain, with F344 rats, a relatively low playing strain (Stark et al., 2021). Stark et

al. found that both male and female rats raised this way exhibit social skill deficiencies and an altered mPFC as adults (Stark et al., 2023; Stark & Pellis, 2020, 2021). Given that a low-playing strain can negatively impact a higher playing strain, it raises the question: can a low-playing strain benefit from a high playing strain? If so, this would have implications for how rats choose partners when living in groups.

To test this, F344 rats were paired with LE rats during the juvenile period. These animals were co-housed until adulthood at which time their social competency was assessed. After behavioral testing, the animals were sacrificed and their brains analyzed. In addition to testing the hypothesis that "more play is better," this study aimed to determine what aspect of the juvenile RTP experience is important to the development of social skills and the mPFC. In other words, is it the frequency of play or the quality of play that matters most? This experiment is presented in Chapter 6.

Building on the findings from Chapter 6, I then reared rats under more naturalistic conditions (Schweinfurth, 2020) in groups of six (Chapter 7). Leaving the rats relatively undisturbed, I quantified the RTP during the juvenile period in the home cage to assess individual differences in RTP experiences. Once the rats reached adulthood, I assessed their social competency skills after which the animals were sacrificed and the neurons in the mPFC were measured. As expected, while all rats engaged in RTP, their RTP experiences varied, and not all rats were equally socially competent as adults. Those with social deficits also showed altered mPFC anatomy, suggesting that not all rats benefit from juvenile RTP.

After finding that some rats do not benefit from juvenile RTP (Chapter 7), a new question emerged: which came first, the chicken or the egg? In other words, do rats develop atypical social skills because they played atypically as juveniles, or did they play atypically as juveniles because they already had emerging social deficits? Indeed, it could be that the

rats that did not benefit from play were treated differently by their mother. While the maternal care of the subjects received in Chapter 7 is unknown, it has been shown that rats that receive less licking and grooming by their mothers engage in more RTP than those that receive more (Parent & Meaney, 2008). It could be that a pre-juvenile experiential deficiency predisposes certain individuals to develop atypically. To begin addressing this, I examined the effects of early life adversity (ELA) on RTP and adult social and behavioral skills (Chapter 8). To induce ELA, the rats underwent chronic, short-term neonatal isolation, whereby the rats were isolated from their siblings and mother each day for three hours from the second day of life until the fourteenth day of life. While not housed in groups like the rats in Chapter 7, the experiment employed group play testing where rats were tested in trios (control + control + isolated or isolated + isolated + control), letting the rats choose their play partners. These choices provided insight into social functioning, revealing not just who rats preferred to play with, but perhaps more importantly, who they avoided.

Taken together, this body of work lays the foundation for understanding how and why partner preferences emerge during group play in rats. It expands the methodological toolkit available for studying play and deepens our understanding of its developmental consequences, highlighting the aspects of RTP most critical for the development of social competence and the prefrontal cortex.

# PART I: WHY CAN'T WE BE FRIENDS? PARTNER PREFERENCES IN RAT ROUGH-AND-TUMBLE PLAY

"*... it is in play that the existing social relations are most typically and completely expressed.*"

— Caroline Loizos, *Primate Ethology*, 1969

"*The patterns of play which involved two or more individuals are forms of activity which relate to the process of conditioning or integrating an individual into the clan. [Indeed,] social relations with animals other than the mother first occur with play-partners.*"

— Clarence Carpenter, *Comparative Psychology Monographs*, 1934

# CHAPTER 2: THE GOLDILOCKS PRINCIPLE: BALANCING FAMILIARITY AND NOVELTY IN THE SELECTION OF PLAY PARTNERS IN GROUPS OF JUVENILES MALE RATS*

## 2.1 Introduction

Social play has been reported in a wide array of mammals (Burghardt, 2005; Fagen, 1981), with play fighting, or rough-and-tumble play, being one of the most commonly reported forms (Pellis & Pellis, 1998). During play fighting, partners compete for an advantage, such as biting or striking a particular body target (Aldis, 1975). For many species, the targets competed over are the same as those in serious fighting, but for many others, the targets can be the same as those contacted during sex, greeting or other amicable interactions, and predation (Pellis, 1988; Pellis, Pellis, Ham, et al., 2022; Pellis & Pellis, 2018). Whatever the advantage sought in play fighting, attack and defense is attenuated, allowing both partners the opportunity to at least sometimes gain the advantage, thus creating a degree of reciprocity (Palagi, Burghardt, et al., 2016; Pellis & Pellis, 2017).

Although most common in the juvenile period, for many species, play fighting can continue into adulthood (Palagi, 2011; Pellis & Iwaniuk, 2000) and in post-pubescent animals, it is often used to assess and manipulate social relationships (e.g., Antonacci et al., 2010; Mills, 1990; Pellegrini, 1995; Pellis et al., 1993), so providing an immediate benefit. In contrast, play fighting in juveniles most likely has delayed benefits, whereby the play experience alters future socio-cognitive performance or other skills (Palagi, 2018; Pellis & Pellis, 2009; Smith, 1982, as discussed in Pisula & Modlinska, 2023). It has been the play

fighting of juveniles that has received the most intense scrutiny regarding the mechanisms that regulate it and the benefits it may confer (Sharpe, 2019; Vanderschuren et al., 2016), but determining the critical experiences derived from such play can be challenging. An important influence that needs to be taken into account is the social setting in which juveniles live.

For species that are typically reared alone with the mother, such as in giant pandas (*Ailuropoda melanoleuca*), play fighting may be preferentially directed to the mother (Kleiman, 1983; Pellis & Pellis, 2009; Snyder et al., 2003), whereas for species living in social groups, not only do juveniles tend to play with other juveniles but they also exhibit preferences as to which juveniles in the group are played with the most (e.g., Cheney, 1978; Ham et al., 2022; Ham, Lilley, et al., 2023; Lilley et al., 2020; Lutz & Judge, 2017; Shimada & Sueur, 2014, 2018; Turner et al., 2020). Several relevant factors have been identified as influencing which partners are preferred. Dominance status influences play in some non-human primates, with animals more likely to play with partners of similar rank (e.g., Biben, 1986; Lutz et al., 2019), although dominance can be less of a factor for species with less rigid dominance hierarchies (Petit et al., 2008; Reinhart et al., 2010). For many species, play is preferred with partners of similar age and sex (e.g., Biben, 1998; Cheney, 1978; Thompson, 1996), although for some, like beluga whales (*Delphinapterus leucas*), these preferences can change with age (Ham et al., 2022; Lilley et al., 2020). In contrast, for some species, play partner preference can remain stable for long periods of time (e.g., Ward et al., 2008), even into adulthood (e.g., Mann, 2006). Given such diversity in play partner preference, it is important to understand the preferences of the species being studied and how these preferences can influence play experiences during the juvenile period.

Laboratory rats (*Rattus norvegicus*) have been key subjects for studying the neurobehavioral mechanisms that regulate play fighting (Siviy, 2016; Vanderschuren et al., 2016), and the delayed functions of this behavior (Pellis et al., 2014; Vanderschuren & Trezza, 2014). In rats, play peaks in occurrence from days 30-40 after birth (Meaney & Stewart, 1981; Panksepp, 1981; Pellis, Pellis, Burke, et al., 2022; Pellis & Pellis, 1990, 1992, 1997; Thor & Holloway, 1984c). Play fighting during the juvenile period improves the development of socio-cognitive skills and alters the neural circuits associated with those skills (e.g., Baarendse et al., 2013; Bell et al., 2010; Bijlsma et al., 2022; Himmler et al., 2013; Schneider et al., 2016; Stark et al., 2023; Stark & Pellis, 2020, 2021; van Kerkhof et al., 2013). As these consequences of play have been reported in some other rodents (e.g., Burleson et al., 2016; Marks et al., 2017), it is likely that the findings on rats may be generalized to many other species that engage in play fighting as juveniles (Pellis et al., 2014; Pellis & Pellis, 2017).

In both male and female rats, play fighting mostly involves competing to access the partner's nape of the neck, which is nuzzled with the snout if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). The most common way to evaluate play fighting in rats is the 'dyadic test,' in which rats are socially isolated, from a few hours to several days, and then introduced into a test enclosure to which they have been habituated (Pellis, Pellis, Burke, et al., 2022). Trials typically last for 5-30 min and play usually commences within the first minute (C. J. Burke et al., 2022). The period of social isolation, even when brief, increases the rats' motivation to engage in play, ensuring a high frequency of encounters to score and evaluate. Different scoring schemes allow differing degrees of details to be recorded from the play that occurs (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis,

Burke, et al., 2022), but a key measure to ascertain how much an individual is inclined to play is how many nape attacks it initiates (Pellis & Pellis, 1990; Thor & Holloway, 1984c).

Juvenile rats can be matched in the dyadic test with either familiar partners, ones with which they share a home cage (Lampe et al., 2017; Pellis & Pellis, 1990), or with strangers, animals they have never met before (e.g., Achterberg et al., 2015; Achterberg & Vanderschuren, 2020). The number of nape attacks are sometimes reported to be higher when play is between strangers than cage mates (e.g., Panksepp, 1981 cf. Pellis & Pellis, 1990), but this is often confounded by the duration of the pre-test social isolation period, as longer periods of separation increase the amount of play (Pellis et al., 1997). Nonetheless, when a rat in a dyadic test is satiated as evidenced by a decline in launching nape attacks, its level of initiating nape attacks rebounds if a novel, unfamiliar partner is introduced (Reinhart et al., 2006), suggesting that strangers may be more attractive play partners. Indeed, in some non-play test paradigms, adult rats tend to have a preference for approaching and socializing with strangers (e.g., Cirulli et al., 1996; Hackenberg et al., 2021; Rogers-Carter et al., 2018; Schweinfurth & Taborsky, 2020).

Additionally, while adult rats may direct prosocial behaviors to both familiar and unfamiliar individuals, they are less likely to interact with strangers of a different strain (Ben-Ami Bartal et al., 2014). This preference for same-strain individuals in prosocial tasks, however, is not present in juveniles (Breton et al., 2022) or in adults that were cross-fostered with the other strain, suggesting that familiarity is important in making social decisions (Ben-Ami Bartal et al., 2014). Given that, in some contexts, adult rats prefer strangers, it is possible that the observations suggesting that juveniles play more with strangers are correct. If so, we predicted that, if rats were given a choice, they would launch more nape attacks toward unfamiliar partners than toward cage mates.

However, observations to date confound two factors: when presenting a subject rat with a play partner in the dyadic test paradigm, an unfamiliar partner is both a stranger and peer with which the subject has never played. That is, it could be either the novelty of the partner's identity or the novel play provided by the stranger that generates increased play. To overcome this confound between familiarity and play experience, groups of three male rats each were housed in cages with a clear, perforated partition between them and three rats on the other side of the barrier. The perforated partition enables the rats across the barrier to see, hear, and smell each other, but not interact physically (Stryjek & Modlinska, 2022). In this way, rats on the same side of the partition are both play partners and familiar, whereas the rats across the barrier are familiar but have never been play partners. To provide a choice, a focal rat from one side of the barrier was placed in a large test enclosure with a cage mate, a rat from across the barrier, and a rat from another cage, so a true stranger. Play within a group, rather than in a dyad, allow rats to initiate play with preferred partners differentially, so permitting a choice to be made and measured (Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2022). If playing with a novel animal is the main reason for choosing a partner, then the focal rat should direct more nape attacks toward the stranger, as it is the least familiar option. Whatever partners are preferred, if there is a preference, the issue is that of how those partners are identified. Different mechanisms are likely important if it is either the novel identity or the novel playfulness of the partner that is the basis for selection (hypotheses and predictions are summarized in Table 2.1).

**Table 2.1.** Summary of the hypotheses and predictions tested.

| Hypotheses | Predictions |
|---|---|
| Due to the greater attractiveness of novel animals, focal rats will play more with strangers than familiar individuals | As a measure of playfulness, the nape attacks launched by focal rats were scored and were predicted to be more frequently directed towards the 'stranger' than a familiar individual |
| As strangers must be identified, probably by olfactory inspection, the onset of play with stranger partners will be delayed as compared to when encountering familiar individuals | The latency to launch playful attacks toward all potential partners would be longer with strangers as compared to with a familiar individual |
| As novel animals are generally more attractive, rats should spend more time with strangers, even when not playing with them, as compared to when with familiar individuals | The time spent in social proximity to strangers, when not playing, is predicted to be longer than time spent in proximity to familiar individuals |
| A reason for why strangers are more attractive play partners is that they play in a way that is more rewarding as compared to play with familiar individuals | Play with strangers should involve more close-quarter wrestling and more role reversals than play with familiar individuals |
| Whether due to recognition or playful feedback, the individual properties of potential partners need to be assessed before play can be focused on strangers | Focal rats would play with all partners early in the trial then mainly play with strangers later in the trial |
| Rats preferentially direct more nape attacks towards dominant animals, therefore, when placed in a group of known and unknown rats, the most dominant rat in the test trail should receive the most attacks | The partner with which the focal rat had the greatest dominance asymmetry would receive the most playful attacks |
| Dominance among rats is positively correlated with size, therefore, as a corollary to the previous hypothesis, the largest rats in the trial should receive the most playful attacks | The partner with which the focal rat had the greatest weight asymmetry would receive the most playful attacks |

If the novelty of potential play partners is their unfamiliarity, then they need to be identified as novel before being selected as a play partner. Like many mammals, rats use scent to identify the sex, dominance status, kinship, and individual identity of other rats

(Barnett, 1975; Brown, 1979; Clemens et al., 2020; Hepper, 1987). Although juvenile rats can play in the absence of olfaction (Siviy & Panksepp, 1987; Thor & Holloway, 1982), when first introduced into a test enclosure, rats will engage in vigorous anogenital sniffing in the first minute before they start engaging in play fighting (Panksepp, 1981; Pellis & Pellis, 1990). If scent is the means by which to determine which partner is which, it should take longer to distinguish between familiar and unfamiliar rats in a group setting: this should lead to different latencies for the focal rat to begin to play with different categories of rats.

Whether identified by smell or some other sensory modality, if focal rats play more with strangers it could be because they are more socially attractive (e.g., Cirulli et al., 1996; Schweinfurth & Taborsky, 2020), and so, focal rats may spend more time in close proximity with them. In turn, this would create greater opportunity to engage in play with strangers. It would thus be expected that the preferred play partners are the ones that the focal animals spend more time with in close proximity, including when not playing. Even though there is no proximity-driven association between play and play partner preferences in groups of familiar rats (Pellis, Pellis, Burke, et al., 2022), the greater attractiveness of strangers could change this pattern (Hepper, 1987).

If the novelty of unfamiliar partners is driven by the different playful feedback they provide, then focal rats should play with all the rats available in the test enclosure before focusing on the one providing the preferred play experience. Indeed, even within a litter or group setting, in which the animals are familiar with one another and all play together, some are preferred as play partners over others (Meaney & Stewart, 1981; Pellis, Pellis, Burke, et al., 2022). This raises the possibility that rats will sample all members of the group for their play, but then focus their play with the preferred members. If this is so, it

would be expected that, early in the trial, focal rats should playfully attack all group members, but then increasingly limit their play to the preferred partners, leading to a different distribution of play with the different group members. Similarly, the style of play should differ among the play bouts with familiar versus unfamiliar partners.

When attacked, a rat can either defend its nape or simply not respond. If the recipient defends the attack, the rat can do so by either fleeing/evading or by turning to face its attacker. In turn, facing defenses can either lead to the defender wrestling on the ground or warding off the attacker by remaining standing, with the former involving more close bodily contact (Pellis, Pellis, Burke, et al., 2022). Moreover, once the nape is successfully defended, the defender can launch a counterattack leading to a role reversal, in which the original attacker becomes the defender (S. M. Himmler, Himmler, Pellis, et al., 2016). Role reversals are important for ensuring that play fighting is reciprocal and lead to more prolonged encounters (Palagi, Burghardt, et al., 2016; Pellis & Pellis, 2017). There are individual differences in how rats play (Pellis & Pellis, 1987; Poole & Fish, 1976), and these differences can lead to different experiences during play depending on the combination of players (Pellis, Pellis, Burke, et al., 2022). Consequently, the differing play experienced with familiar versus unfamiliar partners could be reflected in the behavior performed (e.g., more wrestling versus more evasion, more role reversals versus no response). In this way, partners could be selected based on whether they play in a preferred style (Pellis, Pellis, Ham, et al., 2022).

A final mechanism examined would combine both the identity and behavior of the strangers. Although dominance relationships among cohabiting male rats are not fully established until after sexual maturity (L. K. Smith et al., 1996; L. K. Takahashi, 1986; L. K. Takahashi & Lore, 1983), they do begin to emerge in the juvenile period (Pellis & Pellis,

1991), and this can affect how they play together (Panksepp et al., 1985; Pellis & McKenna, 1992; L. K. Smith et al., 1998). Rats in the home cage will launch more playful attacks toward the dominant male both as juveniles and adults (Pellis et al., 1993; Pellis & McKenna, 1992; Pellis & Pellis, 1991). Given that the focal rats have not had the opportunity to meet the stranger, this rat's dominance status may be the most ambiguous, stimulating more play with them to assess their potential dominance (L. K. Smith et al., 1996). Once the relative dominance of the rats in the play trial were identified, if dominance influences the way that play occurs in established groups, then it would be expected that the focal rats would launch more playful attacks toward the most dominant group member, unless the focal rat is the most dominant. In addition, as dominance is positively correlated with body size (Pellis & Pellis, 1991; L. K. Smith et al., 1996), we also used differences in body weights as a proxy measure of dominance, to assess the partner preferences of focal rats. If consistent with dominance relationships, then the focal rat should direct the most playful attacks to the heaviest member of the test trial. It should be noted that, as male rats form more clearly delineated dominance hierarchies than do females (Barnett, 1975; Ziporyn & McClintock, 1991), and dominance relationships can influence how rats play (Pellis & McKenna, 1992), for the present study only males were used. No matter which, if any, of the above mechanisms may be used, all reflect that, in a situation in which an animal can choose among potential play partners, some degree of social exploration is required.

## 2.2 Method

### 2.2.1 Ethics statement

All care and testing procedures were reviewed and approved by the University of Lethbridge Animal Welfare Committee (protocol #1809) in compliance with guidelines from the Canadian Council for Animal Care.

### 2.2.2 Subjects

Thirty-six weanling Long Evans (LE) male rats were purchased from Charles River Laboratories (Kingston, NY, USA) and arrived at the Canadian Centre for Behavioural Neuroscience at 22 days of age. Upon arrival, the animals were moved into Tecniplast® GR1800 double decker cages (46.2cm x 40.3cm x 40.4cm), which had a Tecniplast® cage divider placed in the middle of the cage creating two spaces to house two, separate groups of rats. The weanling rats were placed on either side of the divider in groups of three, resulting in 12 groups. The home cages had corncob bedding on the floor, and food and water were available *ad libitum*. Animals were housed on a 12-hour light-dark cycle and maintained at a constant temperature of 21°C - 23°C.

### 2.2.3 Apparatus

Animals were tested in a large Plexiglas® play enclosure (80cm x 80cm x 50cm). The play enclosure was filled with a layer of corncob bedding which was around 1.5 cm thick. An ExmourRS 4K Sony Handycam was used for filming the play sessions and was placed over the top of the enclosure, giving a top-down view. Rats were tested in a room illuminated with red lights (B. T. Himmler, Pellis, & Pellis, 2013).

### 2.2.4 Procedure

Starting at 28 days of age, rats from the same side of the divider were habituated to the test enclosure for 30 min in red light over two consecutive days. At 30 days of age, the

rats were tested in groups of four: two from one side of the divider ('cage mates'), one from the other side of the partition ('neighbor'), and one animal from another cage ('stranger'), with one of the cage mates serving as the focal animal (Figure 2.1). Each of the 36 rats were designated as the focal animal on one of the test days. Once designated, their cage mate partner was determined randomly. The neighbors and strangers were picked at random with the only selection criteria being that they could not have previously interacted. Different groups were formed over the course of the six days of testing, ensuring that each of the 36 rats served as the focal animal once.

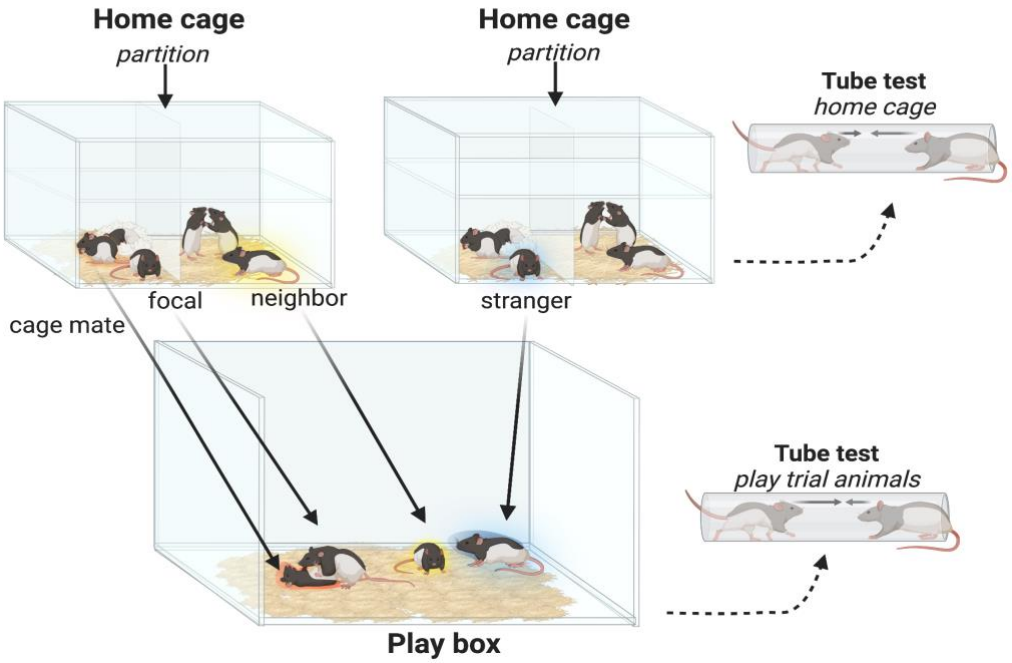


**Figure 2.1** The diagram illustrates how the animals were housed in the partitioned home cage (top), how the partners were combined for testing in the play trials (bottom), and how dominance was determined using the tube test in both the home cage (top right) and the play trial groups (bottom right). Created with BioRender.com.

Each group was placed in the enclosure, in red light, for 20 min and filmed. The bedding was replaced, and the test enclosure was cleaned with Virkon® after each trial to reduce any odors left from the previous rats. To identify individual rats, their tails were marked, using different patterns, with a permanent marker pen (Sharpie®). Prior to testing, the rats were weighed and also socially isolated for 2.5 h to increase playfulness (Pellis, Pellis, Burke, et al., 2022). After testing, animals were rehoused in their respective groups for around 24 h before being socially isolated again for the following play trial.

Even as juveniles, male rats can differ in their dominance relationships, which can affect how they play together (Panksepp et al., 1985; Pellis & Pellis, 1991), so we assessed the dominance relationships among the cage mates. To do so, we used the tube test (Fan et al., 2019; Fulenwider et al., 2021) at 29 days of age (the day before testing began) and at 35 days of age (on the final day of testing). In addition, to assess dominance among the individuals in the groups used in the trials, the tube test was employed at the end of every play trial, which tested the dominance among the focal, cage mate, neighbor, and stranger. Pairs of rats from within the home cage group (e.g., focal + cage mate) and among the play groups (e.g., neighbor + stranger, focal + neighbor, cage mate + neighbor) were placed into a Plexiglas® tube (19.5 cm in length and 4.5 cm in diameter) headfirst at opposing ends. The tube was just large enough to allow one rat through, with the second rat unable to squeeze past its opponent. The 'loser' was thus designated as the rat pushed out of the tube, the 'winner' the rat that remained in the tube. The winning rat was given a point for that round. If neither rat was pushed out, this was considered a tie, and no point was given for that encounter. Each pair was tested five times, and the sum of the points was then used to determine which rat was the most dominant. After testing was completed for a pair, the tube was cleaned with Virkon®, and the next pair was tested.

### 2.2.5 Behavioral analysis

The 20-min video recordings were analyzed using a combination of normal speed and frame-by-frame analysis to score various aspects of the rats' playful attack and defense strategies (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022). Each video was scored in terms of the actions performed by the focal animal. For the present study, we focused on comparing nape attacks as this provided information about the play initiated with each of the potential partners by the focal rat. A playful attack was scored when the snout of one rat contacted the nape of another rat as this is the target in around 90% of playful attacks (B. T. Himmler, Pellis, & Pellis, 2013). Additionally, if a playful attack was directed towards the nape but the defender evaded before it could be contacted, this was also scored as a playful attack. After an attack was launched, the partner could either continue its ongoing behavior (e.g., digging, exploring) or defend against the attack. To defend its nape, the partner could either evade (e.g., swerve or run away from the attacker) or engage in a facing defense, in which the defending rat turns to face its attacker. Depending on the tactics used, a facing defense can lead to wrestling that involves rolling over onto the ground (i.e., supine defense) or remain standing while warding off its opponent (i.e., standing defense) (B. T. Himmler, Pellis, & Pellis, 2013). A simple measure of supine defense is to score the configuration of the pair when one partner is lying on its back and the other is standing over it (i.e., a pin), and a simple measure of standing defense is when members of the pair both stand up and face one another and hold one another with their forepaws (i.e., mutual upright). In addition, the number of role reversals, in which the focal animal is attacked, and launches a counter attack that leads to the attacker becoming the defender, were recorded (Pellis, Pellis, Burke, et al., 2022).

In addition to play, the social proximity of the focal animal to the other rats was calculated. This was done to determine whether the focal rat just played with whichever rat was closest to them or instead seek out a particular play partner within the play box. Animals were considered to be in social proximity if they were within one body length of each other. As some of that time was spent playing together, the total time two individuals spent playing with one another was subtracted from the total time they were in proximity to each other.

### 2.2.6 Statistical analysis

All plots were created using R (R Core Team, 2020) using *ggplot2* (Wickham, 2016) or *ggpubr* (Kassambara, 2019), except for Figure 2.5 and Figure 2.6b, c, which were constructed manually. In addition, all statistical tests were run in R. Post-hoc Tukey tests were done using the base R functions while Dunn tests were done using the *FSA* package (Ogle et al., 2021). Different types of data also required using different types of statistical analyses.

#### *2.2.6.1 Frequency of play directed across partner categories*

Nape attack frequencies were plotted, and the data were not normally distributed (Shapiro Wilks $p < .05$), so a Kruskal-Wallis test was used to compare if rats directed more nape attacks toward each available partner. For *post hoc* comparisons, the Dunn test was used. For data that were normally distributed, an ANOVA was used, with the Tukey test used for *post hoc* pairwise comparisons.

#### *2.2.6.2 Latency, temporal distribution, and order of nape attacks*

Latency to the first nape attack with each potential play partner was assessed using a Kruskal-Wallis test as the data were not normally distributed (Shapiro Wilks $p < .05$). To

determine if nape attacks were directed to each play partner equally across the 20-min play trial, nape attacks initiated by the focal animal were plotted in 2-min time bins. Nape attack events were plotted illustrating which partner they were directed to, in the order in which they occurred. These data show both the number of nape attacks initiated by each focal animal, as well as who the rats first played with, who they played with the most, and the order in which they played with each potential partner. A Chi-square test was used to determine if the partner attacked first differed. A Student's *t*-test was used to compare the first half of the nape attacks, for each focal rat, with the second half of the nape attacks to determine if certain partners were preferred during the first half of the play session over the second half. Clusters of nape attacks (three or more nape attacks directed to one partner) were summed and compared among play partners with a Kruskal-Wallis test after finding the data were not normally distributed (Shapiro Wilks $p < .05$). Additionally, the cluster with the most nape attacks directed toward each partner was compared using a Kruskal-Wallis test, after finding the data were not normally distributed (Shapiro Wilks $p < .05$), to determine if the clusters of nape attacks were greater for some partners over others.

#### *2.2.6.3 Dominance, weight, play style, and proximity*

To determine if weight influences partner preferences, the difference in weight between the focal animal and each play partner was calculated and plotted against the percentage of nape attacks initiated between the pair. Similarly, dominance scores of both the focal animal and their play partners (dominance was assessed in both the home cage grouping and the play trial group) were plotted against the percentage of nape attacks to determine if dominance influences how much the focal animals play with each partner. Pearson correlations were used to determine if there was a significant relationship between the amount of play and weight differences and dominance. The play style, or how

individuals defended a playful attack, as well as the number of role reversals, were compared using a Kruskal-Wallis test among the three partners to determine if partners responded differently, depending on their familiarity.

Finally, the social proximity of the focal animal to other group members when not playing together during the trials was calculated. An ANOVA was used to determine if the focal rats spent more time with an individual rat, as these data were normally distributed (Shapiro Wilks $p > .05$). In addition, social network analyses (Farine & Whitehead, 2015) were conducted to compare the pattern of association when playing and not playing.

## 2.3 Results

### 2.3.1 Partner preferences

A Kruskal-Wallis test revealed that focal rats differed significantly in how many nape attacks they directed toward different partners (Figure 2.2a; $H(2) = 9.16$, $p = .01$), with more nape attacks launched toward neighbors than toward cage mates ($p = .003$) or strangers ($p = .036$). Given the considerable variation across individuals in the total number of nape attacks they launched (see Figure 2.4), the pattern of attacks across partner categories (Figure 2.2a) could have been biased by some overly playful outliers. Therefore, the distribution across partner categories for each focal rat was recalculated as a percentage of its total attacks, and these percentage scores were used to calculate group means (Figure 2.2b). An ANOVA revealed that there was a significant difference across categories of potential partners ($F(2, 105) = 25.97$, $p < .001$), with focal rats launching a significantly greater percentage of attacks toward neighbors than either cage mates ($p < .001$) or strangers ($p < .001$). In addition, focal rats launched a significantly greater percentage of nape attacks toward strangers than cage mates ($p = .034$).

### 2.3.2 Mechanisms influencing partner preferences

#### *2.3.2.1 Latency and temporal distribution*

A Kruskal-Wallis test revealed that there were no significant differences in latency to the first bout of play across partner categories ($H(2) = 0.32$, $p = .85$). In addition, descriptively, no difference was observed in the temporal distribution of play, with play peaking in the first five minutes of the trials in all cases (Figure 2.3).

#### *2.3.2.2 Order of nape attacks*

The order of attacks directed at different types of partners is shown in Figure 2.4. A Chi-square test revealed that the target of the first attack did not significantly differ among the partners available ($X^2 = 1.16$, df = 2, $p > .05$). Comparing the first half with the second half of the nape attacks per trial for all individuals, using a Student's *t*-test, except rat #20, which only launched four attacks, revealed no difference in the percentage of attacks directed at the three categories of potential partners (cage mate: $t(34) = 0.11$, $p = .46$; neighbor: $t(34) = 0.17$, $p = .43$; stranger: $t(34) = -0.39$, $p = .35$). So, partners of all three categories were attacked repeatedly over the course of the trial, although, in many cases, there were clusters, with focal rats repeatedly attacking one partner before moving on to the next. These clusters, of three or more nape attacks in succession, occurred differently among play partners, as revealed by a Kruskal-Wallis test ($H(2) = 9.01$, $p = .01$), with clustered nape attacks occurring toward neighbors significantly more often than cage mates ($p = .008$). Clustered nape attacks did not occur significantly more between cage mates and strangers nor neighbors and strangers. In addition, a Kruskal-Wallis test revealed there was a significant difference in the length of the clusters of nape attacks directed at each partner ($H(2) = 11.57$, $p = .003$), with neighbors having longer sequences of repeated nape attacks

compared to cage mates ($p$ = .004) and strangers ($p$ = .03). However, there was no difference between the length of clusters for cage mates and strangers.

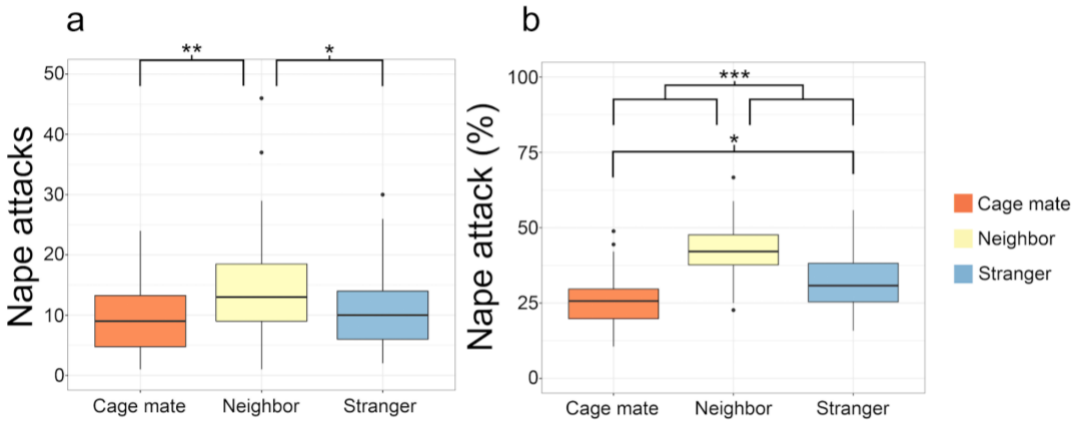


**Figure 2.2** The frequency of nape attacks (a) and the percent of nape attacks (b) by the focal animal towards the cage mate, neighbor, or stranger play partner are shown. Statistical significance is indicated by: * $p < .05$; ** $p < .01$; *** $p < .001$.

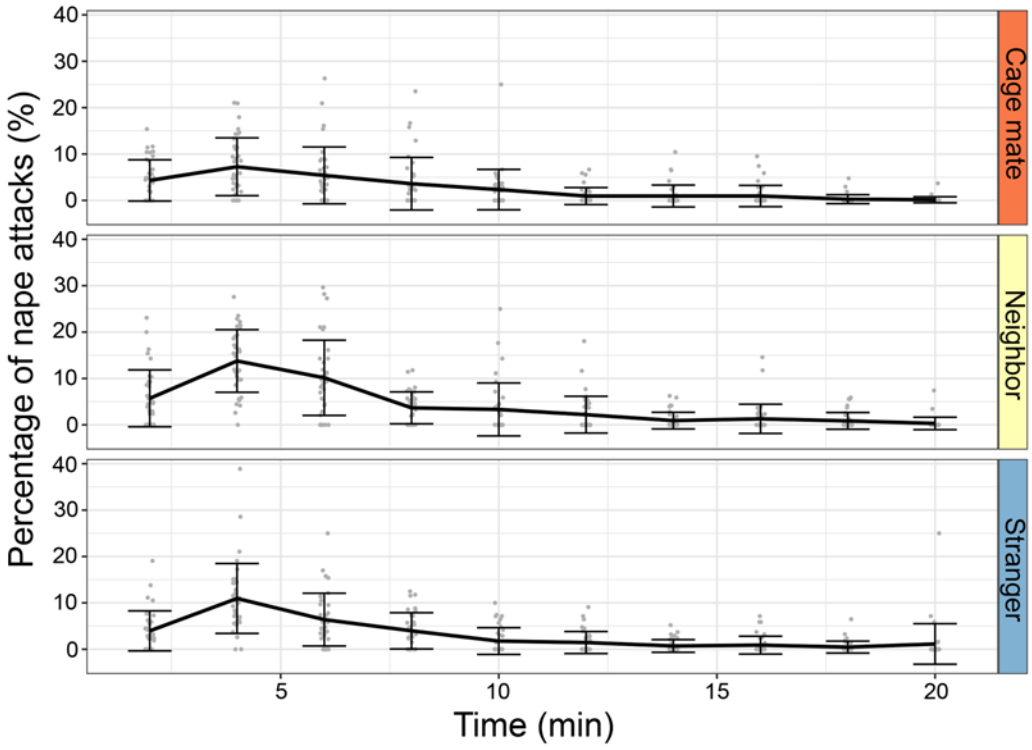


**Figure 2.3** The distribution of play between the focal rat and each category of partner is shown over the 20-min play trial.

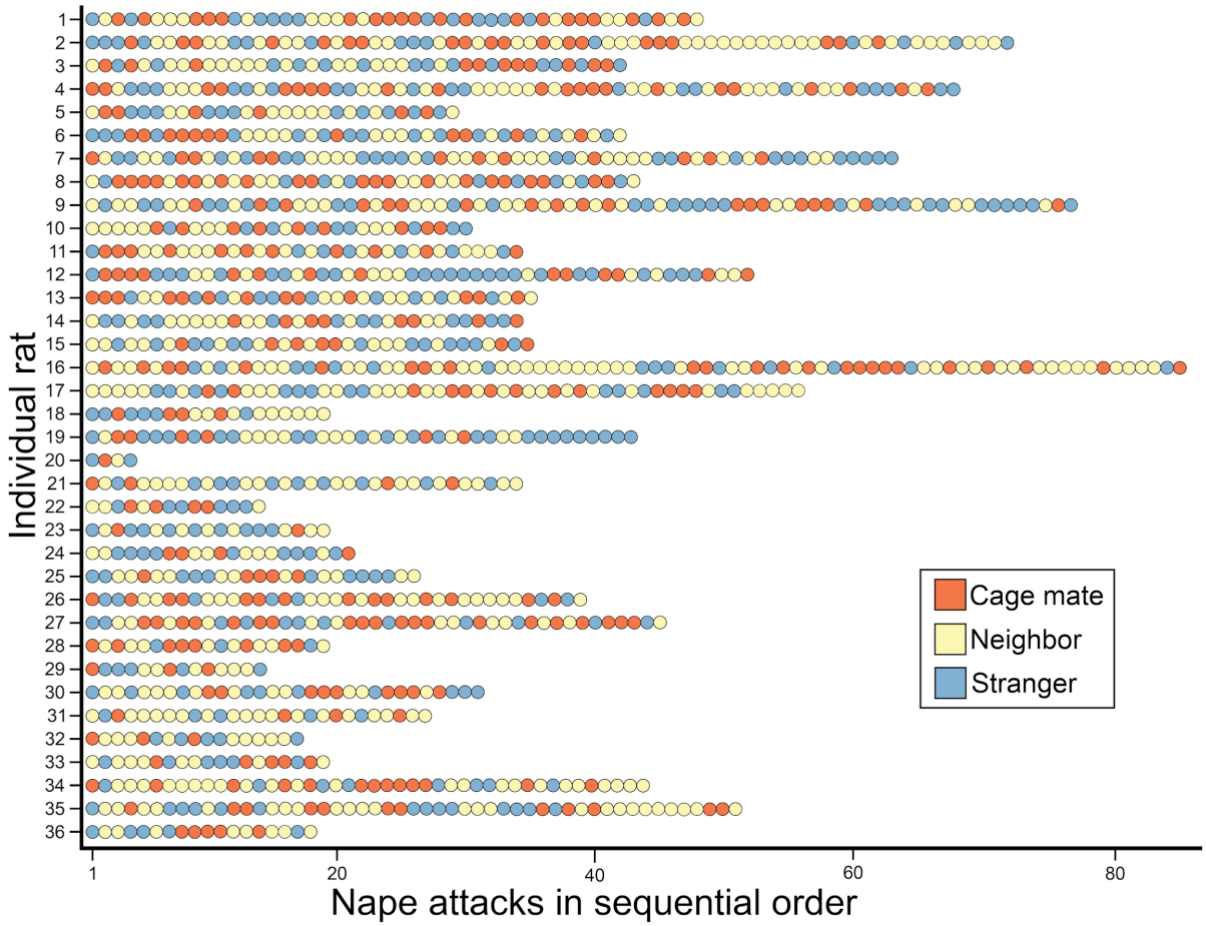


**Figure 2.4** The sequential order of nape attacks initiated by each focal rat with all categories of partners is shown for each trial.

### *2.3.2.3 Weight and dominance asymmetries*

When tested with a Pearson correlation there was no significant relationship between the weight difference between partners and percentage of nape attacks directed toward cage mates ($r(34) = -0.095$, $p = .58$) or neighbors ($r(34) = -0.23$, $p = .18$). However, there was a significant positive correlation between weight difference and nape attacks directed toward strangers ($r(34) = 0.38$, $p = .023$), with heavier, focal rats initiating more play with lighter, stranger rats. Focal rats that weighed around 30-20g less than the stranger

initiated far fewer nape attacks with the stranger (Figure 2.5a), a trend not observed with the cage mates or the neighbors.

Based on Pearson correlation tests, the dominance level of the focal animal in the home cage was not significantly correlated with the percentage of nape attacks directed toward cage mates ($r(34) = -0.042$, $p = .81$), neighbors ($r(34) = -0.18$, $p = .3$), or strangers ($r(34) = 0.2$, $p = .23$). The dominance level of the focal animal in the play trial was not significantly correlated with the percentage of nape attacks directed toward cage mates ($r(34) = -0.035$, $p = .84$), however, there was a negative trend with neighbors ($r(34) = -0.32$, $p = .06$), and a positive trend with strangers ($r(34) = 0.33$, $p = .052$), indicating that more dominant focal animals were more likely to launch attacks toward strangers but not toward neighbors.

Based on Pearson correlation tests, the dominance level of the partners in the home cage was not significantly correlated with either the cage mates ($r(34) = -0.16$, $p = .35$) or neighbors ($r(34) = 0.23$, $p = .18$), however, there was a significant negative correlation with strangers ($r(34) = -0.33$, $p = .049$), whereby focal rats were less likely to attack strangers who were dominant in their home cages (Figure 2.5b). The dominance level of the partners in the play trial was not significantly correlated with the percentage of nape attacks directed toward cage mates ($r(34) = -0.23$, $p = .18$) or neighbors ($r(34) = -0.039$, $p = .82$), however, there was a significant negative correlation with strangers ($r(34) = -0.34$, $p = .041$), whereby focal rats were less likely to attack strangers who were dominant in the play group (Figure 2.5c).

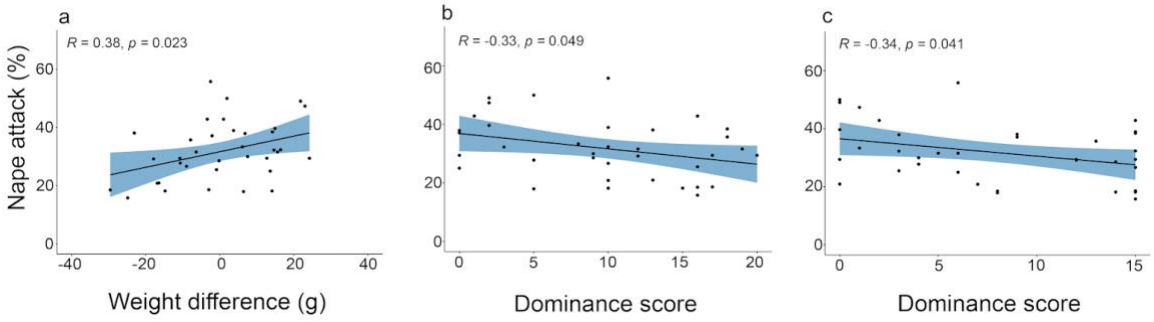


**Figure 2.5** These measures were only significantly correlated with interactions involving strangers. The percentage of nape attacks directed toward strangers is plotted against the weight difference between the focal and the stranger partners (a), the dominance scores of the strangers in their home cage (b), and the dominance scores of the strangers in the groups used in the play trials (c).

#### *2.3.2.4 Play style and quality*

Kruskal-Wallis tests did not reveal significant differences in the percentage of attacks defended that led to pins, evasions, mutual uprights or to role reversals (Table 2.2). That is, the pattern of play fighting appeared to be similar, regardless of the partner.

#### *2.3.2.5 Social proximity*

An ANOVA revealed that the time spent in non-playful proximity did not significantly differ ($F(2, 105) = 2.89$, $p = .06$) across categories of partners (Figure 2.6a). Thus, focal rats spent around 33% of their time in social proximity during the trials with each partner, but, on average, directed 45% of their play toward the neighbor, 30% of their play to the stranger, and only 25% of their play to the home cage mate (Figure 2.6b, c).

**Table 2.2** Results of a Kruskal-Wallis test for several measures of play.

| Behavior | Home cage | Neighbor | Stranger | Kruskal-Wallis test |
|---|---|---|---|---|
| Percentage of attacks directed to each partner that were defended | 58.78 ± 4.19 | 61.48 ± 3.54 | 61.51 ± 3.76 | $H(2) = 0.19$, $p = .91$ |
| Percentage of attacks leading to pins | 30.83 ± 3.43 | 24.77 ± 3.12 | 25.78 ± 3.14 | $H(2) = 1.61$, $p = .45$ |
| Percentage of defense leading to evasions | 21.73 ± 3.03 | 27.72 ± 3.01 | 30.15 ± 3.58 | $H(2) = 3.00$, $p = .22$ |
| Percentage of defenses leading to mutual uprights | 0.56 ± 0.19 | 1.89 ± 0.50 | 2.20 ± 0.79 | $H(2) = 4.29$, $p = .12$ |
| Percentage of defended attacks leading to a role reversal | 2.67 ± 0.50 | 4.09 ± 0.94 | 3.14 ± 0.77 | $H(2) = 1.02$, $p = .60$ |

*Note*. Several behavioral measures of play between the focal rat and each category of partner are shown. Each behavior is represented by the mean and standard error of the mean, and for each the results of a Kruskal-Wallis test is shown.

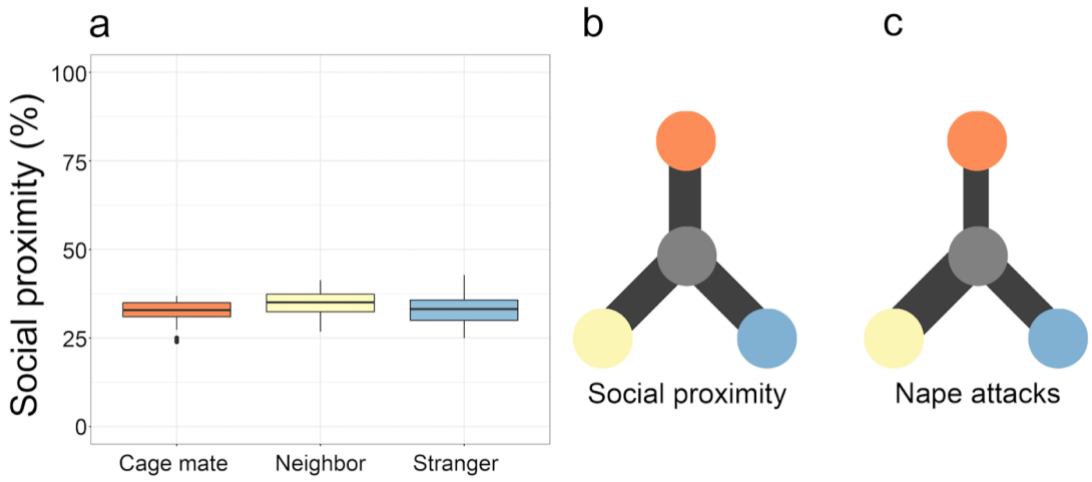


**Figure. 2.6** The percentage of time spent in social proximity when not playing between the focal rat and each category of partner is shown (a). Egocentric unidirectional social networks are presented to compare the time spent in social proximity (b) and playing (c) by the focal rat with each category of partner. There was no significant difference in social proximity but there was for play (see text).

## 2.4 Discussion

Given that, in tests of social preference, adult rats tend to attend more to strangers than familiar partners (e.g., Cirulli et al., 1996; Hackenberg et al., 2021; Rogers-Carter et al., 2018; Schweinfurth & Taborsky, 2020), and that, in dyadic tests, juvenile rats seem to play more when paired with strangers than cage mates (Achterberg et al., 2015; Panksepp, 1981; Pellis & Pellis, 1990; Reinhart et al., 2006), we predicted that, when given the choice, juveniles would initiate more play with strangers than cage mates. To test this, we devised a paradigm that removed three confounds. First, rats were tested in groups, so that the focal rat would have a choice between strangers and cage mates. Second, only a modest amount of pre-test social isolation was imposed on the rats to avoid elevating their motivation to play so high as to produce a ceiling effect that could potentially eliminate preferences. Third, two types of strangers were offered, ones that lived across a perforated transparent

barrier, allowing some degree of familiarization, but no play experience (Peartree et al., 2012), and ones with which they were completely unfamiliar. When so tested, focal rats did show a preference for initiating play with strangers, but while we expected the preference to be stranger>neighbor>cage mate, the data revealed the preference to be neighbor>stranger>cage mate (Figure 2.2 and Table 2.3). Indeed, while focal rats initiated play with all partners during the trials, consecutive play bouts, in which the rats continued to play with one partner repeatedly, occurred more frequently with neighbors than with home cage mates and strangers (Figure 2.4).

**Table 2.3** Summary of the predictions and results.

| Prediction | Supported | Result |
|---|---|---|
| Rats will prefer the most novel partner | No | Neighbor>stranger>cage mate |
| Latency to play will be longer with novel partners | No | No difference in the latency to play |
| Novel partners will be more socially attractive | No | No difference in social proximity |
| Playful feedback will influence preferences | No | Play measures did not differ among partners |
| Distribution of play will be biased for novel partners | Partially | Focal rats did not play with one rat over another at the beginning of the trial, but did engage in more bout repetitions, and longer sequences of repeated bouts with neighbors |
| Rats will prefer to play with the most dominant animal in the group | Partially | Dominance influences play preferences with strangers but not cage mates and neighbors |
| Rats will prefer to play with the heaviest animal in the group | Partially | Weight differences only influenced play preferences with strangers but not cage mates and neighbors |

While excessive novelty or stress dampens the occurrence of non-social play (e.g., play with objects, self-directed locomotor play), more moderate levels of novelty or stress, as induced by novel objects placed in the cage, may stimulate play (see review in Pellis & Burghardt, 2017). Similarly, mild to moderate social stress in some animals, including rats, increases play fighting (Darwish et al., 2001; Norscia & Palagi, 2011; Palagi, 2006; Von Frijtag et al., 2002), whereas more severe stress dampens play (Siviy et al., 2006; Siviy & Harrison, 2008). The neighbor may thus present the right balance between excessive familiarity and excessive novelty and so is the 'Goldilocks' partner—the partner that is 'just right.' Consistent with this possibility is that the weight and dominance of strangers influenced the degree to which play was initiated with them (Figures 2.5). Essentially, an excessive difference in dominance or weight with a stranger increases its novelty-induced stress to a level that play is avoided. Indeed, for the four focal rats that preferred their cage mate over novel partners, both the neighbor and stranger available were more dominant. However, it should be noted that, at this young age, dominance rarely leads to aggression (Pellis & Pellis, 1987; L. K. Takahashi, 1986; L. K. Takahashi & Lore, 1983), and aggression was never observed in this study. The 'threat' is not one of physical attack, but of novelty and strangeness, which leads to stress. Interestingly, in the home cage, with known social partners, greater dominance of a partner attracts more playful contact (Pellis & McKenna, 1992; Pellis & Pellis, 1991), whereas, with strangers, play is avoided if they are more dominant (present study). Clearly, while dominance may be important in influencing partner choice, context may be critical.

Direct measures of changes in stress hormones (D. C. Blanchard et al., 1993; Reinhart et al., 2006; L. K. Takahashi et al., 1992), depending on the quality of the unfamiliar partners presented to rats are needed to test this hypothesis, but the present

findings reveal a strong preference for novel play partners, although ones that are not too threatening. Even so, novelty seems to trump excessive familiarity, as generally rats initiated more play with strangers than with cage mates. While rats preferred novel, but not too novel partners, they initiated play with all members of the group (Figures 2.2 and 2.4), raising the problem of how the rats identified who is a stranger, who is a familiar, and who constituted the 'best' play partner.

### 2.4.1 Social exploration

In the paradigm that we used, the focal rat had never played with two of the potential partners and had never met one of them, so several mechanisms were examined that could be used to identify and focus playful interactions differentially across partners. Since rats engage in anogenital sniffing and other social exploration before they begin to play (e.g., Burke et al., 2022; Panksepp, 1981), we expected that there would be a longer delay in playing with strangers. This was not the case. The onset of play had the same latency for all types of partners and followed the same timeline over the course of the trial, with most focal rats initiating play with all three partners, primarily over the first 3-6 min of the trial (Figure 2.3). That the focal rats did not appear to use odor cues to identify and preferentially target neighbors and strangers appears to be consistent with experiments showing that rats play as normal when olfaction is suppressed or ablated (Beatty & Costello, 1983; Thor & Holloway, 1982). However, those experiments involved rats being tested in dyads in which rats had no choice but to play with the partner provided. Therefore, while olfactory information is not required for rats to engage in play, in a test context in which rats choose to play with some partners more than others, as in the present experiment, olfactory cues cannot be discounted. It could be that the measures we used to infer differences in olfactory information gathering were insufficiently sensitive. After all, rats are not insensitive to

odors encountered during play as juveniles. In one experiment, the choice of male sex partner by adult females was influenced by the odor associated with the play they experienced as juveniles (Paredes-Ramos et al., 2011). Given that rats are able to identify individuals, and their relative dominance, based on their scent (Gheusi et al., 1997; Wesson, 2013), to discount that our rats did not use odor to target the less familiar partners, olfaction needs to be blocked in a group play paradigm.

In whatever way the preferred partners are recognized, a possible way to produce a context resulting in more play with some partners than others, is for the focal rats to have spent more time with the strangers, increasing the opportunity to initiate play. Several studies have shown that for adults, unfamiliar conspecifics attract experimental rats to remain in closer proximity (Hackenberg et al., 2021; C. J. W. Smith et al., 2015). However, our data show that the focal rats spent equal time, when not playing, with all three test mates (Figure 2.6a, b), even though they played more with neighbors and strangers than with cage mates (Figure 2.1, Figure 2.6c). This is the same pattern that occurs in groups of familiar rats when tested together (Pellis, Pellis, Burke, et al., 2022). In both cases, a rat often left the vicinity of one rat to traverse across the cage to initiate play with a more distant rat. These findings suggest that distance sensors are used to orient toward and target preferred partners. As noted above, the indirect measures we used cannot discount olfaction being involved, but other distance sensors need also to be considered. Two possible sensory modalities are vision and audition.

By itself, blocking vision is insufficient to prevent pairs of rats from playing at typical frequencies in the dyadic test (Bierley et al., 1986; Pellis et al., 1996), and indeed, rats tested under red light or in complete darkness play as much if not more than when tested under lighted conditions (S. M. Himmler, Modlinska, et al., 2014; Pellis & Pellis,

1987, 1990; L. K. Smith et al., 1998). Thus, while it is possible that the visual acuity of rats, especially pigmented ones like the Long Evans hooded rats we used, may be sufficient to identify partners at the inter-individual distances afforded by the test enclosure (Prusky et al., 2002), given that our trials were conducted under red light, visual cues would not have been available. Small predatory mammals can use auditory cues, such as footsteps taken in a leaf litter strewn substrate to detect and track prey (Goerlitz & Siemers, 2007; Langley, 1988; Siemers et al., 2007), and similarly, the location of potential partners in the test enclosure could be detected by the sounds made when stepping on the corncob bedding. Masking such sounds with white noise does not prevent play from occurring in dyadic tests, but it does decrease the likelihood of close quarter wrestling leading to pins (Siviy & Panksepp, 1987), suggesting another auditory cue may be used. When playing, rats emit ultrasonic vocalizations, especially frequency modulated 50 kHz calls (Burgdorf et al., 2008; Kisko, Himmler, et al., 2015), and when devocalized pairs of rats are matched, both the frequency of playful attacks and the incidence of role reversals is halved (Kisko, Himmler, et al., 2015). Moreover, different calls are associated with performing different actions during play (C. J. Burke et al., 2018), with some calls being able to attract other rats (Wöhr & Schwarting, 2007), providing the possibility that such calls could be used to target preferred partners. Nonetheless, when triads of rats were tested in play trials, in which one of the three was devocalized, the focal vocalizing rat launched just as many nape attacks to the devocalized partner as the vocal partner (Kisko, Himmler, et al., 2015). These findings do not discount the possibility that ultrasonic calls are used to localize partners, but this may only be detectable when other, more salient cues, such as olfactory ones are not available. Regardless of how they are detected, why are some partners preferred?

Some rats are more playful than others (Achterberg et al., 2023; Lampe et al., 2019; Lesscher et al., 2021; Pellis & McKenna, 1992) which can lead to different patterns of play when rats with different preferences play together (Pellis, Pellis, Burke, et al., 2022; Poole & Fish, 1976). Therefore, it is possible that different partners provide different feedback during play. This led us to make two predictions. First, all potential partners should be sampled in the first few minutes of the trial and then, play should mostly be with the preferred partner in the later phases of the trial. Second, the pattern of play gained between preferred and less preferred partners should be different. In particular, the play with preferred partners should provide more close quarter wrestling and role reversals, the features of play that make it most rewarding (Pellis & Pellis, 2017; Vanderschuren et al., 2016). Neither prediction was supported by the data. The focal rats had the same temporal distribution of play with all three partners (Figure 2.3) and interspersed play bouts with all three across the entire span of the trial (Figure 2.4). Moreover, the pattern of play by the focal rat was the same when playing with all three partners (Table 2.2). While sampling and feedback may not account for why neighbors come to be preferred over cage mates and strangers, the lack of difference in the play experienced may account for why, despite significant preferences being present (Figure 2.2), the focal rats continue playing with all three partners. The finding that there was a significant difference in the length of the string of consecutive play fights initiated with preferred partners (Figure 2.4) suggests that there may be some subtle differences, not detected by our measurements, that make play with preferred partners more rewarding. It could be as simple as the neighbor providing a more exciting balance between novelty and familiarity to make play more stimulating (Pellis & Burghardt, 2017). The role of partner novelty, however, may differ across species.

Unlike the preference for strangers shown by rats, adult mice (*Mus musculus*), spiny mice (*Acomys cahirinus*), and female degus (*Octadon degus*) do not express a preference for either unfamiliar or familiar animals (Beery, 2021; Beery & Shambaugh, 2021; Fricker et al., 2022; Insel et al., 2020), and prairie voles (*Microtus ochrogaster*) and meadow voles (*M. pennsylvanicus*) prefer familiar conspecifics over strangers (Beery et al., 2018; Beery & Shambaugh, 2021). These differences in preference among rodent species may depend on differences in their mating and social systems (Beery & Shambaugh, 2021). While differences in social systems appear to influence variation in styles of play fighting across rodent species (Pellis & Iwaniuk, 1999b), there are no comparative studies to ascertain whether they also influence partner preferences. One comparison suggests that if they do, the effects may be quite subtle and idiosyncratic.

Rats live in colonies in which multiple females, rearing litters of young, live in proximity to each other (Schweinfurth, 2020). Infants mostly interact with their mothers and each other until weaned (Cramer et al., 1990). Once weaned, young rats have the option to play with littermates or with peers from neighboring litters. If our experimental design mimics real-life choices, juvenile rats should prefer to play with neighbors over siblings. Free living Belding's ground squirrels (*Urocitellus beldingi*) also live in colonies, but with females maintaining individual territories around their burrow in which they raise litters. When the young emerge from their burrows and begin to play, they have a choice between their littermates and the young from neighboring burrows (Nunes, Muecke, Lancaster, et al., 2004; Nunes, 2014). Unlike rats in the present study, juvenile ground squirrels play with littermates twice as much together as they do with non-littermates (Holmes, 1994; Nunes et al., 2015; Nunes, Muecke, Sanchez, et al., 2004). As in our study (Figure 2.6), these preferences are not accounted for by the physical distance between littermates and

non-littermates (Holmes, 1994). This suggests that, for the ground squirrels, partner novelty does not influence play partner preference as it does in rats (Figure 2.2). Interestingly though, preliminary data on rats indicate that there are partner preferences when playing with littermates (Pellis, Pellis, Burke, et al., 2022), a pattern also reported for the ground squirrels (Nunes, Muecke, Sanchez, et al., 2004).

In non-rodent species, partner play preferences are often influenced by kin relationships (Cappiello et al., 2018; Thompson, 1996), dominance hierarchies (Biben, 1986), and familiarity (Antonacci et al., 2010; Walker et al., 2015). While the juveniles of some non-rodent species only play with familiar animals or siblings (Antonevich et al., 2020; Drea et al., 1996; Pfeifer, 1980), as adults they may use playful social interactions to assess and manage encounters with strangers (Pellis & Iwaniuk, 1999a). For example, wild male sifakas (*Propithecus verreauxi*) play more with outgroup members than with ingroup members, seemingly to manage and test social relations with unknown individuals (Antonacci et al., 2010). When adult male grizzly bears (*Ursus arctos*) encounter unfamiliar, adult females, they too engage in play to seemingly familiarize themselves with one another (Herrero & Hamer, 1977). In this regard, rats are not atypical, as adult rats similarly use play fighting to familiarize themselves with unfamiliar adults (Stark & Pellis, 2020, 2021). It remains unknown as to how wild juvenile rats associate with peers in large colonies, and some laboratories studies routinely test juveniles with unfamiliar peers (Achterberg et al., 2015; Achterberg & Vanderschuren, 2020). Indeed, our results suggest that rats are not neophobic when young, and even prefer somewhat novel partners. Why different patterns of preferences are expressed across contexts in different species remains to be determined.

### 2.4.2 Future directions

While only males were used in this study, it is likely that females would express a similar pattern of partner preference. Males were selected for this study as dominance hierarchies, which are more prevalent in males (reviewed by Schweinfurth, 2020), influence playful interactions (Pellis & McKenna, 1992). However, because the relative dominance only significantly affected preferences with strangers, and this accounted for very little of the variance, females would likely express a similar partner preference pattern, as relative dominance likely would not influence females. With that said, juvenile female rats are more sensitive to the familiarity of their play partner when playing in dyads, playing more with familiar animals than strangers (Argue & McCarthy, 2015a). However, the rats in this study were not afforded a choice and they were not presented with a 'somewhat' strange partner (i.e., a neighbor). Nonetheless, now that we have found juvenile male rats express partner preferences in a group play testing paradigm, we plan on performing the same experiment in females. Using this paradigm, we also plan to explore how mixed-sex groups interact to determine if juvenile rats express a preference for their own sex in rough-and-tumble play (Argue & McCarthy, 2015a) when given a choice. As the amount and roughness of play changes with age, the sex of the preferred partner may also change (Meaney & Stewart, 1981). Again, the group play paradigm used in the current paper could prove useful in discerning developmental changes in partner preferences.

Additionally, this paradigm may also prove useful in understanding how mixed-strain groups play. Mixed-strain experiments have become increasingly popular and have been used to explore rat prosocial behavior (Ben-Ami Bartal et al., 2014, 2021), the development of play behaviors (Schneider, Bindila, et al., 2016; Siviy et al., 2017; Stark et al., 2021), and social preferences (Kiyokawa et al., 2014; Kogo et al., 2021; Mauri et al.,

2022), to name just a few areas of research. The paradigm employed here could explore how groups of mixed-strain juveniles interact with one another and determine if they prefer their own strain or not. In such a study, the preference for same-strain individuals could be due to dissimilarities in play style between strains (S. M. Himmler, Modlinska, et al., 2014; Siviy et al., 1997) or other factors, such as discordant olfactory cues (Kogo et al., 2021; Nakamura et al., 2016). This is another experiment we plan on conducting in the future.

## 2.5 Conclusion

Our results demonstrate that juvenile male rats express partner preferences when playing in a group. When given the choice by allowing juveniles to play in groups rather than dyads, rats prefer novel partners, but partners that are not too novel (the Goldilocks principle). None of the possible mechanisms used to form partner preferences that we assessed accounted for how these preferences were formed. Whether partner preferences emerge in groups of complete strangers remains to be tested, but if they do, that could be a better paradigm with which to discern how preferences are formed. Although in the current study relative dominance among partners did not account for partner preferences, dominance involving a stranger did influence play. Focal rats were less likely to initiate play with more dominant strangers (Figures 2.5b, c). A potential application of this finding concerns dyadic test paradigms in which strangers are paired together (Pellis, Pellis, Burke, et al., 2022). Rats of markedly different dominance should not be matched. To avoid pre-test or post-test evaluation of each rat's dominance in its home cage or with the test trial partner, body weight, which is correlated with play preferences in the same way as dominance (Figure 2.5a), can be capitalized on by matching rats with as little weight asymmetry as possible (Achterberg et al., 2015; Achterberg & Vanderschuren, 2020). Play partner preference is an understudied feature of play, especially in key animals such as rats

that have served as major laboratory models for the study of play (Siviy, 2016; Vanderschuren et al., 2016; J. W. VanRyzin et al., 2020), but may yield important new insights into the mechanisms of social cognition and exploration (Pellis, Pellis, Ham, et al., 2022, 2023).

# CHAPTER 3: PLAY PARTNER PREFERENCES AMONG GROUPS OF UNFAMILIAR JUVENILE MALE RATS*

## 3.1 Introduction

Play fighting or rough-and-tumble play is one of the most commonly reported forms of play behavior (Pellis & Pellis, 1998), and is observed in many mammals, especially as juveniles (Burghardt, 2005). The defining feature of play fighting is that animals compete for an advantage, which, in many cases, is to contact a particular location on the partner's body, but do so while showing some degree of cooperation (Aldis, 1975). The advantage/targets vary across species and lineages of species (Pellis et al., 2024), being derived from targets typically bitten, rubbed, or otherwise contacted during such adult functional contexts as conspecific aggression, predation, sex, and other forms of affiliative behavior (e.g., grooming) (Pellis, Pellis, & Ham, 2023). The cooperation present in play fighting leads to reciprocity and turn taking, ensuring that the encounters remain playful (Palagi, Cordoni, et al., 2016; Pellis & Pellis, 2017).

Play fighting has been most intensively studied in laboratory rats (*Rattus norvegicus*) (Achterberg & Vanderschuren, 2023; Pellis, Pellis, Ham, et al., 2023), in which the animals compete to nuzzle each other's napes with the tip of the snout (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). Rats are highly motivated to play (Vanderschuren, 2010), and play engages neural circuits spanning the entire neuraxis (Siviy, 2016; Vanderschuren et al., 2016; J. W. VanRyzin et al., 2020). The play fighting experienced in the juvenile period influences the development of socio-cognitive skills and does so by altering the

anatomy and physiology of the prefrontal cortex (Marquardt et al., 2023; Pellis, Pellis, Ham, et al., 2023; Vanderschuren & Trezza, 2014), with similar findings also reported in two other species of rodents (Burleson et al., 2016; Marks et al., 2017). In rats, play fighting continues into adulthood, albeit at a lower frequency (Pellis & Pellis, 1990; Thor & Holloway, 1984c), and is used to assess and manage social relationships, especially dominance relationships (Pellis et al., 1993; L. K. Smith et al., 1999). Both the juvenile function of play fighting as a tool for refining socio-cognitive skills, and its use as a tool for social assessment and management in sexually mature animals, may extend beyond rats and some other rodents to include mammals from many other lineages (Cordoni, 2009; Manitzas Hill et al., 2022; Palagi & Paoli, 2007).

Typically, play in laboratory rats is evaluated with the 'dyadic test,' in which two animals are placed in a neutral arena after a period of social isolation, which can range from a few hours to several days (Pellis, Pellis, Burke, et al., 2022). Play trials last from 5-30 min, with playful attacks to the nape beginning within the first 1-2 min (C. J. Burke et al., 2022). The number of attacks to the nape is an effective measurement to assess the motivation to play (Achterberg et al., 2023; B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022).

While the 'dyadic test' has been a useful tool for studying play in rats (Pellis, Pellis, Burke, et al., 2022), it has a limitation. In the wild, rats live in colonies, with litters from multiple females born in close temporal proximity (Schweinfurth, 2020), affording juveniles the opportunity to play with many other juveniles, both siblings and non-siblings. Thus, unlike in the dyadic test, in more natural settings, rats have a choice of play partners.

Other species reared with littermates or in social groups with multiple similar aged partners exhibit preferences in their selection of play partners (Ham et al., 2022; Lilley et

al., 2020; Nunes, Muecke, Sanchez, et al., 2004). Similarly, when living in groups, rats prefer to play with some members more than others (Mauri et al., 2022; Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2023). Even in the dyadic test, not all partners presented seem equally attractive, with how much play directed at the partner being dependent on their sex, relative difference in age, whether they are of the same or different strain, and whether they are familiar or novel partners (Achterberg et al., 2015; Argue & McCarthy, 2015a; Pellis et al., 2017; Pellis & Pellis, 1990; Stark et al., 2021). This suggests that, when given the choice among different potential partners, rats will show preferences.

In a recent study, we evaluated one factor that seems likely to be important in selecting potential partners: familiarity (Ham & Pellis, 2023). In this study, rats were tested in groups of four, with the focal rats being given a choice of three partners. One partner was a cage mate (i.e., familiar), another was housed on the other side of a transparent and perforated divider (i.e., somewhat familiar), and the third was a stranger from a separate cage (i.e., unfamiliar). The focal rats initiated playful nape attacks differentially: familiar < unfamiliar < somewhat familiar. Focal animals did not vary their play style with animals of varied familiarity, only the amount of play was altered. Moreover, whereas the focal rats did not alter the amount of play they initiated with familiar and somewhat familiar partners with the magnitude of difference in weight and dominance, they did so with fully unfamiliar partners, initiating less play with heavier, more dominant animals (Ham & Pellis, 2023). Given that within a group of familiar rats there are partner preferences (Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2023), and that when given a choice rats prefer to play with less familiar partners (Ham & Pellis, 2023), in this experiment we tested whether within a group in which all the rats are strangers, they would form partner preferences.

Play fighting in the juvenile period influences the development of prefrontal neural connections (Bell et al., 2010) that are important for both coordinating motor actions with a partner and adjusting how they play depending on the identity of the partner (B. T. Himmler et al., 2014). The former depends on the quality of the play (Ham, Szabo, et al., 2024; Stark et al., 2023) and the latter on the diversity of partners to which the rat is exposed (Bell et al., 2010). Therefore, knowing if rats form preferences with unfamiliar rats during a play session has implications on whether juvenile rats choose to socialize and play with partners that maximize the relevant experiences that lead to these socio-cognitive improvements. That is, young rats may actively create a social niche that is functionally beneficial to them (West & King, 1987).

In the present study, groups of unfamiliar male juvenile rats were tested together to determine if rats form partner preferences in play and if they do, what factors they base these preferences on. One rat was selected as the focal animal and its partner preferences was assessed by the relative proportion of nape attacks directed to different members of the group (Pellis, Pellis, Ham, et al., 2023). We predicted that rats would form preferences when playing with unfamiliar partners. After establishing whether or not focal rats had partner preferences, several possible factors that could influence the emergence of those preferences were explored. Rats have individual differences in how much they play (Lampe et al., 2019; Lesscher et al., 2021), but will modify the expression of that playfulness based on the playfulness of their partner (Achterberg et al., 2023). Therefore, we predicted group mates that initiated more nape attacks towards the focal rats would be preferred by the focal animal. Rats that play more also tend to prefer to use different defense tactics than rats that play less (Pellis, Pellis, Burke, et al., 2022). Therefore, we predicted the relative difference in types of defenses used between preferred and non-preferred partners that reflect

differences in the degree of body contact during play would influence partner preferences. In addition, a measure reflecting the degree of cooperation was compared on the assumption that since pairs with more symmetry are more likely to gain the experiences most beneficial for the development of inter-animal coordination skills (Stark et al., 2021; Stark & Pellis, 2020, 2021), rats would select to play with more compatible partners. Given that differences in weight and dominance influenced the amount of play directed towards strangers in another group play experiment (Ham & Pellis, 2023), weight and dominance differences were compared between the most and least preferred partners. In addition to play style, weight, and differences in dominance, rats may select partners based on proximity. That is, rats may pick to play with the individuals that are closest to them. To determine if social proximity influenced partner selection, we measured the time individuals spent close to one another. Finally, the temporal distribution of playful attacks was scored to determine when in the trial preferences were formed or consolidated( Ham & Pellis, 2023). In this way, not only was the existence of partner preferences assessed, but also some of the possible factors contributing to their formation were tested.

## 3.2 Methods

### 3.2.1 Ethics statement

All care and testing procedures were reviewed and approved by the University of Lethbridge Animal Welfare Committee (protocol #1809) in compliance with guidelines from the Canadian Council for Animal Care and comply with the ARRIVE guidelines.

### 3.2.2 Subjects

Forty-eight weanling Long Evans male rats were purchased from Charles River Laboratories (Kingston, NY, USA) and arrived at the Canadian Centre for Behavioural Neuroscience at 22 days of age. Upon arrival, the animals were moved into double decker

cages with corncob bedding and housed in groups of 6 (resulting in 8 groups). Food and water were available *ad libitum*. Animals were housed on a 12-hour light-dark cycle with lights off at 19:30 and maintained at a constant temperature of 21°C - 23°C.

### 3.2.3 Apparatus

Animals were tested in a large Plexiglas® enclosure (80 cm × 80 cm × 50 cm), that was specifically scaled up from our enclosure that is used to test dyads, to accommodate groups of six rats. Corncob bedding was used to sufficiently cover the floor of the enclosure. An ExmourRS 4K Sony Handycam was used for filming the play sessions and was place over top of the enclosure at nearly a 90° angle.

### 3.2.4 Procedure

At 28 days of age, the rats were habituated to the test enclosure for 10 min, with their cage mates, in red light. This was done for two consecutive days between 07:30 and 19:00. At 30 days of age, the rats were sufficiently habituated to the enclosure and testing began. Groups of cage mates were placed in the enclosure, in red light, for 20 min and filmed. The play trials were repeated for eight days. After testing, animals were rehoused with their cage mates for 24 h. Before each play trial, the rats were socially isolated for 2.5 h to increase their playfulness (Pellis, Pellis, Burke, et al., 2022). Before being socially isolated, each rat was weighed. Fresh bedding was replaced, and the test enclosure was cleaned with Virkon® after each trial to reduce any odors left from the previous rats. To identify individual rats, the tail was colored, using different patterns, with a permanent marker pen (Sharpie®). After eight days of testing animals with familiar cage mates, they were tested in two consecutive play trials with unfamiliar animals, when they were 38 and 39 days old. These groupings were assigned randomly so that all the rats in each group were unfamiliar with each other. Even when seemingly redundant, such test trials were run

twice because of the risk of data loss due to instrument failure or some unexpected event, such as load noise in another part of the laboratory, that suppresses play in the rats (Pellis, Pellis, Burke, et al., 2022). As all test trials from the first session when the rats were 38 days old worked, data from the first trial with unfamiliar groups were analyzed for the present study.

Following each play trial, the tube test (Fan et al., 2019) was employed to determine the relative dominance among members of each group tested. To do so, pairs of rats from the play group were placed headfirst into a Plexiglas® tube (19.5 cm in length and 4.5 cm in diameter), simultaneously, at opposing ends. The tube was just large enough to allow one rat through, but not so large that the second rat could squeeze past its opponent. The 'loser' or subordinate rat was determined as the rat that was pushed out of the tube, while the 'winner' remained in the tube. The winning rat was given a point for that round. If neither rat was pushed out after 60 sec, the round was scored as a tie, and no point was awarded for that encounter. Each possible pairing within the group was tested five times and the sum of points was used to determine which was the most dominant. After five rounds, the testing for any given pair was completed, the tube was cleaned with Virkon®, and the next pair was tested.

### 3.2.5 Behavioral analysis of play trials

The first 10 minutes of each video recording was analyzed using both normal speed and frame-by-frame analysis to score various aspects of the rats' playful attack and defense strategies (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022). Only the first 10 minutes was used as the majority of play, following brief social isolation, occurs in the first 10 minutes after partners have been introduced to one another (Bijlsma et al., 2022; Ham & Pellis, 2023). For each video, one of the six rats was selected to be the focal

animal. For group one, rat one was selected, for group two, rat two was selected, so on and so forth until the seventh and eighth group in which rat one and rat two were selected as the focal animal, respectively. Once selected, we used a focal follow scoring approach. Both the attacks initiated and directed toward the focal animal were recorded, with the individuals attacking and defending identified and recorded by their tail markings. A playful attack was scored when the snout of a rat made contact with the nape of another rat as this is the target in rat rough-and-tumble play in around 90% of playful attacks (B. T. Himmler, Pellis, & Pellis, 2013). Additionally, if a playful attack was initiated and directed towards the nape, but the defender evaded before contact could be made, this too was scored as a playful attack. How the rats responded to a playful attack was scored following the protocol described by Pellis, Pellis, Burke, et al. (2022) and is summarized in Table 3.1. The number of playful nape attacks were scored as the number by each animal per trial, whereas how rats responded to attacks were scored as a percentage of the attacks. Thus, defense was calculated the percentage of attacks defended compared to overall attacks, which is a measure of how motivated the recipients are to engage with the attacker. Of the defended attacks, the percentage leading to evasive defense was used as a measure of how reluctant the recipient was in engaging in close quarter wrestling. Similarly, of the defended attacks, the percentage that led to pinning is a measure of the animal's motivation to sustain close bodily contact when playing (Stark et al., 2021). The percentage of defended attacks that lead to role reversals is a measure of both reciprocity and the motivation to continue the ongoing interaction. The relative symmetry in initiating role reversals has been found to be a good measure of coordination between play partners (Pellis et al., 2017; Stark et al., 2021). This is a measure of the pair's behavior, calculated as the number of role reversals initiated by rat A/total role reversals (those by A + B) (Table 3.1). Converted to a

percentage, the resultant value can be interpreted as the closer to zero, the more mutual the interaction, and the closer to 100%, the more asymmetrical the contribution by the partners (Stark et al., 2021).

Finally, the duration of time spent in close social proximity was measured. This was done to determine whether the focal rat played with a partner that was closest and most convenient or instead if they seek out a particular partner within the play box. Rats were considered to be in social proximity if they were within one body length of each other (Ham & Pellis, 2023). As some of the time spent in close social proximity was due to the pair playing, the total time the two individuals spent playing with each other was subtracted from the total time they were in proximity to each other (Ham & Pellis, 2023). All behaviors measured are summarized in Table 3.1.

**Table 3.1** The behavioral measures used in this study.

| Behavior | Definition | Calculation |
|---|---|---|
| Attack | | |
| Nape attack | Playful contact made with the snout on the nape of a partner | ∑ Frequency with each partner = total play |
| Defense | | |
| % Defended | Playful attacks that are responded to | % defended = [(Total attacks launched to a given partner – occurrence when the animal does not respond) / total attacks launched to given partner] × 100 |
| % Evasion | Partner runs away after being attacked | % evasion = [(frequency of defended attacks – frequency of attacks evaded) / frequency of defended attacks] × 100 |
| % Pin | Partner defends nape by rolling into a supine position | % pin = [(frequency of defence attacks – frequency of attacks resulting in a pin) / frequency of defended attacks] × 100 |
| Role reversals | When the role of the defending rat switches to that of attacker (i.e., turn taking) | % role reversal = [(frequency of defended attacks – frequency of role reversals) / frequency of defended attacks] × 100 |
| Asymmetry in role reversals | The degree to which turn taking is shared between a pair. A number closer to 0% indicates that for every role reversal Rat A engages in Rat B engages in a similar amount. If closer to 100%, this indicates one of the rats is engaging in all of the role reversals. | $\text{Asymmetry} = \left(\frac{\lvert(\text{Rat A role reversals}) - (\text{Rat B role reversals})\rvert}{\sum \text{Rat A} \rightleftharpoons \text{Rat B role reversals}}\right) \times 100$ |
| Social proximity | Time spent within one body length of partner | Social proximity = total duration focal and Rat A are within one body length of each other – (time focal spent playing with Rat A + time Rat A spent playing with the focal rat) |

### 3.2.6 Statistical analysis

#### *3.2.6.1 Partner preferences*

To visualize partner preferences, egocentric networks were plotted using *igraph* (Csardi & Nepusz, 2006) in R (R Core Team, 2020). The central node, or central circle, represents the focal animal in each group, with the surrounding nodes representing the potential play partners. The edges, or lines, radiating from the central node are unidirectional and the thickness visualizes the proportion of play the focal animal initiated (N.B., these edges only show focal initiated play and not how much the partners played with the focal rat). The proportion of play, rather than frequency, was calculated and used to make comparisons as there is intra- and intergroup variation in the amount of play initiated by individuals. The proportion of play was calculated by dividing the number of playful attacks directed to a given partner by the focal rat by the total number of playful attacks launched by the focal. To determine if focal rats preferred individuals within their group significantly more than others, Chi-square goodness of fit tests, using the raw play scores for each focal rat, were used to determine if the distribution of nape attacks among the five partners was different from what would be expected if the nape attacks were distributed evenly (i.e., 20% of the focal rats play per partner) for each focal rat. If the Chi-square tests revealed significant deviations from what would be expected by chance, further tests on the other behavioral parameters scored were used to determine what may be driving partner preferences.

#### *3.2.6.2 Potential factors influencing partner preferences*

While measures of the microstructure of play fighting rats have improved in the past decades (Pellis, Pellis, Burke, et al., 2022), they are still too crude to ensure that subtle

differences in style are detected. Sometimes, it is the reluctance of the rats to engage with some partners that provide indirect clues that there is something different in how a specific rat is playing (Stark & Pellis, 2021). Therefore, once the Chi-square test revealed a significant difference in how much play was directed to and from potential partners, trials from the most extreme differences—the most and the least preferred partners—were analyzed using Wilcoxon ranked sign tests in SPSS (Version 29). This analysis included the number of nape attacks launched by the focal rat's partners, preferred tactics of defense, weight asymmetry, dominance asymmetry, social proximity, and latency to first attack by the focal rat to the most and least favorite partner. If there was more than one most or least favorite partner for a focal rat, the measures were averaged. Focal rats that did not express partner preferences were excluded from further analyses.

As rats show a great deal of individual variation in the initiation of playful attacks, a Mann-Whitney U test was used to determine if rats that form preferences initiate more playful attacks than those who do not form preferences. Finally, the temporal distribution of playful attacks directed to each of the five potential partners, over the 10 min play sessions, initiated by the focal rat, was plotted using the *ggplot2* package (Kassambara, 2019).

## 3.3 Results

### 3.3.1 Partner preferences

Egocentric social networks plotting the proportion of nape attacks directed to each of the five play partners showed that all eight focal rats formed preferences (Figure 3.1). To determine if these preferences were significant, Chi-square tests were used to evaluate whether the playful attacks directed to each partner differed significantly from what you

would expect if each partner was attacked equally, based on chance (20%). The Chi-square tests revealed that five of the eight focal rats formed partner preferences (Table 3.2).

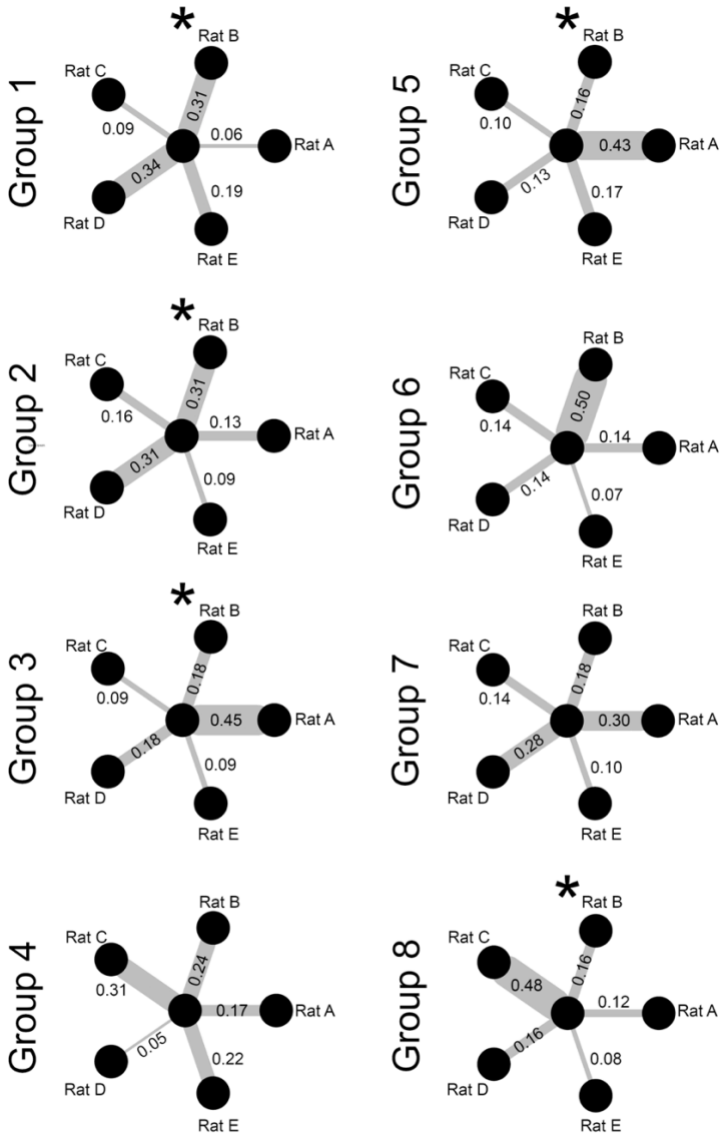


**Figure 3.1** Egocentric social networks are plotted showing the proportion of play focal individuals (center node) directed towards the five other rats in the group. Individual networks are plotted for all eight focal rats, with an asterisk indicating which focal animals differed significantly from a chance distribution (see Table 3.2 for statistical analyses). *Indicates when a focal rat had significant preferences.

**Table 3.2** The number of nape attacks expected, by chance (20%), based on the total number of nape attacks launched is tested against the number of nape attacks actually launched to each partner.

| Focal | | Rat A | Rat B | Rat C | Rat D | Rat E | Total | Chi$^2$ test |
|---|---|---|---|---|---|---|---|---|
| 1 | Nape attacks | 2 | 10 | 3 | 11 | 6 | 32 | **$X^2$ = 10.19, df = 4 $p$ = .037** |
| | *Expected* | *6.4* | *6.4* | *6.4* | *6.4* | *6.4* | | |
| 2 | Nape attacks | 7 | 14 | 6 | 14 | 4 | 45 | **$X^2$ = 9.78, df = 4 $p$ = .044** |
| | *Expected* | *9* | *9* | *9* | *9* | *9* | | |
| 3 | Nape attacks | 10 | 4 | 2 | 4 | 2 | 22 | **$X^2$ = 9.82, df = 4 $p$ = .044** |
| | *Expected* | *4.4* | *4.4* | *4.4* | *4.4* | *4.4* | | |
| 4 | Nape attacks | 7 | 10 | 13 | 2 | 9 | 41 | $X^2$ = 8.15, df = 4 $p$ = .086 |
| | *Expected* | *8.2* | *8.2* | *8.2* | *8.2* | *8.2* | | |
| 5 | Nape attacks | 13 | 5 | 3 | 4 | 5 | 30 | **$X^2$ = 10.67, df = 4 $p$ = .031** |
| | *Expected* | *6* | *6* | *6* | *6* | *6* | | |
| 6 | Nape attacks | 2 | 7 | 2 | 2 | 1 | 14 | $X^2$ = 8.14, df = 4 $p$ = .086 |
| | *Expected* | *2.8* | *2.8* | *2.8* | *2.8* | *2.8* | | |
| 7 | Nape attacks | 15 | 9 | 7 | 14 | 5 | 50 | $X^2$ = 7.60, df = 4 $p$ = .11 |
| | *Expected* | *10* | *10* | *10* | *10* | *10* | | |
| 8 | Nape attacks | 3 | 4 | 12 | 4 | 2 | 25 | **$X^2$ = 12.80, df = 4 $p$ = .012** |
| | *Expected* | *5* | *5* | *5* | *5* | *5* | | |

**Table 3.3** Comparisons from the most extreme differences. Only play from focal rats that had significant preferences (i.e., Focal 1-3, 5, and 8) are compared.

| Behavior | Most preferred (mean ± SD) | Least preferred (mean ± SD) | Wilcoxon Ranked Sign Test |
|---|---|---|---|
| Nape attacks towards focal | 8.0 ± 2.92 | 4.9 ± 2.75 | **Z = 15, $p$ = .042** |
| Weight asymmetry (g) | 11.99 ± 24.90 | 3.94 ± 22.37 | Z = 6, $p$ = .69 |
| Dominance asymmetry | -2.6 ± 10.71 | -1.7 ± 9.58 | Z = 8, $p$ = .89 |
| Social proximity (sec) | 145.54 ± 19.36 | 175.91 ± 21.63 | Z = 13, $p$ = .14 |
| Latency (sec) | 45.35 ± 23.39 | 66.27 ± 40.45 | Z = 12, $p$ = .23 |

### 3.3.2 Potential factors influencing partner preferences

When comparing the most favorite and least favorite partners with Wilcoxon ranked sign tests, we found that the only significant difference was in the amount of play the partner initiated, with favorite partners directing more play towards the focal rat than the least favorite partner (Table 3.3). Weight and dominance asymmetries did not influence preferences, nor did the time spent in social proximity or latency to first attack.

Wilcoxon signed-rank tests comparing the difference in how the focal rats who formed preferences responded to attacks by the most preferred and least preferred partners, and how these partners responded to the focal rats, revealed no significant differences (Table 4). Neither focal rats, nor their partners, differed significantly in the likelihood of defending themselves, using evasive defense tactics, ending in a pin configuration, performing role reversals, or in the symmetry of role reversals.

The temporal profile of attacks by focal rats on each of their partners over the test period revealed there were marked individual differences in the amount of play initiated by each of the eight focal rats (Figure 3.2), but high and low playing focal rats did not differ in the expression of their preferences. Moreover, when comparing the number of nape attacks initiated by focal rats that form preferences ($30.0 \pm 8.87$) against those that do not ($41.0 \pm 18.73$), a Mann-Whitney U test revealed that there was no difference in playful initiation ($z = -0.27$, $p = 0.79$).

**Table 3.4**. Comparisons from the most extreme differences—the most and least preferred partners—are presented below to compare various measures of their play behavior.

| Behavior | Most preferred (% ± SD) | Least preferred (% ± SD) | Wilcoxon Ranked Sign Test |
|---|---|---|---|
| % Defense | | | |
| Focal | 80.49 ± 13.13 | 61.67 ± 26.09 | $Z = 4$, $p = .35$ |
| Partner | 66.29 ± 29.59 | 63.75 ± 37.72 | $Z = 6$, $p = .72$ |
| % Evasion | | | |
| Focal | 42.60 ± 16.41 | 50.00 ± 50.00 | $Z = 9$, $p = .69$ |
| Partner | 59.39 ± 27.07 | 56.19 ± 34.94 | $Z = 4$, $p = .72$ |
| % Pin | | | |
| Focal | 33.49 ± 14.90 | 21.67 ± 21.73 | $Z = 1$, $p = .08$ |
| Partner | 26.35 ± 26.71 | 9.52 ± 14.68 | $Z = 2$, $p = .27$ |
| % Role reversal | | | |
| Focal | 29.19 ± 11.69 | 25.00 ± 43.30 | $Z = 5$, $p = .50$ |
| Partner | 49.44 ± 26.08 | 2.86 ± 6.39 | $Z = 0$, $p = .07$ |
| % Asymmetry in role reversals | 36.67 ± 36.36 | 79.36 ± 43.41 | $Z = 9$, $p = .14$ |

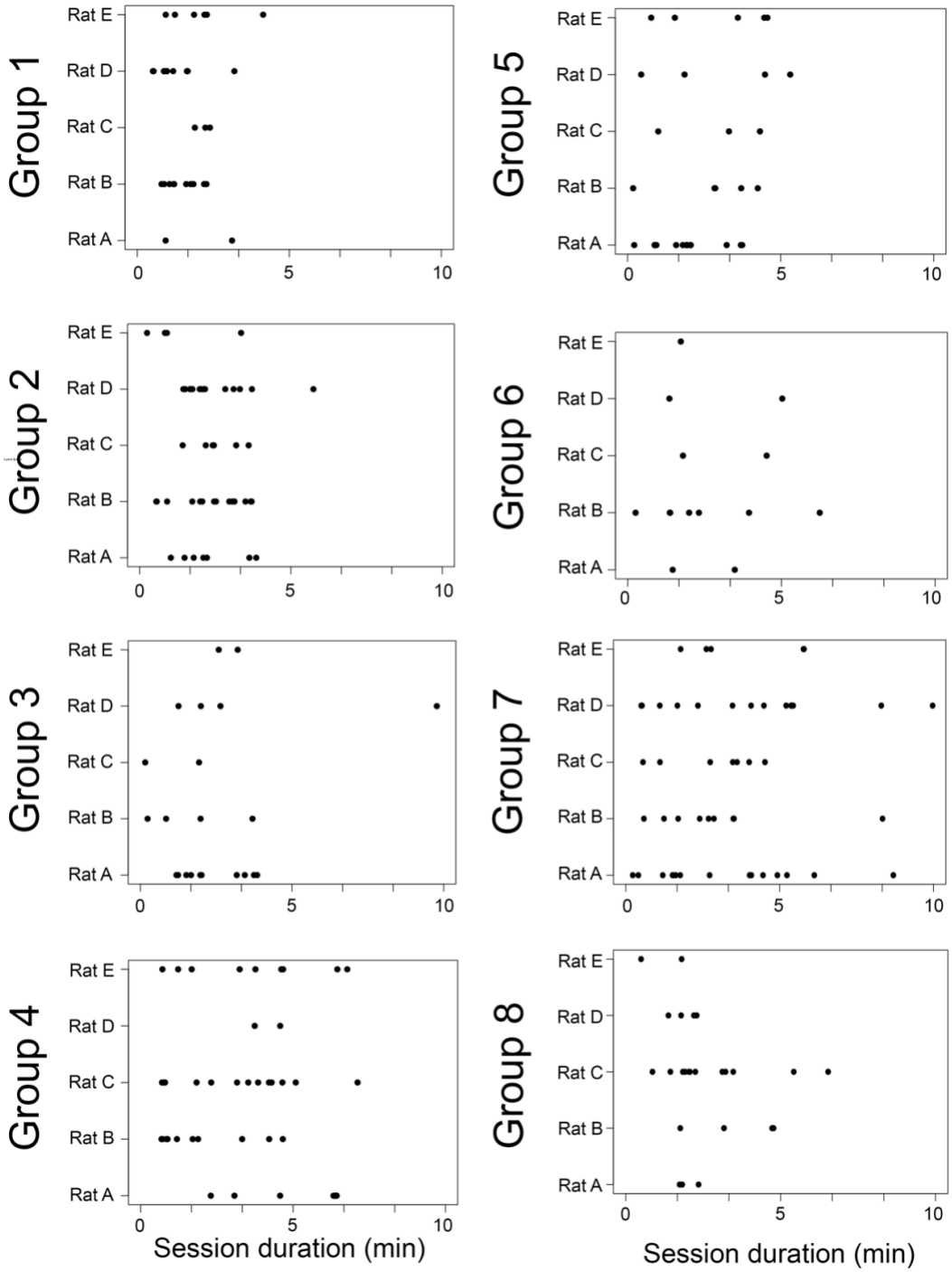


**Figure 3.2** The temporal distribution of playful attacks across the 10 min session is plotted, illustrating when the focal rat-initiated play with each of the five partners, for all eight focal rats from each group.

## 3.4 Discussion

Within groups of rats that are familiar with one another, data indicate that they have partner preferences (Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, & Ham, 2023), and when confronted with familiar and unfamiliar partners, rats have a preference to engage unfamiliar rats in play (Ham & Pellis, 2023). In the present study, even though the groups of six rats had never encountered one another before, five of the eight selected focal rats exhibited partner preferences (Figure 3.1), significantly directing more play to some members of the group than others (Table 3.2). Importantly, these preferences were not an artefact of playing with the nearest available partner. The overall amount of time spent in close proximity when not playing did not account for why one partner was preferred over another. Indeed, a focal rat would leave a rat it was next to and travel to a distant location to launch a playful attack on another rat. So, even though not all focal rats show significant partner preferences, in those that did, the choice involved was an active one.

Of the focal rats with preferences, the preferred partners also directed more playful attacks to the focal rats (Table 3.3). These findings are surprising as the groups consisted of same strain, same sex, same age strangers, and the results presented are based on them interacting for only 10 minutes, so providing little time and no obvious features for individuals to distinguish quickly between more preferred and less preferred play partners.

Despite five of the focal rats showing preferences for certain partners, three did not. This suggests that while some rats are more sensitive to the partners with which they play, others may be more gregarious and so less choosy about play partners. Though it is typical to socially isolate rats before testing play (Pellis, Pellis, Burke, et al., 2022), perhaps the time spent in isolation before testing influenced whether rats formed preferences. By isolating the rats, the individuals are known to have not played before a play session and

thus, should be motivated to play. However, this motivation to play may have influenced the results of this study. If the focal rats are highly motivated to play, due to the social isolation, it may mask preferences, as the rewards of playing may outweigh the motivation to find a suitable play partner. Indeed, even among the focal rats that formed preferences, it remains unknown if these preferences would be even stronger had they not been isolated. Differences in isolation time and its' influence on partner preferences should be investigated in the future.

Nonetheless, given that partner preferences can be established in such situations by some rats, this has a couple of major implications. First, it provides evidence that forming partner preferences may be an important means by which young rats establish a social milieu in whatever context they find themselves that give them the experiences they find most rewarding. That is, they create their own social niche (West & King, 1987). Second, it could explain why there are typically such large variances in scores of play in the dyadic test, even when same sex, same condition rats from inbred strains of rats are used (Achterberg et al., 2023). The random pairing of rats, which are artificially selected by experimenters, may contain preferred and non-preferred partners in the dyads, resulting in uncontrolled influences on both how much and how rats play. What remains to be determined is how rats confronted with multiple unfamiliar partners can so quickly identify which members of the group are preferred.

We assessed some intuitively obvious factors involved in partner selection for the focal rats that formed preferences. First, within established groups of juvenile male rats, the rats that become overtly identifiable as being dominant after sexual maturity tend to receive more playful attacks from the rats that when sexually mature exhibit clear signs of being subordinate, and this tends to be correlated with weight as the to-be dominants, on

average, tend to weigh more (Pellis & Pellis, 1991). Similarly, in trials in which the focal animal could choose between familiar and unfamiliar partners with whom to play, for strangers, preference was given to those which were less dominant and lighter (Ham & Pellis, 2023). That is, in some situations, the dominance of the potential partner, even among strangers (L. K. Smith et al., 1999), can influence whether rats play with them and how they play with them. Again, surprisingly, when all potential partners were strangers, differential dominance relationships, as measured by the tube test, or differences in body weight, seemed to have no influence on which partners became the most and the least preferred play partners.

Second, there are individual differences in how playful rats are, with some consistently launching more nape attacks than others (Achterberg et al., 2023; Lampe et al., 2019; Lesscher et al., 2021), with pairs of high playing versus low playing rats tending to differ in some of the play tactics they predominantly use (Pellis, Pellis, Ham, et al., 2023). Therefore, we predicted that individuals within groups of unfamiliar rats would likely gravitate to rats that play similarly. Focal rats did differ in how much play they initiated (Figure 3.2), but this had no impact on whether partner preferences were formed. In addition to the amount of play initiated, the style and quality of play was evaluated. Neither the focal rats' play style nor the partner's play style significantly influenced the selection of partners. Additionally, the quality of play, as measured by role reversals, play symmetry, and symmetry in role reversals, did not differ between most and least favorite partners.

Over the course of the 10-minute trial, the focal rat continued to interact sporadically with all five potential partners (Figure 3.2) rather than sampling each partner in the first minute and honing in on the preferred individual(s). Therefore, preferences could only be determined from the cumulative effect of counting all the nape attacks over

the entire trial. The same pattern was also seen in the experiment on partner choice when confronted with rats of varying familiarity (Ham & Pellis, 2023). This suggests that, although some partners are preferred, that preference is not exclusive, with even the least preferred partner occasionally being attacked, even towards the end of the trial. Clearly, there is still much to learn about the cues that rats use in choosing which potential partners are preferred—if they form partner preferences—and why they continue to play with all partners, even the least preferred ones when preferred partners are available.

While animals often prefer to play with familiar individuals (Antonevich et al., 2020; Drea et al., 1996; Pfeifer, 1980), and this includes humans (Blurton Jones, 1976; P. K. Smith, 2010), juvenile male rats generally prefer to play with individuals that are novel, but not completely novel (Ham & Pellis, 2023). Indeed, rats also choose to interact with novel partners over familiar partners in non-playful contexts (Hackenberg et al., 2021). Although the salient feature varies from species to species, several factors have been identified as influencing partner preferences in social play. Typically, animals prefer to play with individuals of similar age and of the same sex (Biben, 1998; Shimada & Sueur, 2018). However, for some animals, these preferences change with age (Ham et al., 2022; Lilley et al., 2020) or depending on the type of social play performed (Ham, Lilley, et al., 2023). In addition to preferring animals of the same sex and of a similar age, relative dominance status can also influence play partner preferences, with animals tending to prefer playing with partners of a similar rank (Biben, 1986; Ham & Pellis, 2023; Lutz et al., 2019), although in some situations, rats do the opposite (Pellis & Pellis, 1991). As partners of varying age and sex may provide different experiences (i.e., an older partner, who is more skilled, maybe a more challenging play partner), understanding which partners are

preferred, for a given species, and how these preferences are formed is important as they may reflect differences in the play experiences gained by juveniles.

For rats, it is still unclear what makes a partner 'desirable' and why some rats form preferences and others do not. However, partner choice is context dependent. For example, if preferences are only assessed through dyadic encounters, whereby you allow a focal animal to play with an unfamiliar one day and the next with a familiar animal, you are confounding your results as the rat is only afforded one choice in any given trial. That is, if the rat is motivated to play, it may inflate how much it plays with the partner if that is the only option. Additionally, the partner may alter how much the focal animal plays, with high-playing rats decreasing the amount of play they initiate if they are partnered with a high-playing partner (Achterberg et al., 2023). However, it is still unknown if individuals prefer to play with complementary players (i.e., high players preferring high players, and low players preferring low players) (Lampe et al., 2019). Despite this inflation, dyadic encounters have found that individuals tend to play more with unfamiliar than with familiar animals; however, when rats are given the choice between familiar animals, somewhat familiar animals, and unfamiliar animals, they prefer somewhat familiar animals over strangers and familiar animals, but strangers over familiar animals (Ham & Pellis, 2023).

Comparing the results of this study to a previous study from our lab (Ham & Pellis, 2023) shows yet another example of how preferences and the mechanisms by which they are determined are context dependent. Although the rats had a choice between three individuals of varying familiarity, focal rat partner selection was influenced by relative weight and dominance asymmetry, at least when playing with strangers. However, focal rats in groups of unfamiliar animals are not influenced by differences in relative weight and dominance (present study). Although, it should be noted than in both studies dominance

was measured using the tube test, which may not accurately reflect dominance and instead indicate differences in behavioral strategies. Consequently, the role of dominance will need further testing to be certain in how it may or may not influence play partner preference. Even so, the contrasting findings from these two studies suggest that the salient information used when making decisions on choice of play partners may change when the context changes.

Social play has been linked to the development of socio-cognitive skills in rats and hamsters (Burleson et al., 2016; Ham, Szabo, et al., 2024; Marquardt et al., 2023; Stark & Pellis, 2020) and temperament refinement and reproductive success in ground squirrels (Hurst-Hopf et al., 2023; Nunes, 2014; Shehan et al., 2023). Indeed, the quality of juvenile play experiences in rats significantly alters the development of the medial prefrontal cortex and social skills. It is not the amount of play but instead, the turn-taking or role reversals, and especially the degree of symmetry between partners in such turn-taking, that seem to be driving the brain and behavioral changes (Ham, Szabo, et al., 2024; Stark et al., 2021; Stark & Pellis, 2020, 2021). The focal rats of this study did not have more symmetrical play relationships with preferred partners compared to unpreferred partners. As play is used to familiarize unfamiliar individuals (e.g., Antonacci et al., 2010; Pellis & Iwaniuk, 1999a; Stark & Pellis, 2020, 2021), maintaining relationships with all members of the group may ensure that focal rats familiarize themselves with all individuals equally. An alternative explanation for why some rats do not form preferences, and why even the rats that do continue to play with all members of the groups may be that playing with multiple partners is rewarding and at the same time, it might be beneficial. By playing with a variety of partners, they are maximizing the variation of social experiences they gain. This might serve as a benefit to the orbitofrontal cortex which changes based on the number and

novelty of partners (Bell et al., 2010; B. T. Himmler, Pellis, & Kolb, 2013). Given that there is significant individual variation in play style and frequency, when given a choice, the present findings raise the possibility that rats switch from preferred to less preferred partners to maximize the benefits gained from playing.

Although males and female juvenile rats play using the same behavioral patterns (Pellis, Pellis, Burke, et al., 2022), and gain some of the same benefits from playing in the juvenile period (Pellis, Pellis, Ham, et al., 2023), females also gain some unique benefits (Marquardt et al., 2023). Consequently, studies of play partner choice in group settings should be replicated with females, as their preferred social niche may differ to that of males, and so highlight different influences involved in partner preferences, if such preferences exist.

### 3.5 Conclusions

Our results demonstrate that some juvenile male rats express partner preferences when playing in a group of unfamiliar individuals while others do not. Despite some rats forming preferences, focal rats continued to play with all the available partners throughout the 10-min play session. The focal rat's favorite partner was the partner that in turn directed the most play towards the focal rat, suggesting that being able to form mutual relationships is an important criterion for forming preferences. However, none of the other possible mechanisms (e.g., weight and dominance asymmetry, play style, play quality) that we assessed account for how these preferences were formed. Despite living in groups in the wild (Schweinfurth, 2020), the study of group play and partner preferences is an understudied feature of play in rats, despite laboratory rats serving as a major model of play and its associated neurobiology (Achterberg & Vanderschuren, 2023). Group play should be employed more regularly in studying rat play as it may provide new insights into the

mechanisms of social cognition, development, and individual variation (Pellis, Pellis, Ham, et al., 2022).

# CHAPTER 4: GROUPS OF FAMILIAR MALE RATS FORM UNSTABLE PARTNER PREFERENCES WHEN PLAY FIGHTING DURING THE JUVENILE PERIOD*

## 4.1 Introduction

Play fighting or rough-and-tumble play is one of the most commonly reported forms of social play in mammals, especially among juveniles (Burghardt, 2005; Fagen, 1981; Pellis & Pellis, 1998). Play fighting involves competition to gain a species-specific advantage, which for many species involves contacting a particular body target, but is moderated by some cooperation (Aldis, 1975; Pellis et al., 2024), leading to a degree of reciprocity or turn-taking that does not occur in serious fighting (Palagi, Cordoni, et al., 2016; Pellis & Pellis, 2017). In laboratory rats (*Rattus norvegicus*), a species in which play fighting has been studied most intensively (Achterberg & Vanderschuren, 2023; Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2023; J. W. VanRyzin et al., 2020), the animals compete to gain access to the nape of their partner's neck which is nuzzled with the snout if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987).

Rats are highly motivated to play (Trezza et al., 2010; Vanderschuren, 2010), and while most frequent in the juvenile period, such play continues into adulthood (Pellis & Pellis, 1990; Thor & Holloway, 1984c). Because of this propensity to engage in play fighting spontaneously, studies of rats have been instrumental in characterizing the neural circuits and the neurochemical systems involved (e.g., Achterberg & Vanderschuren, 2023; Siviy, 2016; Vanderschuren et al., 2016; VanRyzin et al., 2020). In addition, the influence

of play fighting on the development of socio-cognitive skills and the anatomy and physiology of the prefrontal cortex has been extensively studied in rats (e.g., Bijlsma et al., 2022, 2023, 2024; Ham et al., 2024; Marquardt et al., 2023; Stark et al., 2023; Vanderschuren & Trezza, 2014).

While rats are highly motivated to play, individuals show a considerable degree of variation in their play behavior both in the amount of play they initiate and in their style of playing (Achterberg et al., 2023; Ham & Pellis, 2023; Lampe et al., 2017; Lesscher et al., 2021; Melotti et al., 2014; Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2022; Poole & Fish, 1976). For example, based on how much play they initiate, some rats can be classified as 'high players' and some as 'low players'(Achterberg et al., 2023; Lesscher et al., 2021). Moreover, pairs of high and low players tend to prefer using different tactics to defend their nape, leading to different styles of play fighting (Pellis, Pellis, Burke, et al., 2022).

For species in which multiple potential play partners are available, not all are equally preferred. In many cases, preferred partners are often age-matched (Cheney, 1978; Ham, Lilley, et al., 2023; Shimada & Sueur, 2014, 2018; Turner et al., 2020), same sex (Biben, 1998; Cheney, 1978; Nunes et al., 2015; Nunes, Muecke, Sanchez, et al., 2004; Thompson, 1996), close relatives (Cappiello et al., 2018; Thompson, 1996), and of similar dominance rank (Biben, 1986; Lutz et al., 2019). These factors affecting play partner preferences may change with age (e.g., preferring older partners when young, but younger partners when older) and/or the type of social play performed (e.g., sexual play versus rough-and-tumble play) (Biben, 1998; Ham et al., 2022; Ham, Lilley, et al., 2023; Lilley et al., 2020; Meaney & Stewart, 1981; Pellis & Pellis, 1991).

Rats are highly social, living in large colonies (Schweinfurth, 2020), with synchronized births from multiple females (Calhoun, 1963). With the average litter size ranging from four to sixteen pups (Schweinfurth, 2020), juvenile rats have many potential partners with whom to play. Despite this, little is known about potential partner preferences when playing (Ham & Pellis, 2023; Lampe et al., 2019). As play fighting is typically dyadic, most research on play in rats has been tested using pairs of rats (Pellis, Pellis, Ham, et al., 2023). But given the diversity in playfulness among juvenile rats, not all peers may be equally attractive play partners when available in a group context. For example, a high playing rat might prefer to play with a high playing rat, or rats may prefer to play with individuals that complement their own play style. Thus, when we select pairs for testing in dyads, we could be constraining rats to play with incompatible partners. That is, if the rats were given a choice, there may be partners with whom they would not play in a group.

Given the individual variation in play, and that rats form preferences with partners with whom they associate in various non-playful social contexts (Calhoun, 1963; Hakataya et al., 2023; Mauri et al., 2022; Proops et al., 2021; Schweinfurth et al., 2017), in prior work we investigated whether juvenile male rats form partner preferences when playing in groups (Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2023). In the first study, focal rats had a choice of a cage mate, a neighbor, from a cage in which he could be seen, smelt, and heard, but not played with, and a stranger which he had never seen before. Even though the focal rats initiated play with all test subjects, they had a clear preference for the less familiar partners over cage mates (Ham & Pellis, 2023). But what if all the potential partners in the test cage are strangers, will some be preferred over others? This led to the second experiment in which groups of six, completely unfamiliar partners were tested together. We found that, even among strangers, some rats form play partner preferences

over the course of a 20 min play trial (Ham & Pellis, 2024). Together, these findings suggest that, while less familiar rats are preferred, not all unfamiliar rats are equally valued as play partners. In a third line of experiments, we investigated whether rats have preferences within groups of familiar, co-habiting, animals. As preliminary data suggested that they did, with rats leaving the vicinity of the closest rat to traverse the test cage to launch a playful attack on a distant, preferred partner (Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2023), in the present study we further explored play partner preferences in groups of familiar rats.

We investigated play in groups of six, familiar juvenile male rats, to verify whether co-habiting rats form play partner preferences. Moreover, as individual rats tend to be consistently high or low players with associated preferences in style of play (Achterberg et al., 2023; Ham & Pellis, 2023; Lesscher et al., 2021), we predicted that play partner preferences should remain stable over the juvenile period. The reason for this prediction is that we hypothesized that rats select partners that most closely complement their individual play styles. Consequently, regarding mechanisms for partner selection, we predicted that the same individuals should remain as the most preferred partners and central to the play initiation network (as calculated by the animals' eigenvector centrality score). Similarly, as turn taking and symmetry seems to be what influences the development of socio-cognitive skills in rats (Ham, Szabo, et al., 2024; Stark et al., 2023), we predicted that rats would prefer to play with partners that engaged in more turn taking and with whom they had more symmetrical play relationships, thus maximizing the occurrence of these experiences. Indeed, this might even lead to sub-communities forming within the group with complementary play partners playing more with one another. Therefore, play trials were run over eight consecutive days during the peak juvenile play period, sampling multiple

days during this eight-day period (Figure 4.1). Partner preference predictions were tested using social network analysis methods (Ham et al., 2022; Lilley et al., 2020; Pellis, Pellis, Burke, et al., 2022).

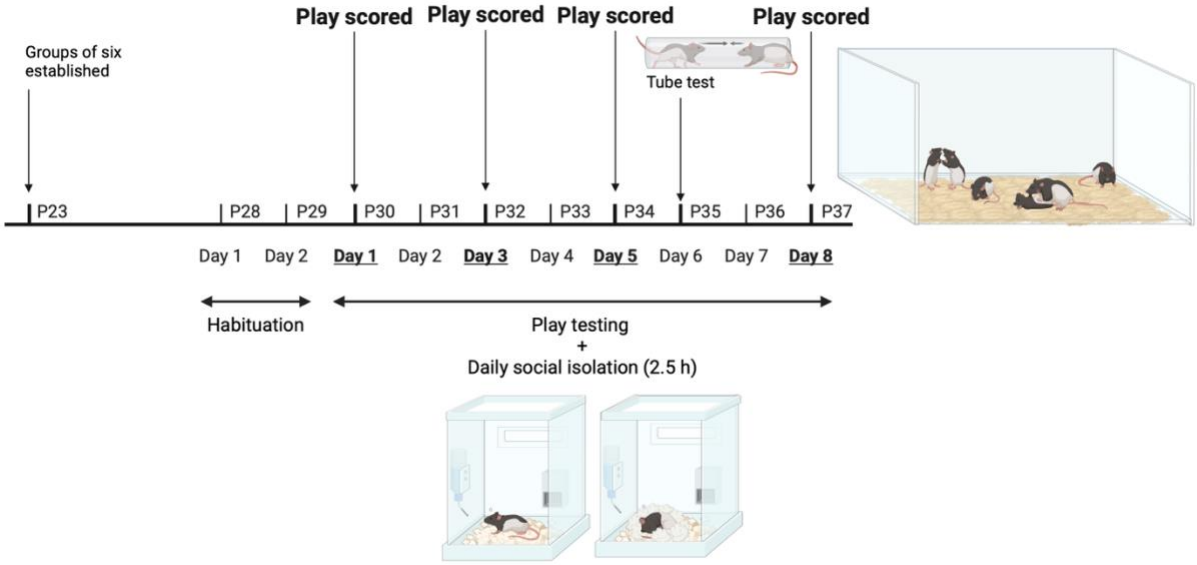


**Figure 4.1** The timeline of when the groups were established, habituated, and tested. Note that the rats were isolated and tested each day, but only days 1, 3, 5, and 8 were scored. The age of the rats, in postnatal days (P), is listed beside each hash in the timeline. Created with BioRender.com.

## 4.2 Methods

### 4.2.1 Animals

We purchased 48 Long Evans male weanlings from Charles River Laboratories (Kingston, NY, USA) which arrived at the Canadian Centre for Behavioural Neuroscience at 22 days of age. On arrival, we housed the rats in Tecniplast® GR1800 double decker cages in groups of 6, resulting in a total of 8 groups. The floor was covered in corncob bedding and food and water were available *ad libitum*. The rats were housed on a 12-hour light-dark cycle (lights on at 7:30 a.m.) and maintained at a constant temperature of 21°C-23°C and humidity. All care and testing procedures were reviewed and approved by the University of Lethbridge Animal Welfare Committee (protocol #1809) in compliance with guidelines from the Canadian Council for Animal Care.

While studying play in groups of rats is still in its infancy, we chose to form groups of six as this would allow for a great deal of selection and variation, while still being manageable to score. By constructing groups of six, the rats could choose to play with five different individuals, and even if a pair was already playing, rendering them unavailable, an individual still had the choice between three other animals.

### 4.2.2 Apparatus

We tested the juvenile male rats in a large Plexiglass® enclosure (80 cm x 80 cm x 50 cm). Corncob bedding was used to cover the floor of the enclosure sufficiently. We used an ExmourRS 4K Sony Handycam for video recording the play sessions. We placed the camera at a 90° angle above the enclosure.

### 4.2.3 Procedure

Before play testing began, starting at 28 days of age, we habituated the rats to the test enclosure for 10 min per day, with their cage mates, in red light, for two, consecutive

days (Figure 1). At 30 days of age, testing began. Prior to the play sessions, we socially isolated the rats (food and water was available *ad libitum*) for 2.5 h to increase their playfulness (Achterberg et al., 2023), and the groups of six cage mates were placed in the enclosure, in red light, for 20 min and filmed. We repeated this testing procedure over eight consecutive days, over the peak play period (Panksepp, 1981; Pellis, Pellis, Burke, et al., 2022). Indeed, this is the period when the play influences both behavior and brain development (Pellis, Pellis, Ham, et al., 2023). Between each trial, we cleared the cage with Virkon® and we replaced the bedding. To identify the individuals within the group, we drew tail patterns on each rat with a permanent marker pen (Sharpie®).

### 4.2.4 Behavioral analysis

Days 1, 3, 5, and 8 were analyzed and compared. The trials on days 2, 4, 6 and 7 were run in case of mishap in the targeted trials (such as camera malfunction). As all targeted trials were successful, only those were scored and compared across all eight groups. Alternating days were selected so we could sample throughout the peak juvenile play fighting period, while still determining whether rats form preferences and, if they did, whether those preferences remain stable over the peak play period. We analyzed the 20 min video recordings using both normal speed and frame-by-frame analysis to score the microstructure of the rat play (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022). We scored each video six times, following one focal rat at a time, noting the attacks initiated and directed toward each of the individuals within the group. A playful attack was scored when the snout of a rat made contact with the nape of another rat, as this is the target in rat rough-and-tumble play in around 90% of playful attacks (B. T. Himmler, Pellis, & Pellis, 2013). In addition, if the playful attack was launched but was evaded before contact could be made, this too was scored as a playful attack.

A rat defending itself in play can either defend its nape or ignore the attack and continue with its ongoing behavior (e.g., grooming, exploring, playing with another partner). In this case, the response to an attack was scored as 'no response.' If the attacked rat defended itself, it could do so by either evading its attacker (e.g., swerve, jump or run away) or by engaging in a facing defense in which the defending rat pivots to face its attacker. Once facing its attacker, the playful defense could result in wrestling on the ground if the defender rolled onto its back and was pinned there by the attacker, or in an upright 'boxing' position if the defender remained standing on its hind feet. In addition, we measured the number of role reversals (i.e., when the defender launches a successful counterattack and becomes the attacker) (Pellis, Pellis, Burke, et al., 2022). Measuring the number of role reversals provides a gauge on the quality of play as turn taking is a fundamental aspect of keeping play cooperative and reciprocal (Palagi, Cordoni, et al., 2016; Pellis et al., 2005).

Another marker of reciprocity, and so the quality of play, is the degree of symmetry in the playful actions of the partners (Cordoni et al., 2016, 2022; Llamazares-Martín et al., 2017; Nolfo et al., 2021; Stark et al., 2021). To assess the degree of symmetry in playful attacks, we subtracted the number of attacks by one partner from those by the other, and the absolute difference was divided by the sum of the nape attacks by both partners (see equation below). This value is subtracted from 1, with values closer to 1 indicating a high degree of symmetry, while values closer to 0 indicate a high degree of asymmetry (Pellis, Pellis, Burke, et al., 2022).

$$\text{Symmetry} = 1 - \left( \frac{|(\text{Rat A playful attacks} \rightarrow \text{Rat B}) - (\text{Rat B playful attacks} \rightarrow \text{Rat A})|}{\sum \text{Rat A} \rightleftharpoons \text{Rat B play}} \right)$$

To assess the relative dominance among animals, we employed the tube test (Fan et al., 2019; Fulenwider et al., 2021). Although the animals were juveniles, at this age, male rats start forming dominance relationships which can affect how they play with one another (Panksepp et al., 1985; Pellis & Pellis, 1991). We ran the tube test at 35 days of age using a Plexiglas® tube that was just large enough to allow a rat through, but not so large that the rat could turn around or a second rat could squeeze by (19.5 cm in length and 4.5 cm in diameter). The tube test compared each possible pairing in the group of six. Between testing each pair, the tube was cleaned with Virkon®. For each pair, a 'winner' and a 'loser' was designated based on who out of the pair stayed in the tube. In other words, the winner pushes the loser completely out of the tube (i.e., all four paws of the 'loser' outside of the tube). The winning rat was given a score of one. If both rats remained in the tube for 60 sec, this was considered a tie, and no point was awarded. Every pairing was tested consecutively five times. We used the sum of wins from each trial to rank the rats from most dominant to least, with the most dominant rat having the highest score.

Given that rats typically play in pairs, even if multiple individuals are present, a factor influencing partner preferences could be partner availability. That is, rats might not have the choice of playing with a preferred partner, but instead whichever rat is available. To assess if partner availability influenced partner selection, we calculated how many nape attacks occurred when two or more partners were available. Additionally, we calculated how many nape attacks occurred when all five partners were available.

One possible explanation for partner preferences is social proximity, in which rats play with whoever is closest and so most convenient to play with. Alternatively, rats may seek out a particular partner with whom to play within the box. As such, we measured the time the rats spent in close social proximity. Time spent in social proximity was measured

if they were within one body length of each other (Ham & Pellis, 2023, 2024). As some of the time spent in close proximity was due to the animals playing with one another, we subtracted the time the individuals spent playing with each other from the total time they were in proximity to each other.

### 4.2.5 Statistical analysis

To visualize partner preferences, we constructed social networks using the *igraph* package (Csardi & Nepusz, 2006) in R Studio (R Core Team, 2020). We plotted directed social networks, with the size of the nodes or vectors representing the total amount of play (frequency) each rat initiated. The thickness of the edges or lines connecting the nodes represents the proportion of nape attacks each rat directs towards its partner. The proportion of play, and not the frequency, was plotted so that comparisons of preference strength could be made despite there being a considerable degree of variation in the amount of play each rat initiated.

We evaluated the social networks of each group, for each day, for randomness using a Mantel test (Mantel, 1967) in R with the *ade4* package (Bougeard & Dray, 2018). To do so, we constructed 'real' data matrices using the proportion of play directed toward each of the five potential partners on each day. We used the proportion of play to account for variation in play frequency among individuals. We then created a 'hypothetical' matrix consisting of the expected values if the rats played with each partner equally. If the distribution of play among partners was random, the 'real' and 'hypothetical' matrices should be correlated. Both matrices were transformed into distance matrices. We performed 9999 permutations to generate a null distribution and assess the observed correlation between the real and hypothetical datasets. If, on a given day, a group's proportional play matrix was correlated with the hypothetical data, those days were excluded from further

partner preference analyses (i.e., preference index scores, generalized linear mixed model comparisons).

When the two matrices differed significantly (Mantel test), we calculated a preference index score for each possible dyad using Thompson's calculation (Thompson, 1996) (see below). The index scores range from 0 to infinity and indicate the strength of preference for each of the potential play partners within the group (Thompson, 1996).

$$I_{ij} = \frac{B_{ij}}{B_i / k - 1}$$

Values greater than 1 indicate more play interactions than expected by chance, while those equal to or above 2 indicate a strong preference (Thompson, 1996). Scores equal to or below 0.25 indicate fewer interactions than expected by chance, and so likely avoidance of those individuals. The formula, where $k$ is the number of rats in the group (6), $B_{ij}$ is the number of nape attacks initiated by the $i^{th}$ rat with $j^{th}$ rat as the recipient (Thompson, 1996). $B_i$ is the total number of nape attacks initiated by the $i^{th}$ rat (Thompson, 1996). After calculating the preference index scores, their distribution was plotted using the package *ggpubr* (Kassambara, 2019).

To illustrate the strong partner preferences on any given day, as determined by play partner index scores (described above), directed social networks were created. Only strong positive and negative preferences are depicted in these networks (play index scores $\leq 0.25$ or $\geq 2$), with solid edges depicting a preferred partner and hashed edges indicating an unpreferred partner.

We constructed additional networks to assess if sub-groups or -communities were formed (Newman, 2006). When sub-groups are present, networks have non-zero modularity scores varying between -1 and +1. When sub-groups or communities are not

detected, or, in other words, when the distribution of edges are not different from what would be expected in a randomized network, the modularity values are equal to zero. When scores are equal to zero, this suggests that no sub-groups were formed. Where plotted, asterisks represent days when the associations between sub-groups were significant.

To calculate the stability of partner preferences, we calculated the degree of change in favorite and least favorite partner for each rat between days 1-3, 3-5, and 5-8. To calculate a change in rank, the rank on each day was subtracted from the prior day. For example, if Rat A's favorite partner on Day 3 (rank = 1) was his least favorite partner on Day 1 (rank = 5), this would result in a large change in rank (rank 5 – rank 1 = 4), this being the maximum change in rank. However, if the rat changes from playing with his favorite rat to his second favorite rat (rank 2 – rank 1 = 1) between Days 1 and 3, this would be a small rank change. If the rat's favorite partner remained the same, the rank change score would be 0 as there was no change. With this simple calculation, we assessed whether the preferences were stable between days and if not, to what degree they had changed. We plotted the distribution of changed values in R using *ggplot2* (Kassambara, 2019).

We used repeated measures ANOVAs with Bonferroni corrections to compare if certain rats within a given group were played with more often than others (i.e., if some rats are more popular than others). The overall preference by other group members for a given individual was tested by comparing the percentage of play directed to each rat in the group. Although certain individuals might be preferred across the four days, it could be that their popularity on any given day, but not all days, drives this effect. As such, if we found that certain individuals were significantly preferred across the four test days, a repeated measures ANOVA with a Bonferroni correction was used to test if the percentage of play

directed towards a preferred rat changed from day-to-day. In both cases, where plotted and when significant, asterisks are used to indicate significance (**$p < 0.01$, ***$p < 0.001$).

As play varies from individual to individual (Ham & Pellis, 2023; Lesscher et al., 2021), we predicted that some groups might also vary, with some being high playing while others were not. To visualize group variation, we plotted the total play observed in each group (i.e., the sum of all playful attacks by each of the six rats). We used a repeated measures ANOVA with a Bonferroni correction to compare whether certain groups consistently played more than others across the four days. To assess whether certain individuals within the group played more than others, we calculated *z*-scores for the total play initiated on any given day. In doing so, we assessed whether certain individuals consistently played above or below the mean. To visualize the *z*-scores, heatmaps were plotted.

Other factors, such as play quality and style, were modelled with a generalized linear mixed model (GLMM) using the "glmer" function from the *lme4* R package (Bates et al., 2015). More specifically, we tested whether variation in the frequency of playful attacks launched by the partner rat and directed toward the focal rat, play style (i.e., the percentage of responses to playful attacks, the percentage of attacks defended by evasion or rolling into a supine pin position), and play quality (i.e., percentage of role reversals) predicted partner preference. To model these aspects of play style and quality (predictor variables), the partner preference, using only the not preferred ($n$ = 27) and preferred partners ($n$ = 31) (as determined by the play partner preference index (Thompson, 1996), see Figure 3B for the inter-animal relationships tested), was set as the dependent variable. Given that play partners could either be "not preferred" or "preferred," a binary GLMM was used. We set the initiating rat's respective identification number as a random error term

to account for the repeated measures across days. Thus, we compared the frequency of play initiated by the partner and percentage of response, evasion, pin, and role reversals (predictor variables) between not preferred and preferred partners (dependent variable). A total of 58 lines of data were compared.

We also tested pair measures using a GLMM. In our second model, we tested whether play symmetry, relative differences in weight and dominance, and social proximity, were associated with our dependent variable (partner preference). As with the first model, we modelled only the play between not preferred ($n$ = 27) and preferred partners ($n$ = 31), which was set as the fixed effect, with the initiating and receiving rat's respective identification number set as a random error term to account for the repeated measures. A total of 58 lines of data were compared. For both models, Wald's confidence intervals are reported.

To correct for partner availability, the number of nape attacks launched when all partners were available was subtracted from the total number of nape attacks originally scored for significantly preferred and not preferred partners. If these partners were truly preferred or avoided, they should still be above or below the median despite not correcting the other, non-significant play relationships. We used sign tests to assess if preferred and non-preferred rats were attacked above or below the median, respectively, after correcting the number of nape attacks for availability.

All the figures generated, and statistical analyses reported, were done using R Studio.

## 4.3 Results

### 4.3.1 Partner preferences

Directed social networks, which plot the proportion of nape attacks initiated and received by all six members of the group, revealed that, on any given day, rats form partner preferences (Appendix A, Figure A1). These preferences are illustrated in the networks from two groups (Figure 4.2).

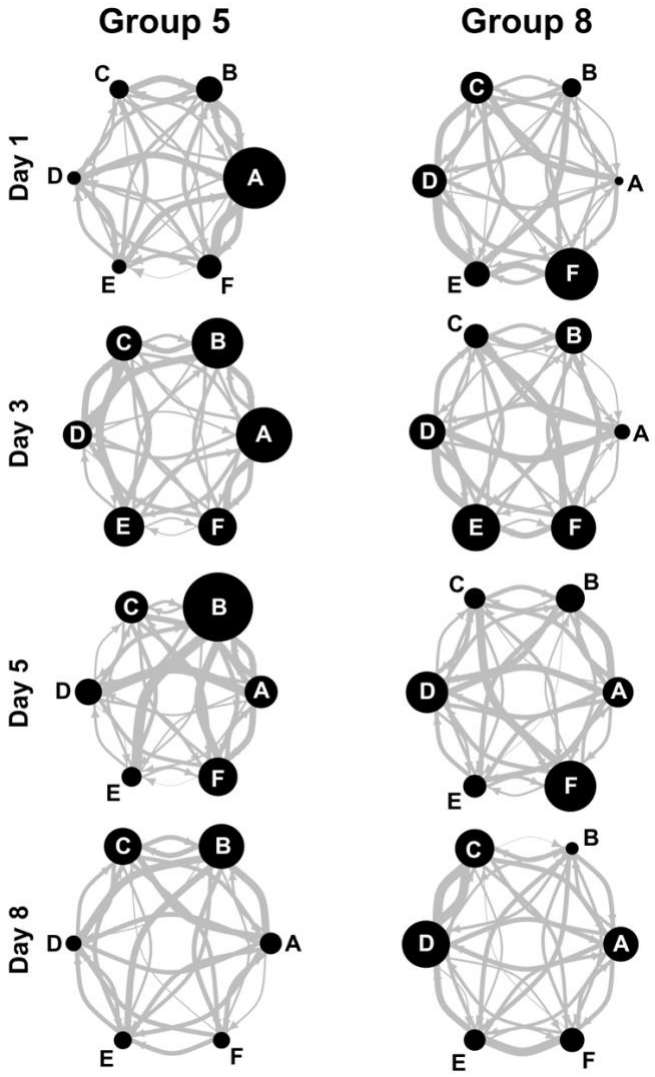


**Figure 4.2** Directed social networks are plotted illustrating the play preferences of two groups across the four test days. Each circle or node represents an individual in the group. The size of the node depicts the amount of play that individual initiated. The lines or edges connecting the nodes illustrates the proportion of play that individual directed towards the rats in the group. See also Appendix A, Figure A1.

In addition to the visualized networks (Figure 4.2; Appendix A, Figure A1), we used Mantel tests to compare the play proportion matrices from each group on each day with a hypothetical equal distribution matrix. If the model found that matrices were correlated, that would indicate that the play relationships were random, and so animals did not have preferences. In other words, individuals within the group were the target of playful attacks as often as would expect by chance. We found that only two of the matrices (Group 3, Day 3 and Group 5, Day 8) were correlated with the hypothetical matrix ($r = 0.55$, $p = 0.044$; $r = 0.49$, $p = 0.015$, respectively).

Using Thompson's play partner preference index calculation (Thompson, 1996), we calculated the strength of partner preferences for each possible dyad, in each group, for each day, except for those excluded by the Mantel test (see above). With scores greater than 1 indicating play preferences that are greater than chance and those greater than 2 being strong preferences for a particular play partner (Thompson, 1996), we found that there were 395 play relationships in which the preference scores were higher than expected by chance. Of the 395 play scores, 31 were equal to or greater than a score of two, suggesting some rats had strong preferences (Figure 4.3A). Conversely, we found that there were 27 relationships that had a score of 0.25 or less, suggesting that certain individuals within the group were not preferred as they were played with far less than expected by chance. These results are represented graphically in Figure 4.3B, showing only the strongly preferred and strongly not preferred relationships. These directed social networks illustrate that not all rats in the group express preferences and that who they prefer changes from day-to-day. Together, the results from the Mantel test and from the play partner preference calculations

(Thompson, 1996), suggest that the distribution of play is non-random, indicating that some rats are preferred or avoided when rats are playing in groups.

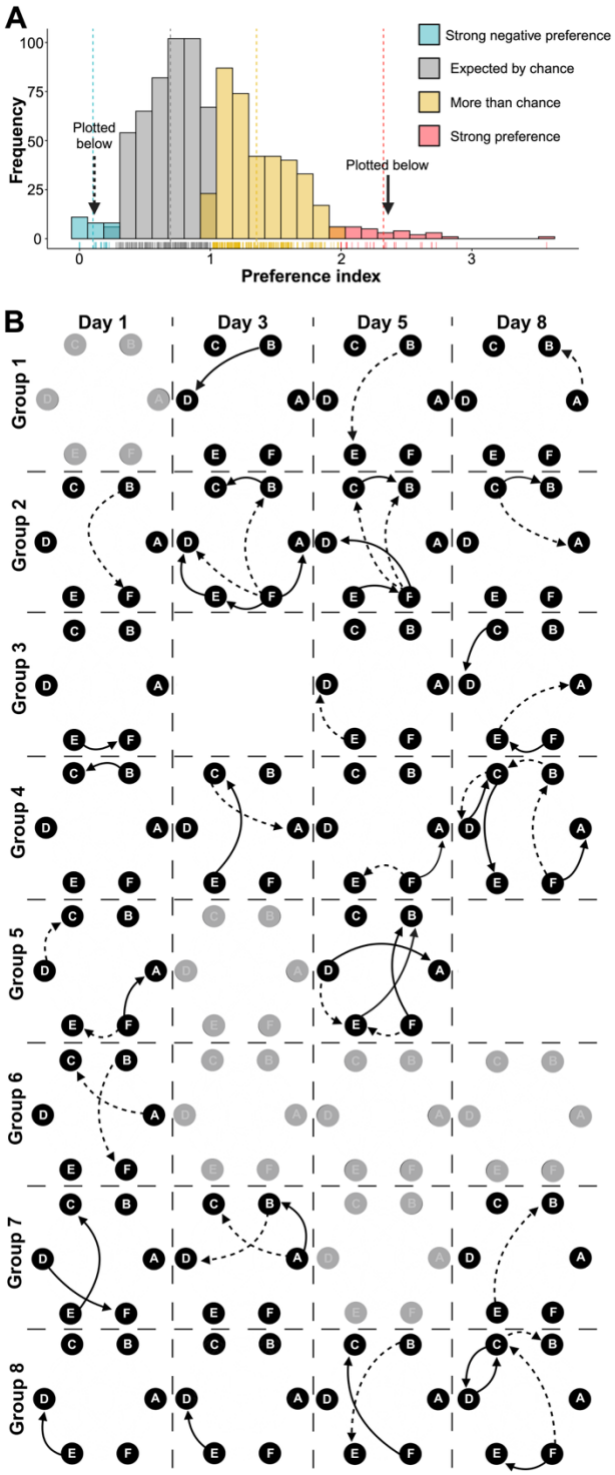


**Figure 4.3** The distribution of preference indices, or preference strength, for all possible combinations of initiator and recipient (A). Strong preferences are those that are ≥ 2, while weak preferences are ≤ 0.25 (A). Directed social networks are plotted illustrating the significant play preferences, of all eight groups across the four test days, except for Group 3, Day 3 and Group 5, Day 8 as we found the preferences on these days in these groups to be randomly distributed (B). Each node represents an individual in the group. The solid lines represent partners toward which playful attacks were launched significantly more than expected by chance and the dash lines the partners that received attacks significantly less than expected by chance.

Social network analysis revealed that only three of the eight groups formed sub-groups or communities (Figure 4.4). Of these three, sub-groups were only formed every day in two of the groups, and these sub-groups were not stable over the four days. Therefore, on most days, any partner preferences that were formed were assessed by factors specific to each day, not long-term group influences.

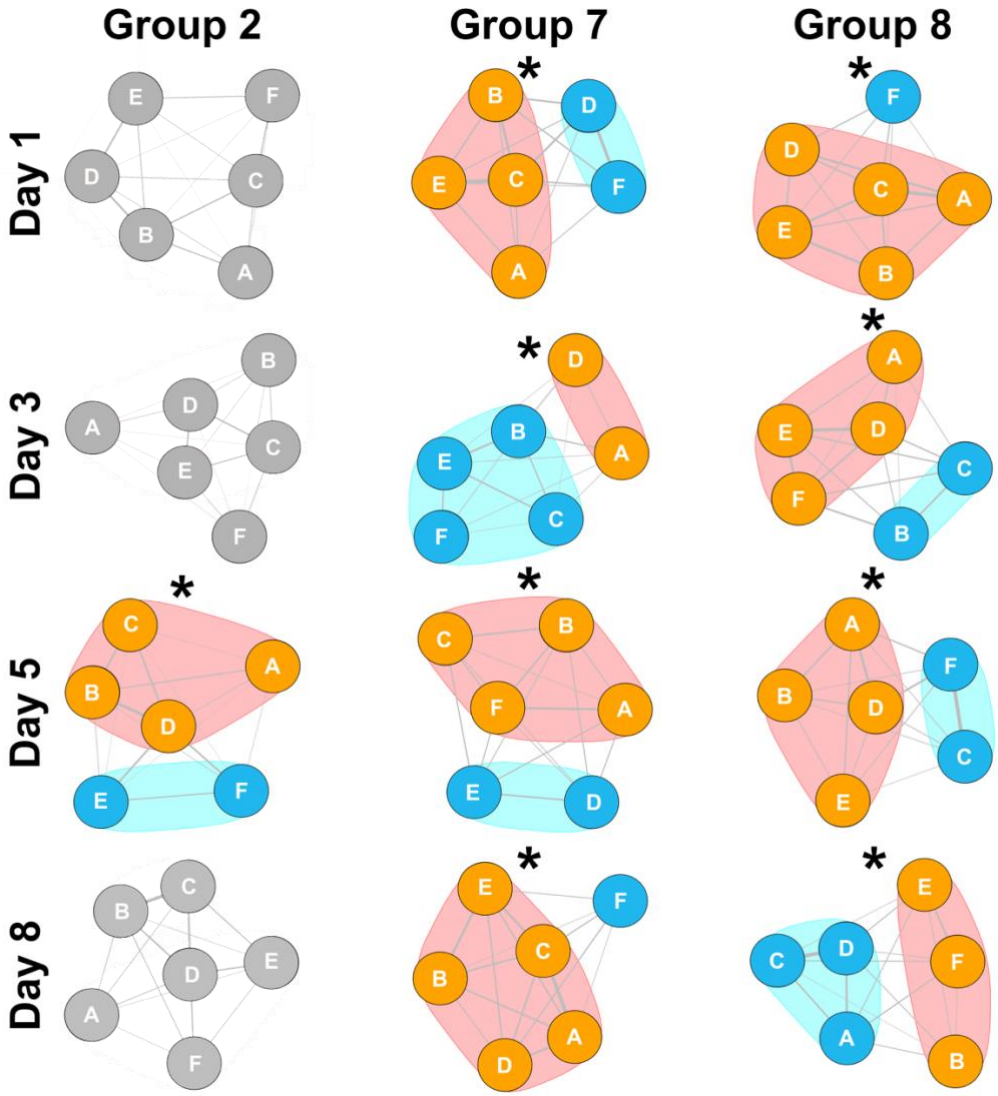


**Figure 4.4** Network modularity or sub-group formation is plotted using undirected social networks. The lines or edges connecting the nodes represent the amount of play (and not the proportion of play) directed between pairs of rats. Asterisks represent days when the associations between sub-groups were significant. The sub-group associations are illustrated through the use of polygons.

### 4.3.2 Partner preference stability

Although rats formed preferences on any given day, these preferences, on average, were not maintained. The degree of partner change reveals that, on average, rats do not prefer to play with the same individual from day-to-day. Their favorite partner on a test day was typically their second favorite or middle favorite partner the previous day (Figure 4.5, left panel). Similarly, their least favorite was their second least favorite or middle favorite partner the previous day (Figure 4.5, right panel).

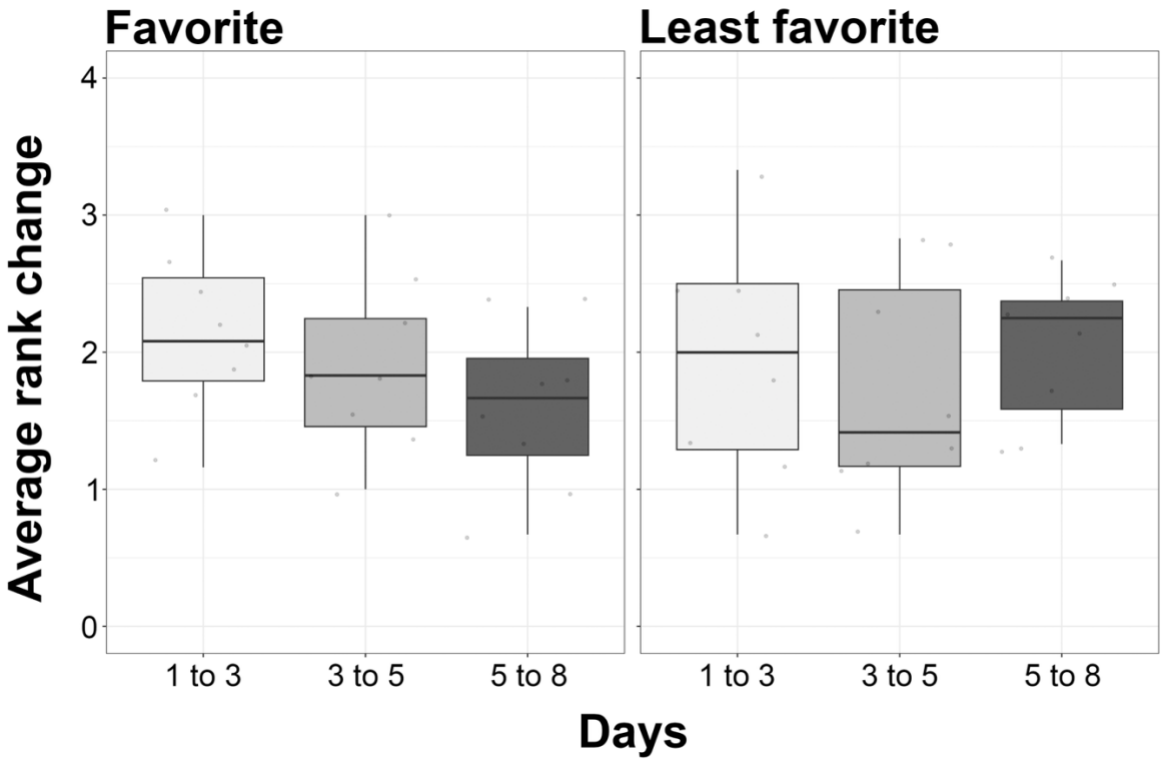


**Figure 4.5** The average change in rank, for each group, of the favorite (left) and least favorite (right) partner. The boxplots depict that between each of the test days, the preferences are unstable. A score of 0 would indicate no change in rank and partner stability, while a score of four would indicate a major change in rank and no partner stability. Data are represented as median +/- max and min.

### 4.3.3 Popularity

While some rats form partner preferences on any given day, repeated measures ANOVAs with a Bonferroni correction revealed that certain individuals in Group 2 ($F(5,95) = 3.06$, $p = 0.013$), Group 3 ($F(5,95) = 3.31$, $p = 0.008$), Group 5 ($F(5,95) = 6.53$, $p < 0.0001$), Group 6 ($F(5,95) = 4.48$, $p = 0.0009$), and Group 8 ($F(5,95) = 2.96$, $p = 0.02$) were consistently favored over others across the four test days (Figure 4.6).

Although certain individuals were preferred across the four days, it could be that their popularity on a given day, but not all days, drives this effect. To assess if certain individuals were equally popular each day, repeated measure ANOVAs with a Bonferroni correction were used to compare the individuals that were significantly preferred or avoided (Figure 4.6). The popularity of some rats fluctuated across testing days, whereas the popularity of other rats was stable. These models revealed that in Group 2, neither Rats A, C, D, nor Rat E changed from day-to-day. In Group 3, Rats D and E did not fluctuate in popularity from day-to-day. In Group 5, Rat F ($F(1,3) = 5.47$, $p = 0.01$), but not Rats A, B, C, E, changed from day-to-day. In Group 6, Rats C and D did not fluctuate from day-to-day, nor did Rats B and D in Group 8.

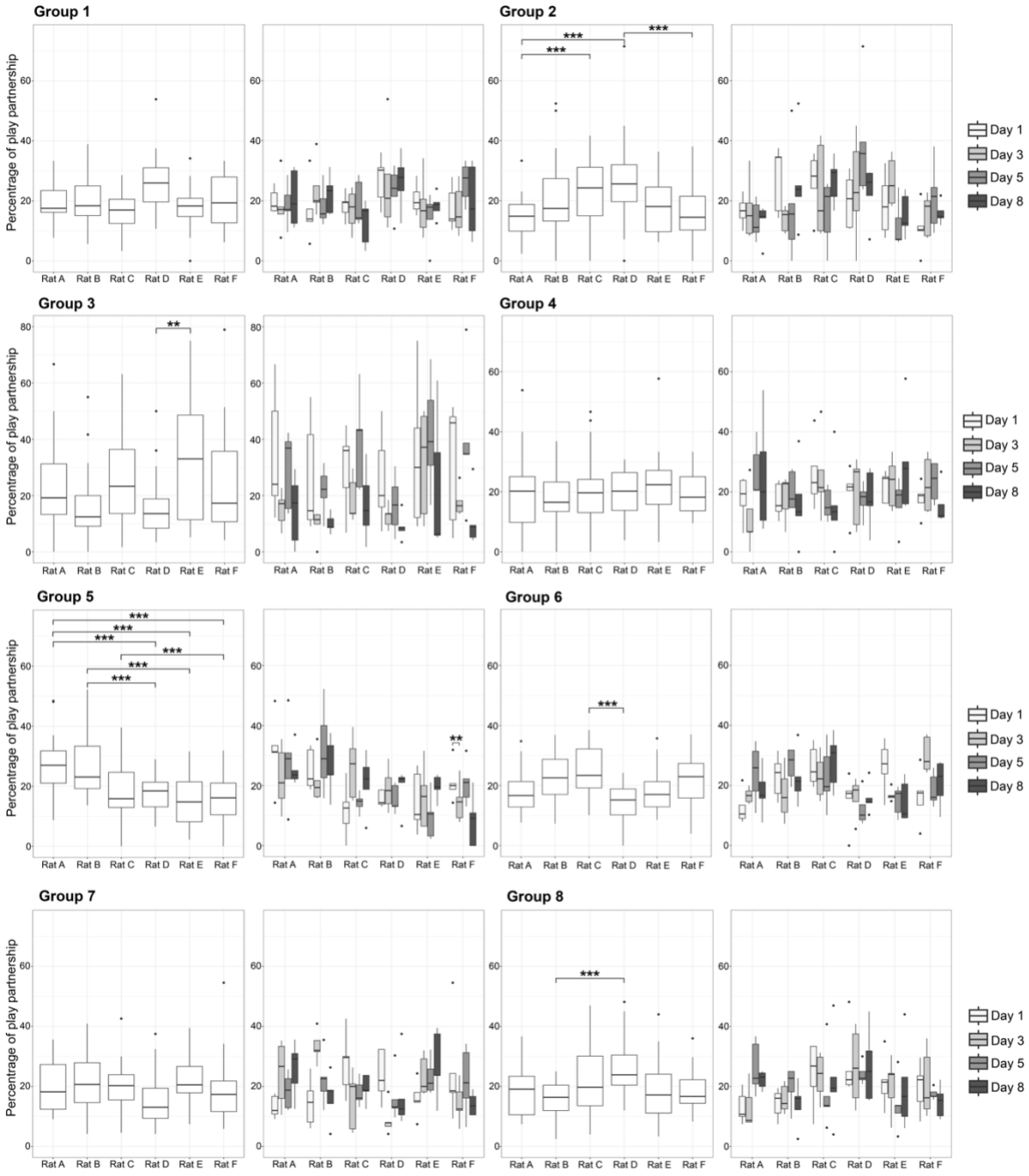


**Figure 4.6** The overall preference by other group members for a given individual is plotted for each group. For some groups, certain individuals are consistently, and significantly, preferred over others. For each individual in every group, the preferences are plotted for each day. To test if the individuals that were significantly preferred or avoided changed from day-to-day, we used a repeated measures ANOVA. Data are represented as median +/- max and min with outliers plotted as individual points. **$p < 0.01$, ***$p < 0.001$

### 4.3.4 Group and individual variation

When summed for the four days, we found that groups did not show different frequencies of play interactions ($F(7,21) = 1.67$, $p = 0.166$). That is, there was no clear evidence for some groups being 'high playing' and others 'low playing.' A day-to-day analysis (Figure 4.7) revealed that the pattern of play varied among groups, with some groups playing at relatively consistent levels on all days (e.g., Group 8) and others fluctuating daily (e.g., Group 4). Corresponding heat maps of attacks initiated by individual members of groups (Figure 4.7) show that while some animals consistently play above the expected mean for the group (e.g., Rat A, Group 4), many animals fluctuate, playing above the mean on certain days and below on others (e.g., Rat C, Group 4).

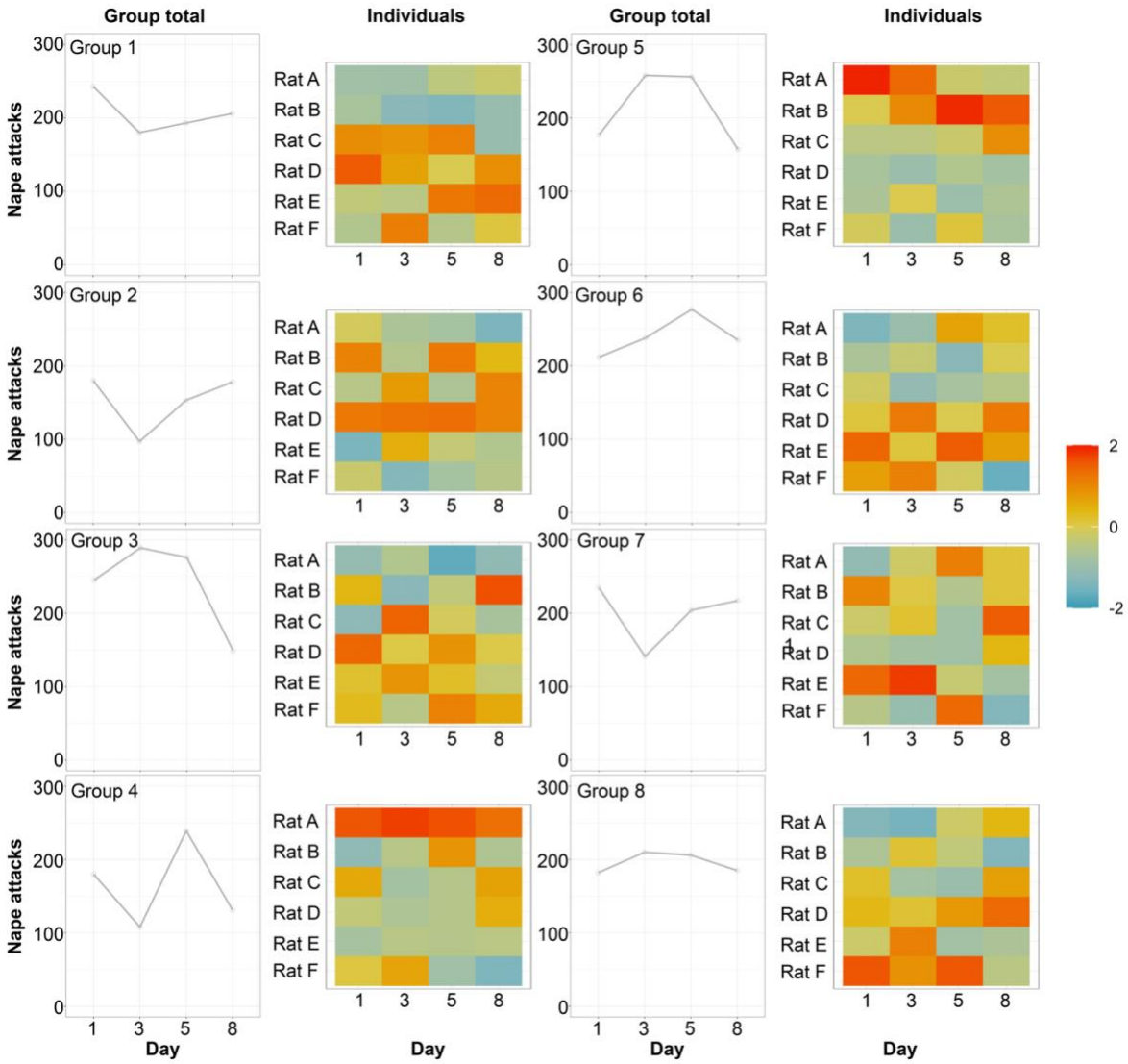


**Figure 4.7** The total number of nape attacks initiated by all group members is plotted for each of the four test days, for each group. Beside the total nape attack plots are heat maps illustrating whether individuals (y-axis) on a given day (x-axis), from their respective group, play above or below the mean of that day. Only deep red scores indicate individuals are playing significantly above the mean while only deep blue represents individuals playing significantly below the mean.

### 4.3.5 Potential mechanisms influencing preferences

#### *4.3.5.1 Play frequency, style and quality*

Given the degree of variation in play across individuals (see Figure 4.2), a possible factor making some individuals more attractive play partners is that they initiate more nape attacks. We found that the number of nape attacks launched by a partner and directed towards the focal rat did not predict whether a partner was the preferred versus the not preferred partner (Table 4.1), as determined by the play partner preference scores (Figure 4.3A) (Thompson, 1996) .

When we compared the percentage of defensive tactics used by preferred partners and non-preferred partners, we found that preferred partners were predicted to defend themselves more from a playful attack but did not use different defensive tactics (Table 4.1). Role reversals were predicted to occur more often with preferred partners (Table 4.1).

#### *4.3.5.2 Dominance and weight*

We found that dominance relationships and weight asymmetries did not predict whether a partner was the most versus least preferred favored partner (Table 4.2). The results of the tube test are tabulated in Appendix A, Table A1.

#### *4.3.5.3 Proximity*

The amount of time that rats were in close proximity (within a body length) did not predict whether a partner was the most versus least preferred partner (Table 4.2). These data suggest that opportunity is not a key driver of partner choice, as on many occasions a rat left the company of group mates to traverse the cage to initiate play with another, more distantly located partner.

**Table 4.1** Comparisons of the non-preferred ($n$ = 27) and preferred ($n$ = 31) partners (dependent variable) with a binary generalized linear mixed model are presented below to compare various measures of the rats play behavior (predictor variables). The frequency of play initiated by the partner and directed towards the focal rat, as well as the percentage (± standard error of the mean) of the various measures are presented along with the results of the model.

| Fixed effect | Not Preferred | Preferred | Estimate | SE | z | p | OR | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|---|---|
| Intercept | | | -2.533 | 1.630 | -1.555 | 0.120 | 0.079 | -5.728 | 0.660 |
| Partner play frequency | 5.11 ± 0.63 | 7.84 ± 1.05 | -0.252 | 0.145 | -1.732 | 0.083 | 0.777 | -0.537 | 0.033 |
| **% no response** | **14.81 ± 6.97** | **29.58 ± 3.59** | **0.042** | **0.019** | **2.269** | **0.023** | **1.043** | **0.006** | **0.078** |
| % evasion | 29.63 ± 8.96 | 31.61 ± 2.44 | 0.022 | 0.019 | 1.160 | 0.246 | 1.022 | -0.015 | 0.058 |
| % pin | 11.11 ± 6.16 | 30.53 ± 3.72 | 0.030 | 0.020 | 1.557 | 0.119 | 1.031 | -0.008 | 0.067 |
| **% role reversal** | **2.38 ± 1.43** | **47.28 ± 7.15** | **0.136** | **0.052** | **2.598** | **0.009** | **1.146** | **0.033** | **0.239** |

Note. SE = Standard error; OR = Odds ratio; CI = Confidence interval.

**Table 4.2** Comparisons of non-preferred ($n$ = 27) and preferred ($n$ = 31) partners (dependent variable), with a binary generalized linear mixed model, are presented below to compare various measures of behavior, measured as a pair (symmetry, proximity, dominance), as well as the difference in weight between pairs of rats (predictor variables). The percentage (± standard error of the mean) of the various measures are presented along with the results of the model.

| Fixed effect | Not Preferred | Preferred | Estimate | SE | z | p | OR | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|---|---|
| Intercept | | | 1.774 | 1.493 | 1.188 | 0.235 | 5.892 | -1.152 | 4.700 |
| **Play symmetry** | **0.51 ± 0.06** | **0.75 ± 0.05** | **-2.911** | **1.058** | **-2.750** | **0.006** | **0.054** | **-4.986** | **-0.837** |
| Proximity (sec) | 292.65 ± 11.86 | 281.77 ± 10.46 | -0.001 | 0.005 | 0.170 | 0.865 | 1.001 | -0.009 | 0.011 |
| Weight | -3.31 ± 3.57 | -2.64 ± 3.35 | -0.005 | 0.016 | -0.296 | 0.767 | 0.995 | -0.036 | 0.027 |
| Dominance | 1.92 ± 2.28 | 0.88 ± 1.51 | -0.024 | 0.029 | -0.820 | 0.412 | 0.976 | -0.082 | 0.033 |

Note. SE = Standard error; OR = Odds ratio; CI = Confidence interval.

#### *4.3.5.4 Partner availability*

Given that when two rats are playing together, they are no longer available as potential partners, the partner preferences we detected could have been driven by partner availability. Therefore, we combed through the data set and selected the nape attacks by the focal animals (i.e., those rats found to have a significant positive or negative preference based on the analyses presented above) when all five potential partners were available. On average, this resulted in 60.8% of the original attacks, occurring when all partners in the group were available. We used the previously found positive and negative play associations to predict whether the adjusted association should be above or below the median value. For preferred and non-preferred partners, the focal rats attacked as previously predicted (sign test: most preferred, $n = 24/31$, $p = 0.0033$; least preferred, $n = 26/27$, $p < 0.0001$). In other words, preferred rats were played with above the median, even when around 40% of the playful attacks were not included. Likewise, non-preferred rats were still avoided. Moreover, even when all five potential partners were not available, in over 98.8% of cases, the focal rats had at least two other rats available to direct their attacks. These analyses suggest that the partner preferences found were not simply byproducts of partner availability.

## 4.4 Discussion

Given our preliminary findings (Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2023), we predicted that rats living in groups should form play partner preferences, and that these preferences should remain stable over the juvenile period. Moreover, given that individual differences in playfulness tend to be stable over time and context (Achterberg et al., 2023; Lesscher et al., 2021; Melotti et al., 2014), and that the

quality of play, especially turn taking and symmetry, seems to be important in rewarding playful interactions (Pellis et al., 2017, 2019), we predicted that rats should prefer high players as partners, who tend to play more reciprocally. Our findings support some, but not all, of these predictions. Many rats did exhibit play partner preferences (Figures 4.2 and 4.3). Preferred rats tended to engage in less defensive actions but engaged in more role reversals than unpreferred partners (Table 4.1). Contrary to our prediction, we found that partner preferences were unstable, with different partners being preferred on different days (Figure 4.3). In addition, there was considerable variation at a group level, with some groups showing clear preferences and others not, similar to the group variation in play preferences reported for domestic pigs (*Sus scrofa domesticus*) (Turner et al., 2020).

Together with our previous findings, the mechanisms used to form play partner preferences among juvenile male rats are becoming clearer. Weight and dominance asymmetries do not influence preferences when playing in groups in which all of the individuals are familiar (present study) or when all individuals are unfamiliar (Ham & Pellis, 2024). However, when the choice is between a stranger and more familiar partners, these asymmetries do influence partner preferences, with a more dominant or larger stranger being a less attractive play partner (Ham & Pellis, 2023). Still, this is a relatively minor influence as strangers that are closer in dominance to the subject are preferred over cage mates. Therefore, in the juvenile period, dominance asymmetries are, at best, modifiers under some partner choice situations, not a main determinant for partner choice. Unlike when rats are playing with unfamiliar partners (Ham & Pellis, 2023, 2024), preferred partners among groups of familiar individuals are those which have a more symmetrical play relationship and engage in more role reversals.

A prediction that was not supported was that, in groups of familiar animals, the play preferences should be stable, as in wild Japanese macaques (*Macaca fuscata*), where play relationships are correlated over a long period of time (Shimada & Sueur, 2018). This was not what we found as preferences varied from day to day (i.e., they were unstable). Indeed, even at a group-level, the sub-communities formed also varied from day to day, suggesting there is instability both in individual preferences and in group dynamics (Figure 4.4). However, the day to day changes in partner preferences were not random. If rats changed partner preference, they were most likely to switch to the second or third most preferred partners, not to the least preferred rats (Figure 4.5). Moreover, in many groups, while the status for an individual as the most preferred partner could change from one day to the next, it continued to rank among the preferred partners (Figure 4.6). That is, there tend to be clusters of high players within groups (Figure 4.2), with partner preferences switching among these group members over days. If this is the case, then two further issues need to be explored. First, what underlies the daily changes among the preferred partners, and second, what is it about the least preferred rats that make them so undesirable?

### 4.4.1 Potential factors associated with or influencing partner preferences

One possible factor influencing why the most preferred status can switch from one day to the next is that an individual alters its behavior. We found that the amount of play an individual engages in fluctuates from day to day (see Figure 4.7). So, the preferred high player on Day 1 may become less preferred on Day 3 as it initiates less play on that day, thus, leading the other high players in the group to pick a new "favorite partner." Even though an individual high player (i.e., a rat that initiates relatively more nape attacks) may decline in its playfulness on any given day, overall, the same individuals tend to remain the group members initiating the most play in each group (Figure 4.7), suggesting that the same

coterie of rats are likely the ones that remain among the preferred play partners. Furthermore, with changes in playfulness, subtle changes in how those rats play could tip the balance on the attractiveness of the previous favorite partner, so explaining the significant influence of defensive behavior and role reversals (Table 4.1, see also Ham & Pellis, 2023, 2024). Alternatively, rats could be actively seeking diversity in play partners and interactions, making partner preferences relatively unstable over time (discussed further below).

Another potential explanation is that preferences are artificially driven by partner availability. That is, a rat may play with a given partner simply because all the other partners are busy playing. Our data, however, do not support this hypothesis. Rather, we found that in nearly all cases, an attacker had two or more potential partners to which to direct their play. Moreover, while scoring the attacks, casual observations indicated that, on many occasions, rats waited for their preferred partner to be available instead of attacking a non-preferred partner which was available.

A simpler, alternative explanation could be that, on any given day, rats that remain in closer proximity to one another are more likely to play together. This is unlikely because neither in this study, nor in two previous ones (Ham & Pellis, 2023, 2024), in which we measured proximity, was there any evidence for an association between proximity and play partner preference. In addition, our observations during all these studies revealed that the least preferred rats would often be bypassed to reach a distant location and initiate play with one of the preferred partners.

It should be noted that although some group members were played with rarely, each animal in each group engaged in play with everyone in the group in almost every session (Figure 4.2; Appendix A, Figure A1). So, it is possible that play is being used, to some

degree, as a means of maintaining social contact among group members, and that least preferred partners may still be selected for other kinds of social interactions. For example, allogrooming is used to manage social relationships among familiar animals in a home cage context (D. C. Blanchard & Blanchard, 1990; Draper, 1967). While allogrooming was rarely observed in the present study, given the pre-test session social isolation used to increase the likelihood of play, this is not surprising. Spontaneously occurring social interactions in groups of rats living in their home cages need to be monitored to determine whether trade-offs are being made between playing with group mates and grooming them. That is, a partner rarely played with may receive more allogrooming. So, while studies such as this one indicate that some individuals are not preferred as play partners, it cannot be assumed that they are completely ignored or treated as unattractive partners in other social contexts.

Regarding play fighting, rats may select partners in a way to regulate some preferred features of the play experience, such as some combination of the frequency of play (Achterberg et al., 2023) and the balance between cooperation and competition during play (Pellis et al., 2024). The role of homeostasis in shaping the amount and content of play is a relatively unexplored avenue of study (Achterberg et al., 2023; Baldwin & Baldwin, 1976). Moreover, the selection of play partners may also be influenced by a variety of subtle cues, such as odor, vocalizations, how they are contacted, or the complementarity of their actions relative to that of their partners. These mechanisms and factors remain to be explored.

It was clear that one or two rats per group never rose to being popular, with little play directed at them and they, in turn, directed little play to the others (Figure 4.2 and 4.6; Appendix A, Figure A1). Whether these rats were 'ostracized' (Table 4.1) because they were unattractive partners for some reason, or because they had a low motivation to play,

remains to be determined. However, that each group had at least one outcast suggests that, in naturally occurring colonies of rats, not all rats may receive the same level of play experience, and this could affect the ability of the animals to gain the benefits typically associated with play (Pellis, Pellis, Ham, et al., 2023). Indeed, naturally occurring variation in the amount of play experienced during the juvenile period has been found to have repercussions later in life in several species (e.g., Blumstein et al., 2013; Fagen & Fagen, 2009; Nunes & Monroy Montemayor, 2023; Perret, 2021, including rats: Ham, Iwaniuk, et al., 2023; Parent et al., 2013). The occurrence of natural variation in the play expressed by rats in the juvenile period (Achterberg et al., 2023; Lampe et al., 2019; Lesscher et al., 2021; Melotti et al., 2014), and the finding that the play experienced in the juvenile period, especially its quality, can affect the development of socio-cognitive skills and their underlying neural substrates (Bijlsma et al., 2024; Ham, Szabo, et al., 2024; Schneider, Bindila, et al., 2016; Stark et al., 2021, 2023; Stark & Pellis, 2020, 2021), raises the possibility that rats may regulate partner choice as a way of gaining experiences that ensure appropriate developmental outcomes (Ham, Iwaniuk, et al., 2023; Pellis, Pellis, Ham, et al., 2023).

### 4.4.2 Maximizing two different kinds of experiences?

Specifically, it seems that it is the degree of symmetry and turn taking that individuals experience during play fighting as juveniles that influences the development of social skills and the dendritic arbors of the pyramidal neurons in the medial prefrontal cortex (Ham, Szabo, et al., 2024; Pellis et al., 2019; Stark et al., 2021, 2023). These same features of the play experience also seem to be critical for keeping the playful behavior enjoyable (Mitchell, 1990), with rats engaging in behavior that facilitates turn taking (Foroud & Pellis, 2003; Pellis et al., 2005). Given that young animals can create a social

niche that is most beneficial to them (Kaiser et al., 2024; Trappes et al., 2022; West & King, 1987), it is possible that niche creation may account for both our expected and unexpected findings about play partner preferences.

As expected, rats did prefer some partners over others, but unexpectedly, rather than direct all their playful attacks to one or two preferred partners, over the course of the test trial, they also attacked less preferred partners, including the least preferred ones (Figure 4.2 and Appendix A, Figure A1). This is a pattern that is consistent with the study of play involving different group membership (e.g., all strangers, a mixture of strangers and familiar rats (Ham & Pellis, 2023, 2024). To gain the benefits derived from turn taking, juveniles appear to need to experience a minimum threshold level (Pellis et al., 2017, 2019), with too little or too much leading to an excessive lack of symmetry in play fights, either of which can lead to detrimental developmental outcomes (Ham, Szabo, et al., 2024; Stark et al., 2021). Therefore, it is possible that an individual plays with suitable partners to reach that minimum threshold, so explaining the above expected levels of play directed at a coterie of group members (Figures 4.4 and 4.6). But what accounts for the play directed at the less optimal group members? Previous studies comparing being reared in a group versus being reared with a single partner during the juvenile period found that having more than one partner influences the dendritic arbor of the pyramidal neurons in the orbitofrontal cortex (Bell et al., 2010; B. T. Himmler et al., 2018). Since damage to the orbitofrontal cortex in normally reared rats reduces their ability to modulate their responses with the identity of the partner in both playful and non-playful social interactions (Pellis et al., 2006), it is possible that by interacting with multiple partners, rats indirectly train their orbitofrontal cortex to match responses to specific partners better (Pellis, Pellis, Ham, et al., 2023). In contrast, the improvements in social skills and the anatomical changes in the

medial prefrontal cortex can be achieved by playing with a single partner over the juvenile period (Baarendse et al., 2013; Bell et al., 2010; Bijlsma et al., 2022, 2023, 2024; Ham, Szabo, et al., 2024; B. T. Himmler, Pellis, & Kolb, 2013; Stark et al., 2023; Stark & Pellis, 2020). Damage to the medial prefrontal cortex in normally reared rats reduces their ability to adjust their movements to those of their partner in both playful and non-playful interactions, but does not disrupt the ability to modulate actions with the identity of the partner (Bell et al., 2009; B. T. Himmler et al., 2014). These findings suggest that the play experiences train rats in how to modulate behavior with the actions of their partner (Pellis, Pellis, Ham, et al., 2023). If so, then in a natural setting with multiple potential partners available, the rats should follow two simple rules: (1) play with partners that provide the minimum turn taking experiences needed to train the medial prefrontal cortex and (2) play with a sufficiently diverse number of partners to train the orbitofrontal cortex. The pattern of play partner preferences in the rats in the present study is consistent with this hypothesis and so worth further testing.

### 4.4.3 Implications

With a push to study more ethologically relevant behaviors and test animals under more naturalistic conditions (d'Isa & Gerlai, 2023; Dennis et al., 2021; Kondrakiewicz et al., 2019), we propose that, depending on the question being asked, group play may be a more suitable paradigm to explore juvenile social play and social development (Pellis, Pellis, Ham, et al., 2022).

With that said, it is clear from our results that the choice of partners should be provided to rats, and that rats should be tested on more than one day as individual idiosyncrasies in play change day-to-day. For example, Figure 4.2 reveals that while certain animals maintain a relatively stable play frequency (e.g., Rat D), others vary greatly from

session-to-session (e.g., Rat A). Therefore, scoring only one day may provide a misleading snapshot of Rat A's behavior. While this requires more effort, we do not feel this is a limitation but is instead beneficial, especially when phenotyping individual differences and studying social development. Indeed, these individual differences more closely resemble those of human children engaging in play (Humphreys & Smith, 1987).

Elucidating individual differences with the group play paradigm may prove particularly useful when studying rat models of diseases and psychological disorders (C. J. Burke et al., 2021; Lonstein et al., 2023). By giving the rats a choice of partners, certain individuals may be avoided, revealing nuanced social deficits. For example, if groups of three are tested, in which two of the three are 'control' rats and one is an 'experimental' animal (e.g., a disease or social deficit model strain), determining the frequency and style in which a control plays with the control partner versus the experimental partner may reveal the severity of the deficits (Kisko, Himmler, et al., 2015; Pellis, Pellis, Ham, et al., 2022). However, if a control rat is playing under dyadic conditions, the rat has no choice but to play with its prescribed partner, and this could potentially mask such deficits. This is particularly true when the rats have been socially isolated for a period of time, a standard practice of studying play (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022), and so are highly motivated to play.

### 4.4.4 Limitations of the study

One limitation of this study is that it was limited to males. We used male rats in this experiment as dominance hierarchies are more prevalent in males (Schweinfurth, 2020) and these hierarchies may have influenced their partner selection. However, dominance asymmetries did not influence the selection of play partners, and so females would likely express partner preference patterns similar to males (Ham & Pellis, 2023). Given that

females are sensitive to the identity of their play partner (Argue & McCarthy, 2015a), future studies should use females as well, as these may highlight some of the factors not readily discerned in all male groups. Additionally, studying mixed-sex groups or groups of mixed kin and non-kin animals may be worthwhile as this would more accurately reflect the natural variation in play partners to which wild rats would have access. Indeed, in group living Belding's ground squirrel (*Urocitellus beldingi*) juveniles prefer to play with relatives rather than neighbours, but have no preference for full versus half siblings (Nunes et al., 2015). Even so, specific individuals are preferred over others (Nunes, Muecke, Sanchez, et al., 2004). Whether kin based preferences are formed in wild and/or domesticated rats remains to be determined.

Another potential limitation is that the rats came from general holding pens from Charles Rivers, so some of the rats may have been related. While we have no way of knowing how many, if any, were related, this could potentially drive some of the play partner preferences present. Future studies should examine whether rats have preferences for kin, using in-house breeding to ensure that the relatedness of the group members are known, and compare those results to other communally living rodents, such as Belding's ground squirrels.

While we chose to sample play in the typical experimental fashion (i.e., animals are isolated and then tested in an enclosure), to be sure that the preferences observed in these snapshots reflect the rats' preferences throughout the day, home cage records are necessary. Indeed, we are currently analyzing the data from such a project. However, since most studies of play in rats involve play sessions that occur once a day, and only last between 5 and 10 minutes, we wanted to use a similar approach here. That way we could determine

if, during that time frame, partner preferences emerge. Whether they are maintained over a long period of time, and throughout the day, remains to be determined.

Finally, the tube test, though frequently used, may not provide accurate results as to the animals' dominance relationships. Instead, 'wins' and 'loses' during the tube test could simply arise due to differences in motivation, behavioral strategies, or size. As this was our only measure of dominance in the present study, we cannot be sure that we effectively tested the influence of dominant-subordinate relationships within the groups of rats. These findings need to be corroborated using other measures of dominance.

### 4.5 Conclusions

The results from this study demonstrate that rats have partner preferences when playing with familiar cage mates. However, these preferences vary from day-to-day. While dominance and weight asymmetries do not influence preferences, the likelihood that the partner defends itself, the degree of symmetry between rats, and the degree to which they take turns was correlated with partner preference formation. In most of the groups, certain individuals were significantly preferred over others each day, suggesting that some rats are more *popular* than others. Together, these data suggest that animals are not at the mercy of their environment but can create a social niche that is preferred. The created social niches may be selected to maximize both the high quality and diversity of play experiences. By giving the rats, a choice of partners, and so a choice in experiences and quality of play, we let the rats *tell us* which aspects of their play they value (i.e., play frequency, style, quality, the individual). In turn, this more naturalistic approach to studying play and social behaviors offers insight into group dynamics and nuanced individual differences which may, in turn, offer new insights when studying rat models of disease and development.

# CHAPTER 5: JUVENILE MALE RATS FORM PREFERENCES BASED ON STRAIN WHEN PLAYING IN GROUPS BUT NOT IN PAIRS*

## 5.1 Introduction

Though there are many forms of play behavior (Burghardt, 2005; Fagen, 1981; Pellis & Pellis, 2009), rough-and-tumble play (RTP) or play fighting is the most common form of social play reported and studied, especially in young mammals (Pellis et al., 2024; Pellis & Pellis, 1998). Regardless of the species, RTP is centered around animals competing for an advantage—such as access to a particular location on the partner's body—while also showing some degree of cooperation, making the interactions somewhat reciprocal (Pellis et al., 2024). Laboratory rats have been the most intensively studied species and have provided many of the major insights into the neurobiology and developmental consequences of RTP (Achterberg & Vanderschuren, 2023; Pellis et al., 2023).

Rats engage in RTP both as juveniles and adults (Pellis & Pellis, 1990; Thor & Holloway, 1984c), although its occurrence peaks during the juvenile period, between 30-40 days after birth (Panksepp, 1981; Pellis & Pellis, 1990; Thor & Holloway, 1984a). Juvenile male rats are frequently reported to engage in RTP more than females (e.g., Meaney, 1988; Meaney & Stewart, 1981; Thor & Holloway, 1983), however, this is not always the case (Northcutt & Nwankwo, 2018). Indeed, it seems that other factors, particularly how the rats are reared as juveniles can greatly modify the prominence of this sex difference. For example, when reared in multi-animal, mixed sex groups, males play more than females. However, when rats are reared with one, same-sex partner, sex differences in RTP frequency are greatly diminished (S. M. Himmler, Himmler, Pellis, et

al., 2016). Regardless of sex differences in the frequency of RTP, in rats of both sexes RTP involves attack and defence of the nape of the neck, which is gently nuzzled with the snout if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). Typically, for 80% or more of nape attacks the recipient uses defensive tactics to prevent contact, such as rolling to supine so that its nape is pressed to the ground and cannot be contacted (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022). While sex differences emerge in how rats play as they age (Pellis, 2002), during the juvenile period, males and females adopt similar defensive tactics (Pellis & Pellis, 1990). Though most intensely studied in domesticated strains of rat in the laboratory (e.g., Achterberg et al., 2014, 2019; S. M. Himmler, Modlinska, et al., 2014; Siviy et al., 2017; Veenema et al., 2013), this pattern of attack and defence of the nape is also observed in wild populations (Calhoun, 1963) and wild-caught rats observed under laboratory conditions (Barnett, 1958; B. T. Himmler, Stryjek, Modlinska, et al., 2013a).

Given that in rats RTP has been shown to be a naturally motivated behavior (Siviy, 2016; Vanderschuren, 2010), does not require training (Pellis, Pellis, Ham, et al., 2022), and has a considerable degree of individual variation (Achterberg et al., 2023; Ham & Pellis, 2023; Lesscher et al., 2021), this behavior has become increasingly used as a means to study social behavior and its development. For example, play has been implicated in the development of executive functions, social skills, and cognitive flexibility, with anatomical and physiological changes of the areas of the prefrontal cortex associated with these capabilities (Bijlsma et al., 2022, 2024; Ham, Szabo, et al., 2024; Schneider, Bindila, et al., 2016; Stark & Pellis, 2020, 2021).

Though every strain of laboratory rat studied to date targets the nape of the neck during RTP (S. M. Himmler, Himmler, Stryjek, et al., 2016; S. M. Himmler, Lewis, et al., 2014; S. M. Himmler, Modlinska, et al., 2014), there is variation in how much they play. For example, Sprague Dawley rats (SD) rats typically initiate around 1.5 times more play than Long Evans (LE) rats (S. M. Himmler, Modlinska, et al., 2014). Fischer 344 (F344) rats typically launch half as many attacks as LEs when tested with partners from other strains (Siviy et al., 1997, 2003; Stark et al., 2021), but more than LE rats when tested in same-strain pairs (Ham et al., 2024). As well as the variation in play frequency both within and among strains, how the rats play also varies from strain to strain. For example, compared to LE rats, SD and F344 rats are more likely to evade a playful attack, by swerving or running away, than rolling to supine (Ham et al., 2024; S. M. Himmler, Modlinska, et al., 2014; Orsucci et al., 2024; Siviy et al., 2023).

Given the differences in playfulness and defensive tactics, some combinations of strains are more compatible than others. For example, LE, SD, and F344 rats tend to retain their strain-typical preferences in tactics of defense when tested with unfamiliar partners from other strains (S. M. Himmler, Lewis, et al., 2014; Schneider et al. 2016; Siviy et al., 1997, 2003, 2011, 2017, 2023; Stark et al., 2021). If reared together, some strains, such as LE and SD, tend to modify their play styles to more closely match the style of the other strain (S. M. Himmler, Lewis et al., 2014), but others, such as F344 rats, maintain their strain-typical style regardless of the strain with which they are reared (Schneider, Bindila, et al., 2016; Siviy et al., 2017; Stark et al., 2021). Critically, F344 rats, unlike SD and LE rats, seem unable to create the opportunities for role reversals and symmetry in playful attacks when playing with a partner from a different strain (Ham et al., 2024; Schneider et al., 2016; Stark et al., 2021), and so ensure the experiences of reciprocity that are essential

for play to provide its developmental benefits (Pellis et al., 2023). Because of these strain differences, given the choice among potential play partners, we predicted that LE rats should prefer their own strain over SD and F344 rats, but likely prefer SD over F344. Partner preferences among adults in non-playful contexts support this prediction. LE and SD rats prefer to socialize with their own strain or even each other, before socializing with a F344 partner (Kiyokawa et al., 2022; Kogo et al., 2021).

Typically, when testing play in rats, the 'dyadic paradigm' is used, where two rats are placed in a neutral arena after some period of social isolation (Achterberg & Vanderschuren, 2023; Pellis, Pellis, Burke, et al., 2022). A limitation with this paradigm is that rats are forced to play with partners selected by the experimenter, and play ensues even if the partner is not optimal (Achterberg et al., 2023). Indeed, if given the choice of multiple partners, in a group testing paradigm, rats will tend to preferentially initiate more play with some members of the group than with others (Ham & Pellis, 2023, 2024, 2025). Such a group play paradigm more closely resembles the situation in which rats evolved, where they not only have multiple littermates as potential partners, but also peers from the litters of other females in the colony (Meaney & Stewart, 1981; Pellis & Pellis, 1997; Schweinfurth, 2020). Therefore, to test our prediction, we used a group paradigm to assess play preferences in partner selection across strains.

Groups of four rats, comprising two LE rats and one each of SD and F344 rats, were tested. One LE rat was selected as the focal rat and the frequency of nape attacks it initiated toward the other three rats was used as a means to determine partner preference. Because of the quantitative (number of attacks) and qualitative (preferred defense tactics) differences among these strains discussed above, our prediction was that the order of preference would be LE>SD>F344. The focal LE rats were also tested in dyads with the

three different strains, and we predicted that LE rats would play with LE, SD, and F344 rats at similar frequencies as they satisfy their motivation to play with the only partner available (Ham, Szabo, et al., 2024; S. M. Himmler, Lewis, et al., 2014; Stark et al., 2021). Finally, we used socially conditioned place preference (sCPP) (Achterberg et al., 2012, 2014; Trezza et al., 2009, 2011), and predicted that the focal LE rats would prefer to spend time in a context in which they had played with a same-strain partner over one in which they had played with a less compatible F344 partner.

To assess the LE rats' preference in the sCPP test further, their ultrasonic vocalizations were recorded. As 50 kHz calls are emitted in positively affective contexts, including play (Burgdorf et al., 2008), and their rate of emission is increased in a play anticipation paradigm (C. J. Burke, Kisko, Swiftwolfe, et al., 2017; C. J. Burke et al., 2021; Knutson et al., 1998) but reduced during dyadic play encounters between LE and F344 partners (Stark et al., 2021), we predicted that more calls would be emitted when accessing the preferred box in the sCPP trials.

## 5.2 Materials and methods

### 5.2.1 Ethics

All care and testing procedures were approved by the University of Lethbridge Animal Welfare Committee (Protocol #2408) in compliance with guidelines from the Canadian Council for Animal Care.

### 5.2.2 Subjects

Sixteen LE, eight SD, and eight F344 male rats were purchased from Charles River Laboratories (Kingston, New York) and arrived at the Canadian Centre for Behavioural Neuroscience at 24 days of age. Upon arrival, the rats were housed in same-strain pairs.

Animals were housed in double decker cages with corncob bedding. Two days after arriving, the animals were handled and weighed daily by the experimenters to habituate the rats to the experimenters, being handled, and being weighed. Food and water provided were *ad libitum*. The rats were housed on a 12-h light-dark cycle (lights on between 7:30 a.m. and 7:30 p.m.) in a room maintained at a constant temperature of 21-23˚C.

### 5.2.3 Apparatus

#### *5.3.3.1 Group play*

Play in groups was tested in a clear Plexiglas enclosure (80 × 80 × 50 cm). The floor of the enclosure was covered with corncob bedding. Interactions were filmed with a digital video camera (Sony Handycam FDR-AX53) which was placed above the enclosure at a 90° angle so that all four rats could be visualized simultaneously. Red lights were used to illuminate the enclosure.

#### *5.2.3.2 Dyadic play*

Play in pairs was tested in a clear Plexiglas enclosure (50 × 50 × 50 cm). The floor of the enclosure was covered with corncob bedding. Interactions were filmed with a digital video camera which was place outside of the enclosure obliquely, at a 45° angle. Red lights were used to illuminate the enclosure.

#### *5.2.3.3 Social conditioned place preference*

sCPP was tested in an apparatus comprised of three compartments, each with a removable Plexiglas lid. Two of the compartments were large and equal in size (41 × 41 × 50 cm) separated by a smaller alleyway (16.5 × 11 × 11 cm). The two large enclosures, where the animals were conditioned to a stimulus, had different visual and tactile cues (Figure 5.1). One of the contexts was a white box with a black floor, with stainless-steel

bars spaced 1.5 cm apart lining the floor. The other was a black box with a white floor that did not have bars along the floor. Both chambers had Pettersson M500-384 USD ultrasound microphones (Pettersson Elektronik AB, Sweden) affixed to the Plexiglas lids so that USV could be recorded. Sessions were filmed with a digital camera which was placed above the enclosures at about a 70° angle.

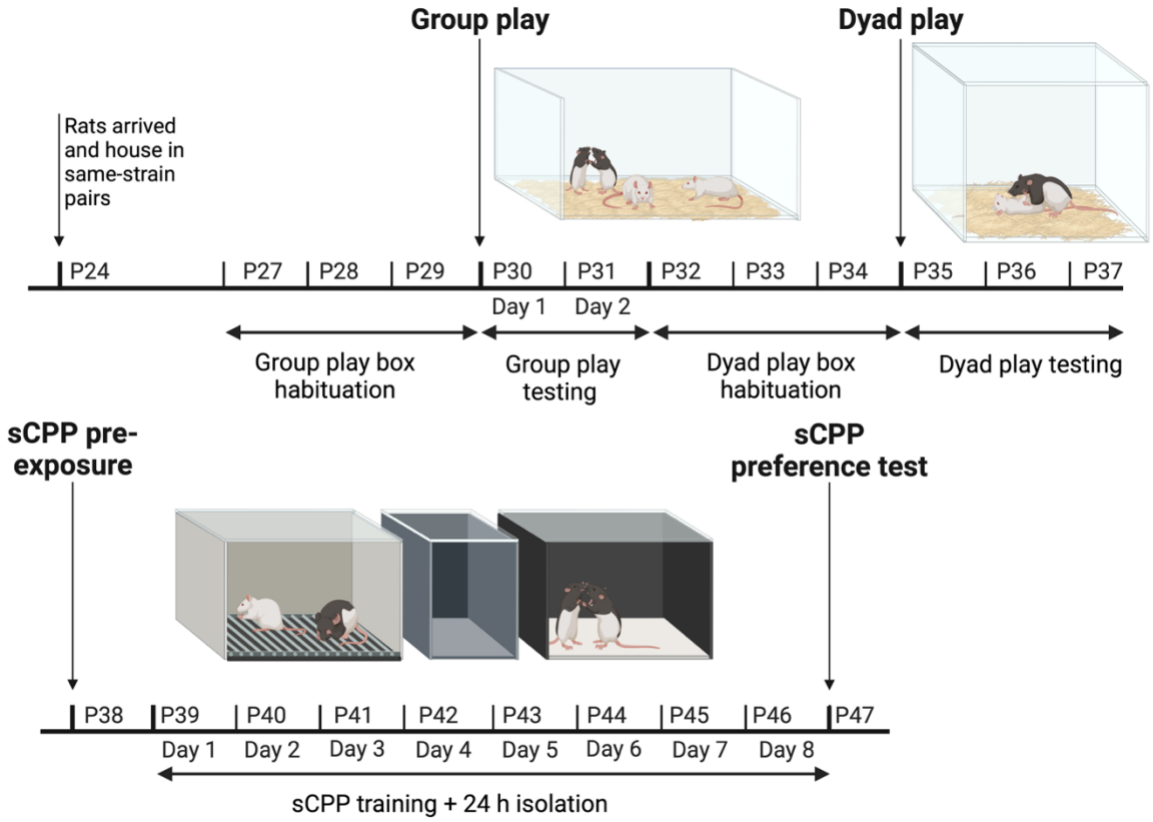


**Figure 5.1** The experimental paradigm illustrating how the animals were tested in groups on postnatal day (P) P30 and P31, tested in pairs between P35-37, and pre-exposed, trained, and tested using social conditioned place preference (sCPP) on P38, P39-P46, and P47, respectively. Created with BioRender.com.

### 5.2.4 Procedure

Eight of the 16 LE rats ordered were designated as 'focal' rats and were the primary subjects of this study. These eight focal rats were housed together in pairs, and tested and scored in both the group and dyadic contexts for play, and finally, they were tested in the sCPP paradigm (Figure 1).

#### *5.2.4.1 Group play*

At 27 days of age, the rats of each strain were habituated to the test enclosure for 20 min, with their cage mates, in red light. This was done for three consecutive days between 7:30 a.m. and 3:00 p.m. At 30 days of age, group play testing began. Before testing, rats were weighed, their fur marked with a permanent marker pen (Sharpie®), and socially isolated for 2.5 h to increase their playfulness (Pellis, Pellis, Burke, et al., 2022). Food and water were provided *ad libitum* during social isolation. Groups consisting of two LE (one being the focal LE), one SD, and one F344, all of which were unfamiliar with one another, were placed into the test enclosure, in red light, for 20 min and filmed. The group play trials were repeated for two days, switching the individuals within each group so that they were again all unfamiliar on the second test day. Following a test session, the animals were placed back into their respective home cages.

#### *5.2.4.2 Dyadic play*

As the dyadic enclosure was located in a different room and is a different size, at 32 days of age, the rats were habituated to the dyadic test enclosure for 10 min, with their cage mates, in red light. This was done for three consecutive days between 7:30 a.m. and

3:00 p.m. At 35 days of age, dyad play testing began. Before testing, rats were weighed, marked with a permanent marker pen (Sharpie®), and socially isolated for 2.5 h. Again, food and water were provided *ad libitum* during social isolation. Pairs of either two LEs, one LE and one SD, or one LE and one F344, who were unfamiliar with one another, were placed into the test enclosure, in red light, for 10 min and filmed. The dyadic play trials were run over three days, counterbalancing the strains paired with the focal rats. Following all test sessions, the animals were placed back into their respective home cages.

#### *5.2.4.3 Social conditioned place preference*

*Pre-exposure.* Using a modified version of sCPP (see Achterberg et al., 2012, 2014; Trezza et al., 2009, 2011), at 38 days of age, the rats were placed into the middle alley chamber, which was connected to both large boxes, one at a time. Under red-light conditions, the rats were allowed to explore both chambers freely for 15 min while being video recorded. Time spent in both chambers, and the middle alley, was measured. According to the pre-exposure results, the rats were counterbalanced such that any innate preferences for either context was balanced for partner × context pairing. Following pre-exposure, all rats, including the partner rats, were socially isolated with food and water provided *ad libitum*.

*Training.* Training began approximately 24 h after pre-exposure. Two of the chambers were sealed, making the middle alleyway and the opposing chamber inaccessible. Half the rats experienced an LE partner in the white box while the other half experienced an LE partner in the black box. In the opposite context, they were paired with a F344. On days 1, 3, 5, and 7 of the training, rats were placed in one of the chambers for 15 min with an unfamiliar partner (either LE or F344). In the afternoon, they were placed in the opposite

box, for 15 min, with the opposite partner. On days 2, 4, 6, and 8 of training, the order of the two daily sessions was inverted. For example, on day 1, focal Rat A would be partnered in the morning with an LE rat in the white box. In the afternoon, they would be partnered a F344 rat in the black box. On day 2, focal Rat A would be partner with a F344 rat in the black box in the morning, and an LE in the white box in the afternoon. Morning and afternoon sessions, on any given day, were separated by at least four hours. Partners were counterbalanced so that half of the partners experienced an LE in the morning and half experienced a F344 in the morning. Similarly, the focal rats starting a training session was counterbalanced so that if Rat A started first on day 1, he would start last on Day 2. Partners were rotated each day so that they were always unfamiliar with the focal rat to preclude the formation of partner preferences and ensure that any preferences formed were for the strain and not the particular individual with which they were interacting. Training was done for 8 consecutive days. Following morning and afternoon training session, the rats were placed back into social isolation.

*Preference test*. Twenty-four hours after the eighth and final day of training, the preference test was conducted (Figure 1). Both boxes were unsealed, and the grey alleyway was replaced so that the two boxes were connected. Like pre-exposure, rats were placed in the middle alleyway and allowed to explore both boxes freely for 15 min under red-light conditions. The test sessions were filmed, and the recordings were subsequently used to score the time spent in the two boxes and the alleyway.

### 5.2.5 Behavioral analyses

#### *5.2.5.1 Group play*

Following group play trials, the videos were analyzed using a combination of normal speed and frame-by-frame analysis to score play behavior (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022). As we were interested in how a focal rat would distribute its play among the potential play partners, we used a focal follow approach (Bateson & Martin, 2021) and scored the distribution of nape attacks directed towards each partner by the focal LE rat. Playful attacks were scored when the snout of one rat was in contact with, or directed towards, the nape of another rat (Pellis, Pellis, Burke, et al., 2022). The frequency of play, and latency to first and last attack, were scored for both days.

#### *5.2.5.2 Dyadic play*

We measured playful RTP bouts to determine if the focal LE rats played with one strain more than the others. Like above, a playful attack was scored when the snout of one rat was in contact, or directed towards, the nape of another rat (Pellis, Pellis, Burke, et al., 2022).

#### *5.2.5.3 Social conditioned place preference*

To calculate if there was a preference for a particular chamber during the pre-test, the time spent in each of the three sections of the apparatus, was calculated. Time in a box was accumulated when both forepaws and half the body were past the threshold of the doorway into one of the boxes (McDonald et al., 2023). Similarly, time spent in each box was scored by an observer blind to the training conditions after the preference test to determine if a certain box was preferred.

Based on previous studies, we assumed that LE-F344 pairs would have less reciprocal play relationships than LE-LE pairs; thus, we expected an LE partner would be

more compatible than a F344 partner despite the absolute frequency of play being similar (Ham, Szabo, et al., 2024; Stark et al., 2021). However, to be certain that the play experiences differed when paired with a F344 vs a LE during training, the encounters in both the morning and afternoon on training days 1, 5, and 8 (beginning, middle, and end of the training, respectively) were scored and averaged. The number of attacks launched by the focal rat were scored. Additionally, the amount of play the partner initiated was scored so we could quantify how playful the partner rats were, and thus, how rewarding they were as a play partner (Lampe et al., 2019). If bedding is not provided in the play enclosure, rats switch defense strategies, reducing the number of rotations to supine (Pellis & Pellis, 2021). As we used two different floors, we scored the number of attacks resulting in a pin to determine if the floor biased how rats played.

#### *5.2.5.4 Ultrasonic vocalizations*

The vocal recordings from the preference tests were analyzed using Raven Pro 1.6 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). Raven Pro generated spectrograms with a 256-sample Hann window. Using this window, vocalizations were manually selected and labeled by a scorer blind to the play partner context pairing. Each of the USV was scored according to the 14 category schema provided by Wright et al. (2010). These data were used to compare the number and type of calls made in each context. The total number of vocalizations and call types were compared. In addition, the percentage of total calls, and each call type, was calculated to determine if more calls or more calls of a certain type were made in either context. Finally, the rate at which calls were made was calculated (number of calls / total time spent in the context).

### 5.2.6 Statistical analyses

All plots were created using Prism version 10 (GraphPad Software).

#### *5.2.6.1 Group play*

The frequency of nape attacks and latency to first and last attack of each partner, on each day, were compared using two-way repeated measures ANOVAs with Bonferroni corrections.

#### *5.2.6.2 Dyadic play*

The frequency of nape attacks initiated by each pair in all strain combinations, as well as the frequency of nape attacks initiated by the focal rat in each of those pairings was compared using Freidman's tests in Prism. After plotting the data, we noted a large degree of variation in the latency to first attack on Day 1, and so we performed a robust regression and outlier removal (ROUT) test, with a false discovery rate (Q) set to 1% using Prism. However, no outliers were detected.

#### *5.2.6.3 Social conditioned place preference*

Using Prism, paired *t*-tests were used to determine if the rats had an inherent bias for either the black or white box during the pre-test. After conditioning, we used a paired *t*-test to test if the rats formed a preference for the LE or F344 context, as well as for either the black or white box. The frequency of play behavior initiated by the focal rat and how they defended themselves from playful attacks by the LE and F344 partners, and whether this differed in the black and white boxes, was tested with paired *t*-tests. We used unpaired *t*-tests to determine if the frequency of play behavior initiated by the partner rats in either the LE or F344 context and the black or white box was significantly different. This was repeated to determine if the partner rats defended themselves differently from playful attacks by different partners or different boxes.

***5.2.6.4 Vocalizations.*** Two-way repeated measures ANOVAs were used to compare the number, distribution, and rate of vocalizations in the contexts with different partners and different boxes.

## 5.3 Results

### 5.3.1 Group play

We found that the focal rats did not play with each strain equally ($F(2,14) = 64.18$, $p < 0.0001$) (Figure 5.2A), however, there was no effect of day ($F(1,7) = 2.40$, $p = 0.166$) or strain × day ($F(2,14) = 0.70$, $p = 0.515$). Post hoc tests showed that focal rats played with LE partners more than SD ($p < 0.0001$) and F344 ($p < 0.0001$) partners on Day 1. Similarly, on Day 2 (Figure 5.2B), focal rats played more with LE partners than SD ($p < 0.0001$) and F344 ($p < 0.0001$) partners.

When the latency to first attack was compared, we found that there was a significant effect of strain ($F(2,14) = 6.815$, $p = 0.009$), but not day ($F(1,7) = 0.77$, $p = 0.411$) or strain × day ($F(2,14) = 1.968$, $p = 0.177$). Post hoc tests showed that there was no difference in time to first attack between LE vs SD, but F344 partners were the last to be attacked compared to LE and SD ($p = .002$; $p = 0.03$, respectively) on Day 1 (Figure 5.2C). No differences were found in latency to first attack on Day 2 (Figure 5.2D). When the latency to last attack among partners was compared, we found a significant effect of strain ($F(2,14) = 5.07$, $p = 0.022$), day ($F(1,7) = 6.38$, $p = 0.040$), and strain × day ($F(2, 14) = 6.21$, $p = 0.012$). Post hoc tests showed that there was no difference in latency to last attack between strains on Day 1 (Figure 5.2E). However, on Day 2 (Figure 5.2F), LE and SD were attacked later in the test session than the F344 rats ($p = 0.0005$; $p = 0.010$, respectively). Post hoc

tests also revealed that F344 rats were attacked sooner in the test session on Day 1 compared to Day 2 ($p = 0.0003$).

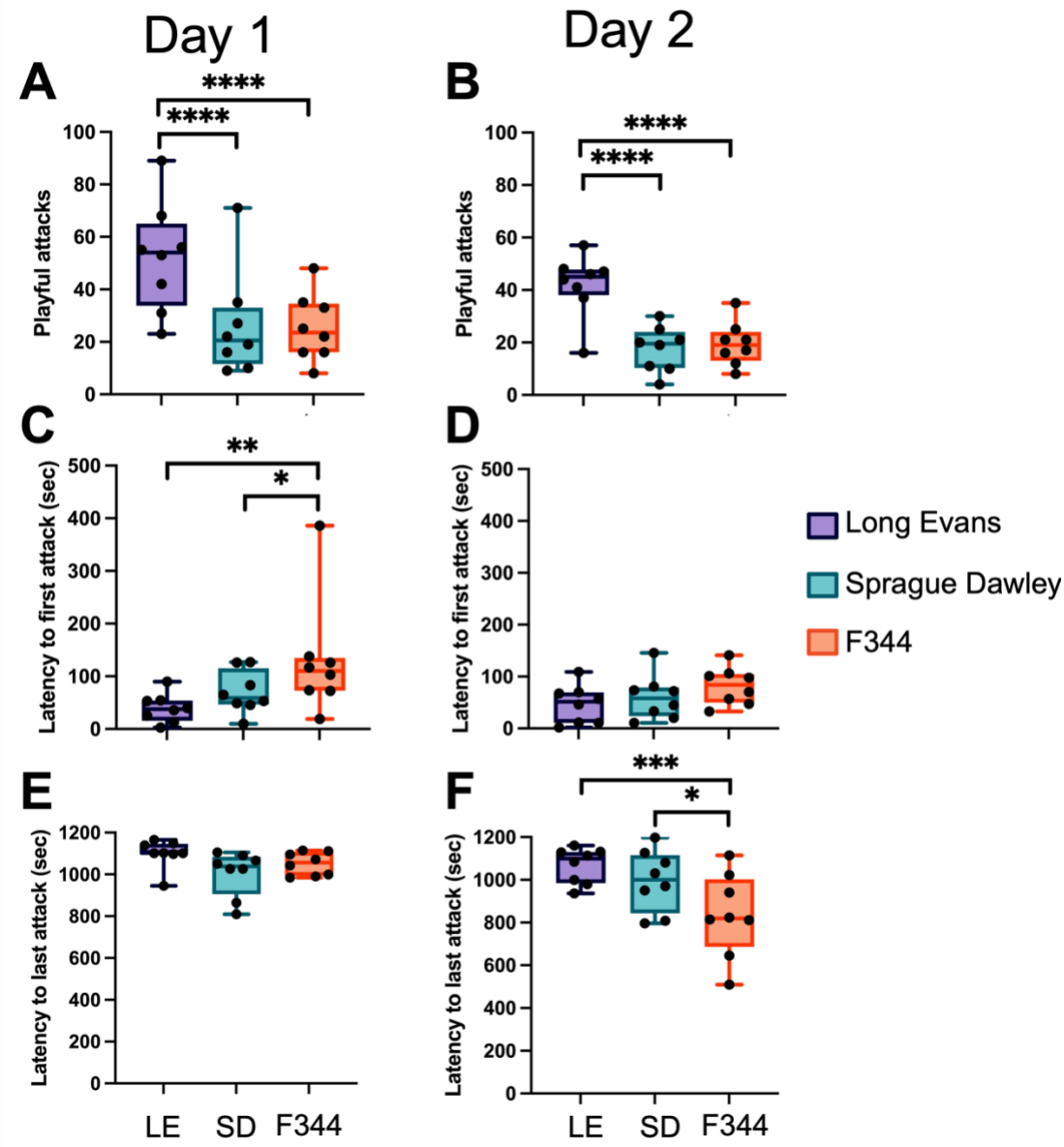


**Figure 5.2** On both day 1 (A) and day 2 (B) of group play, the frequency of nape attacks initiated by the focal rat and directed toward a LE, SD, and F344 varied with focal rats directing more of their play toward LE partners than SD and F344s. LE partners were attacked faster than SD and F344 partners on day 1 (C) but not day 2 (D). No difference was found in latency to last attack on day 1 (E), but on day 2 (F), LE rats were attacked later in the session than both SD and F344s. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

### 5.3.2 Dyadic play

The total play between pair mates did not differ significantly in the any of the strain combinations (Figure 5.3A) ($X^2(2) = 5.87$, $p = 0.053$), although there was a trend for less play in the pairs with F344 partners. Similarly, LE focal rats launched a comparable number of nape attacks (Figure 5.3B), regardless of the strain with which they were partnered ($X^2(2) = 1.75$, $p = 0.531$).

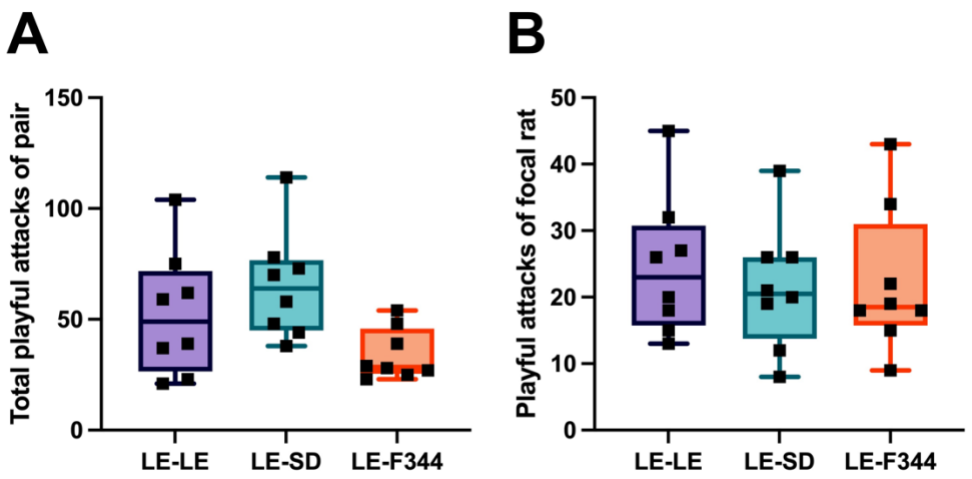


**Figure 5.3** When the frequency of attacks by both partners in the pair was summed for dyadic play, no difference was found between LE-LE, LE-SD, nor LE-F344 pairs (A). Similar, the frequency of play initiated by the focal rat when playing with either an LE, SD, or F344 partner did not differ (B)

### 5.3.3 Socially conditioned place preference

During the pre-test, we found that there was no bias for the box that was to-be-paired with an LE nor the box that was to-be-paired with a F344 ($t(7) = 0.216$, $p = 0.835$) (Figure 5.4A). After 8 days of training, we found that the focal rats did not form a preference for either the LE or F344 paired contexts ($t(7) = 0.773$, $p = 0.465$) (Figure 5.4B). However, when further analyzing the data, we found that the rats significantly preferred the

white box during the preference test ($t(7) = 6.261$, $p = 0.0004$), regardless of whether they were partnered with a LE or a F344 in the white box (Figure 5.4C).

After scoring the interactions on training Days 1, 5, and 8, we found that the focal rats did not play with LE partners more than F344 partners across the three sample days ($t(7) = 1.431$, $p = 0.196$) (Figure 5.4D). The focal rats, however, did defend attacks by rolling over into a supine position when playing with an LE partner (mean number of supine defenses: 6.29) more than when with a F344 partner (mean number of supine defenses: 1.17) ($t(7) = 0.230$, $p = 0.0131$). LE partners, on average, initiated more play than the F344 partners ($t(14) = 3.102$, $p = 0.0078$) (Figure 5.4E), but did not defend themselves with more supine defences when playing ($t(14) = 1.772$, $p = 0.098$).

Given that focal LE rats preferred the white box over the black box, we compared whether the focal rats initiated more play in the white box. We found that LE rats launched more playful attacks in the white than the black box, across the three days measured ($t(7) = 4.336$, $p = 0.0034$) (Figure 5.4F). The focal rats did not, however, defend themselves by rolling to a supine position more in either the white or black box ($t(7) = 0.865$, $p = 0.416$). No difference was found in the frequency of play initiated by the partner in either the white or black box ($t(14) = 0.652$, $p = 0.525$) or in the number of supine defences they used ($t(14) = 1.104$, $p = 0.288$).

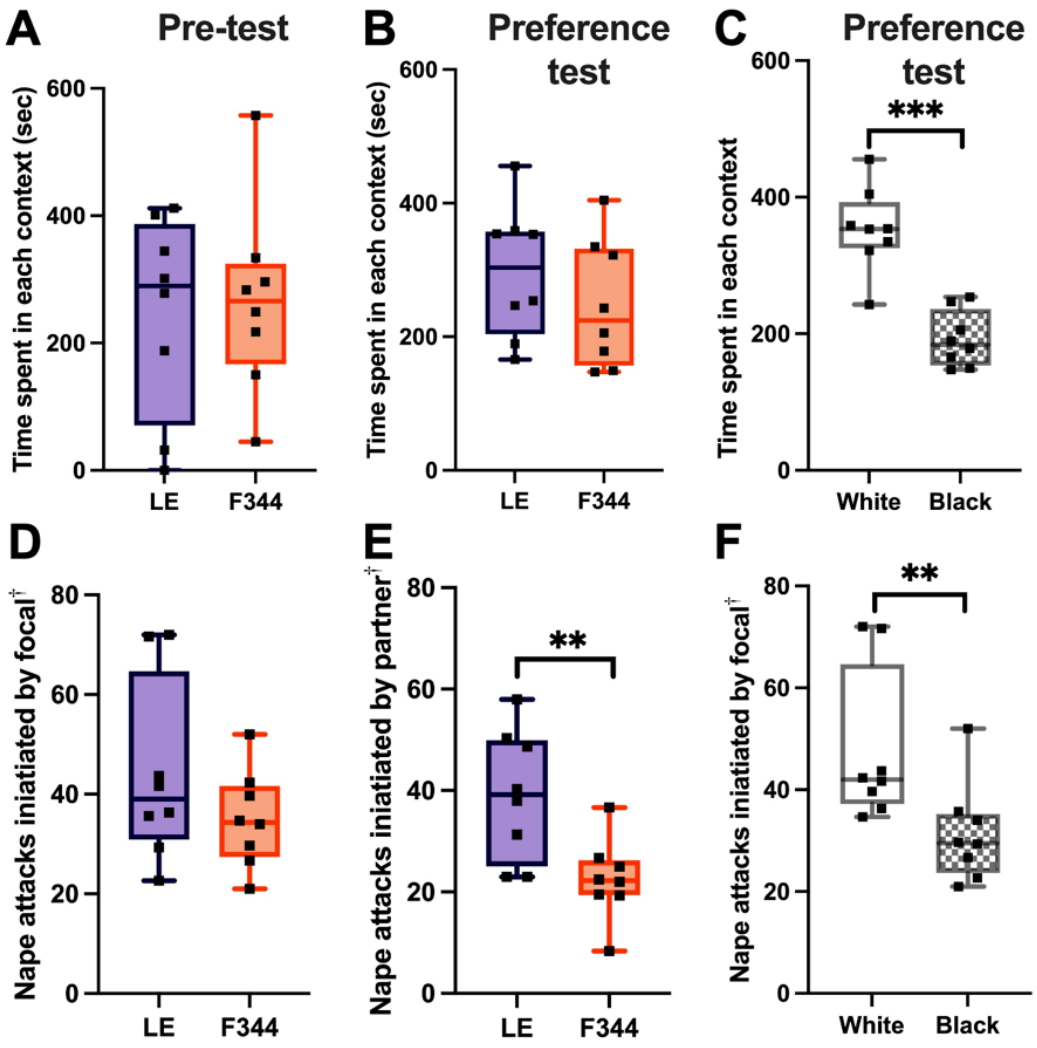


**Figure 5.4** The time spent in each box was not different between the to-be-paired LE and to-be-paired F344 contexts (A). After eight days of condition place preference training, focal rats did not prefer the LE social context over the F344 context (B). The focal rats did, however, prefer the white box over the black box, regardless of the social partner they experienced in the box (C). The focal rats did not initiate more play with LE or F344 rats (D). However, LE partners played more than F344 partners (E). Though focal rats did not play more depending on the social context, the focal rats initiated more play in the white box compared to the black box (F). †Attacks were measured on three different days (Day 1, 5, and 8) and the average frequency of attacks across the three days was used. $^{**}p < 0.01$; $^{***}p < 0.001$

Of all the USV emitted, the majority (93%) were trills, trill with jumps, and flat calls, so these were the call types we analyzed further. There was no interaction effect between the number of ultrasonic vocalizations recorded and the context paired with either strain ($F(3,42) = 0.165$, $p = 0.919$) (Figure 5.5A) nor for the white or black boxes ($F(3,42) = 0.622$, $p = 0.605$) during the preference test (Figure 5B). When we compared the percentage of vocalizations emitted in the LE vs F344 contexts, no interaction effect was found in the distribution of calls ($F(3,42) = 1.524$, $p = 0.222$) (Figure 5.5C). However, an interaction effect was found between the color of the box and the type of vocalizations emitted ($F(3,42) = 8.450$, $p = 0.0002$), with the focal rats emitting more trill with jumps in the white box and more flat calls in the black box (Figure 5.5D). Given that the focal rats did not spend equal amounts of time in each chamber, we also assessed the rate at which each vocalization type was used in each context. Focal rats vocalized at a different rate depending on the context ($F(3,42) = 72.85$, $p < 0.0001$), vocalizing at a higher rate in the F344 paired context (Figure 5.5E). Similarly, focal rats vocalized at a different rate depending on whether they were in the white or black box ($F(3,42) = 7.777$, $p = 0.0003$), emitting vocalizations at a higher rate in the black context (Figure 5.5F). However, for neither partner pairing nor box type pairing were there any significant differences in call types emitted.

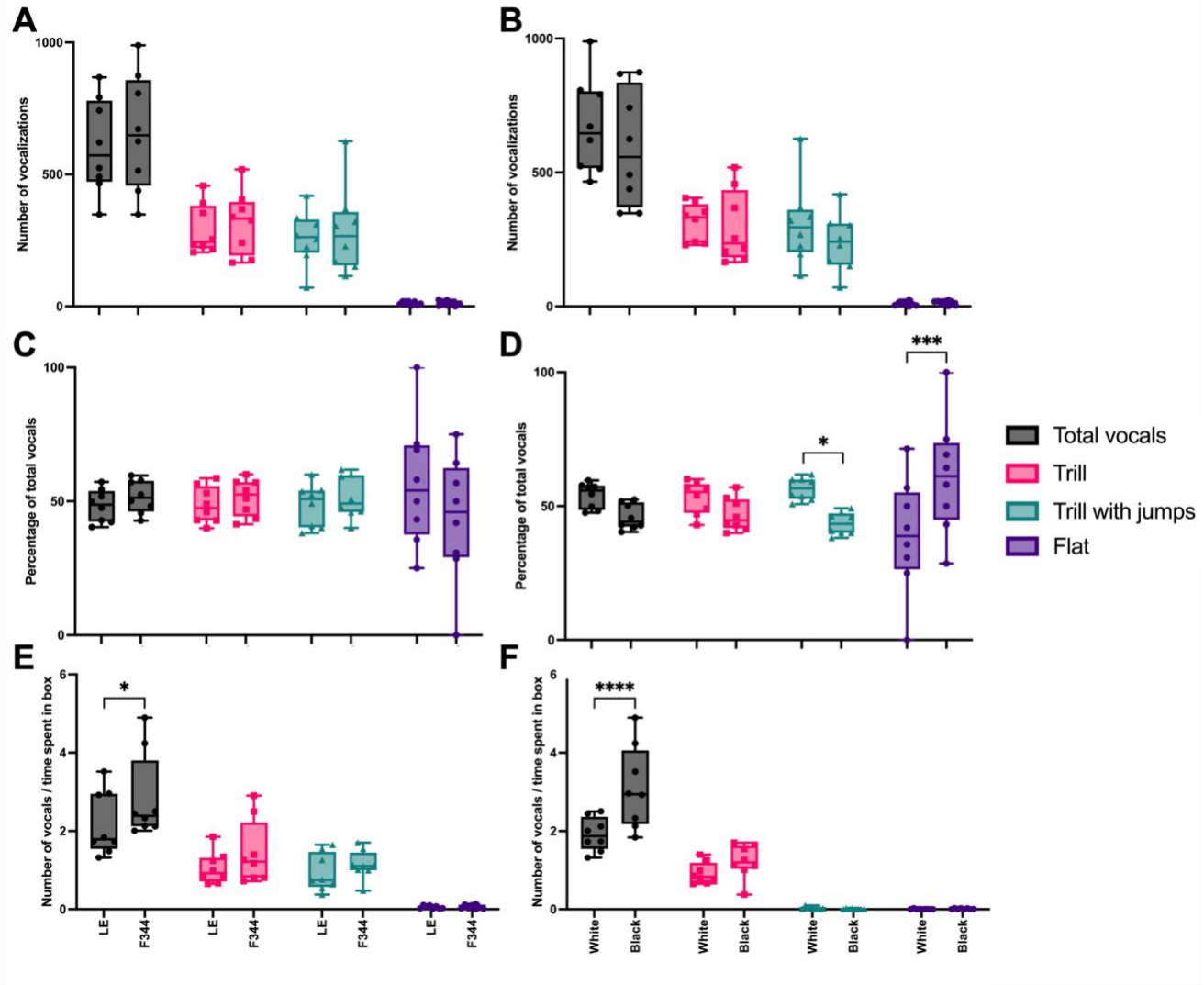


**Figure 5.5** The total number of vocalizations, trills, trill with jumps, and flat calls emitted in each paired context (i.e., LE or F344) did not significantly differ (A). Similarly, the number of calls did not differ depending on the box (i.e., white or black) (B). When calculated as a percentage, the total number of calls and the types of calls emitted did not differ between paired contexts (C). However, focal rats did emit more trill with jumps in the white box, compared to the black box, and more flat calls in the black box, compared to the white box (D). When calculated as a rate, more calls were made in the F344 context than the LE context (E) and more calls were made in the black box than the white (F), however, the call type did not differ between either context or box. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$

## 5.4 Discussion

Given that there is a considerable degree of variation in both the amount of play and the style of play among strains of rats (S. M. Himmler, Modlinska, et al., 2014; Siviy et al., 2023), we aimed to determine whether rats have play partner preferences based on strain identity in different contexts. To do so, we tested juvenile male LE rats and in three conditions. First, we tested the rats with unfamiliar partners in a group setting where they had the choice to play with either an LE, SD, or F344 rat. In the group play test, which was repeated twice on two consecutive days, focal rats consistently played more with the same-strain LE partner over the SD and F344. Unexpectedly though, SD were not preferred over F344 rats. However, both LE and SD partners were playfully attacked before the F344 rats were on Day 1 of testing, suggesting some preference of SD rats over F344 rats. Following the group play testing, the focal rats were paired with each strain, one at a time, in a dyadic play encounter. We found that the focal rats directed the same amount of play to all partners, suggesting that when rats do not have a choice, they will play with partners of different strains equally. That is, despite there being considerable differences in both the amount and the style of RTP among strains of rats (S. M. Himmler, Modlinska, et al., 2014; Siviy et al., 2023), when playing with members of a different strain in a dyadic context, the strain-typical differences are reduced, leading to a convergence in both frequency and style of play (Ham, Szabo, et al., 2024; Reinhart et al., 2006; Siviy et al., 1997, 2003; Stark et al., 2021). Although, it should also be noted that prior rearing with a different strain can affect the degree of such convergence (S. M. Himmler, Lewis, et al., 2014). That is, subject to modifying influences of the test paradigm used (see also Argue & McCarthy, 2015b), the dyadic test context can lead to the partners accommodating to each other's play styles, but again, some strains, such as the F344, are more resistant to such accommodation than others

(Ham, Szabo, et al., 2024; Siviy et al., 1997, 2003, 2017). When restricted to playing with a particular partner in the dyadic test paradigm, rats seem to adjust their play (Achterberg et al., 2023) in a manner that may not be necessary when multiple partners are available (Ham & Pellis, 2023, 2024, 2025), and, as shown here, a partner from a less suitable strain, can be avoided in preference to members of more suitable strains. Finally, we used a modified version of the play-induced socially conditioned place preference paradigm (Achterberg et al., 2014; Trezza et al., 2009, 2011) to determine if the focal LE rats would prefer the context in which they played with a compatible, same-strain play partner over an incompatible, F344 partner (Ham, Szabo, et al., 2024; Stark et al., 2021). The focal rats preferred the white box over the black box, and this was the box in which they played more, irrespective of the strain of the play partner. Interestingly, the focal rats vocalized at a higher rate in the black box, and in boxes that had been rewarded with F344 partners, indicating that while some parameters of 50 kHz, such as the number and types of calls may reflect positive affective states (Burke et al., 2017, 2021; Knutson et al., 1998), others, like rate of emission, may reflect overall level of arousal (e.g., Gaub et al., 2016; Raza et al., 2015). Consequently, it would seem that, at some level, the rats are distinguishing between both the box and the strain of the training partner.

### 5.4.1 Play is better than no play

When given a choice, we found that focal rats significantly prefer their own strain, however, when the choice in play partner is removed, the rats play equally with all partners (F344 or SD). That is, in a dyadic set-up, as is done in most play research, the focal rat will play with a less preferred partner as much as with a preferred partner. This may be in part due to the rats having been socially isolated (even if just for 2.5 h) before testing play. Though briefly isolating the rats to ensure they are motivated to play before testing is a

commonly used technique (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022), this increased motivation to play might influence the results from the dyadic tests. For instance, if the rats are highly motivated to play, and the only partner they have is a suboptimal one, the motivation to play may outweigh the reluctance to play with such a partner. Perhaps, if they were not isolated at all, and so not as motivated to play, they would only play with the LE partners and avoid both the SD and F344 partners during the group test. How the duration of social isolation and motivation to play affects partner selection will be the focus of future studies; however, preliminary results from our laboratory indicate that LE focal rats are not less selective with increased isolation time suggesting that the time spent in isolation did not bias the strength of their partner preferences. Alternatively, the lack of discrimination between SD and F344 rats in the group test may be due to the presence of an LE rat. As in the non-play social tests using adults, when given the choice, LE rats preferred to interact with other LE rats over other strains, but when the only choices available were SD rats, they preferred the SD rats over the F344 rats (Kiyokawa et al., 2022; Kogo et al., 2021). Similarly, if a group test for play only involved a choice between an SD and a F344 rat, LE rats may show a preference for SD play partners. That is, cross-strain preferences may still exist, but to characterize them, the appropriate testing paradigm needs to be identified.

That context is critical for identifying the occurrence of behaviors is one implication from this study, as LE rats tested in pairs did not show the same play partner preferences as when tested in groups. It should be noted that we chose F344 and SD rats because their style of play differs markedly from that of LE rats (S. M. Himmler, Lewis, et al., 2014; S. M. Himmler, Modlinska, et al., 2014; Siviy et al., 1997; Stark et al., 2021), which may possibly exaggerate the effects of partner preference in mixed-strain group play.

Indeed, we selected the three strains we did as they each play differently. However, their differences are graded. SD share a more similar play style to LE than F344 (S. M. Himmler, Himmler, Stryjek, et al., 2016; S. M. Himmler, Lewis, et al., 2014; S. M. Himmler, Modlinska, et al., 2014). Had we used strains that play more similarly to LE rats, such as Brown Norway and Wistar rats (S. M. Himmler, Modlinska, et al., 2014), we may not have observed such a strong preference during the group play testing. Still, even subtle differences within a strain appear to be sufficient for rats to form partner preferences in a group setting. For example, LE rats playing in groups of same-strain, same-sex partners, form partner preferences whether playing with strangers (Ham & Pellis, 2024), cage mates (Ham & Pellis, 2025), or with partners of mixed familiarity (Ham & Pellis, 2023). Nonetheless, as stated above, when rats do not have a choice and are motivated to play, they will play with whatever partner is available. However, when provided the opportunity to choose, they will select a same-strain partner (present study), or a samestrain partner with particular characteristics (Ham & Pellis, 2023, 2024, 2025). These contradictory results highlight the need to study ethologically relevant behaviors and to test animals under more naturalistic conditions (d'Isa & Gerlai, 2023; Dennis et al., 2021; Kondrakiewicz et al., 2019) as, depending on the question being tested, the paradigm used could bias the results. Indeed, had we only tested the rats using the dyadic paradigm, we may have concluded that LE rats do not prefer their own strain. For general or fundamental phenomena, such as the effect of pharmacological manipulation and deciphering which brain regions are involved in play, using play frequency, duration, and the microstructure of play as tested under the dyadic test set-up is sufficient for now (Achterberg et al., 2016, 2019; Achterberg & Vanderschuren, 2020; Trezza & Vanderschuren, 2008; J. W. VanRyzin et al., 2019; Veenema et al., 2013). However, to answer more ethologically

relevant questions, such as partner preference due to experimental manipulations or treatments, the group set-up may be better suited (Ham & Pellis, 2023, 2024; Holman et al., 2019).

In addition to partner discrimination, the focal LE rats played far more when in a group setting than when in a dyadic setting. On day 1, the focal rats launched on average 102.38 attacks, and 78.88 on day 2, that is roughly 4.2 times and 3.3 times more play than when tested in the dyadic context (Figure 3B). This large difference between test conditions suggests that there may be a group contagion effect whereby rats are more motivated to play when more individuals are present. Alternatively, it could be that the novelty of choice is motivating given that the animals were pair housed and so were never offered the opportunity to choose a partner. In either case, the increased frequency of play when in groups cannot be explained by the natural increase and decrease in playfulness with age. Play in rats is most frequent between 30-40 days of age, typically peaking around 35-36 days of age (Panksepp, 1981; Pellis & Pellis, 1990; Thor & Holloway, 1984a). In the present study, group testing occurred when the rats were 30 and 31 days old, and dyadic testing around 35 days, so, if anything, play should have been more frequent when tested in pairs.

### 5.4.2 Socially conditioned place preference: Partner or context?

Previous studies have shown that isolated rats prefer contexts that are paired with RTP over contexts without the opportunity to play (Achterberg et al., 2012, 2014; Trezza et al., 2009, 2011). Here, a slightly altered methodology was used to determine if rats would prefer a context in which they encounter a playful same-strain partner over a context where they encounter a playful partner from a different strain. We predicted that rats would prefer

the context in which they had a same-strain partner because the play experiences would be more compatible, both in style and frequency.

To our surprise, we found that the focal LE rats did not prefer the context that was paired with a same-strain partner. Instead, they preferred a particular box, regardless of the strain of the partner. The focal rats in our study significantly preferred the white box. Following previous protocols (Achterberg et al., 2012, 2014; Trezza et al., 2009, 2011), we varied the color of the enclosure and the texture of the floor so the rats could easily distinguish between the two contexts. However, unlike the previous sCPP studies where fine metal mesh and wide metal mesh flooring was used to distinguish the two contexts, we had one floor with metal rods lining the surface while the other floor was smooth. Despite us wanting the floor texture varied so to provide the rats an additional cue, we may have inadvertently biased the play experiences.

Depending on the context or the enclosure in which rats are tested, both the frequency and structure of play can be altered. For example, when tested in circular arenas, rats play significantly less than when tested in square or rectangular ones (Hole & Einon, 1984). If no bedding is provided in the enclosure, the rats will switch defense strategies, favoring evasive strategies over rotating to a supine position (Pellis & Pellis, 2021). Given that RTP in rats can be rough, it is suspected that having bedding to cushion the wrestling ensures that the play remains comfortable (Pellis & Pellis, 2021). Knowing this, we selected chambers that were both square (and of the exact same dimensions) and devoid of bedding, so as not to have one with bedding and one without. We believe this was achieved as we did not find a difference in the number of supine defences performed between boxes.

In addition to the texture of the floor, the color of the walls and floors of the boxes were varied and this could have possibly biased the animals even though it is standard to

both alter the floor and wall color (e.g., Martínez et al., 1995; McDonald et al., 2021; Roux et al., 2002). In the white enclosure, the floors were black while in the black enclosure the floors were white. It remains unclear if the color of the walls and/or floors, texture of the floor, or both biased the rats' behavior. For instance, considering the box preference that we observed, it is possible that a black floor is less alterting to rats and hence favors play. In future experiments, these factors (wall color, floor color, and floor texture) should be separated and tested singularly for their effects on RTP.

Regardless of the environmental cues that were salient to the rats, they played less in the black box during the training days and emitted different vocalizations during the preference tests depending on the context. In the white box, rats emitted more trills with jumps than in the black box. These trills have been associated with positive experiences (C. J. Burke et al., 2022; Reinhold et al., 2019) and seem to be used in coordinating play behavior (C. J. Burke et al., 2018, 2020; Kisko, Euston, et al., 2015). Conversely, 50 kHz flat calls, which are often used as contact calls (Brudzynski, 2015), frequently emitted after having been socially isolated (Wöhr et al., 2008), were emitted more in the black, unpreferred box.

Given that the animals did prefer a box, and they preferred the box they played in more, it seems that like previous studies (Achterberg et al., 2012, 2014; Trezza et al., 2009, 2011), the rats in this study formed preferences based on their play experiences. This preference, though, was not due to the social context (i.e., not the strain × context association). Instead, it seems that the attractive nature of the white box, whatever that may be, overpowered any effect of the play partner despite the F344 partners playing less than the LE partner, providing hence a different play experience. Rather, it is the environmental

context that facilitated the preferred experiences that is selected and not the partner during the sCPP preference test. In any case, this suggests that rats do prefer contexts in which they had experienced more attractive play experiences. However, this can be driven by the context/enclosure and not necessarily the identity of the partner. Nevertheless, because the rats were isolated for 23.5 h a day for eight consecutive days, they were highly motivated to play (Panksepp, 1981). After such chronic social isolation, socializing, regardless of the quality or nature of the interaction, may be so rewarding that the possible effect of the identity of the partner was masked. Consequently, the present findings do not preclude the possibility that in a different testing paradigm, rats would show a preference for one strain over another. Even so, as noted above, the focal rats had a higher rate of vocalizing when in both the least preferred black box and in the box, either white or black, that had involved playing with a F344 partner, which suggests that there may be subtle aspects of behavior that reflect preferences, including social ones. Again, this reinforces our earlier point that constructing the appropriate test paradigm and identifying the most appropriate behavioral markers are central to drawing firm conclusions about what is and what is not salient to the animals.

One limitation of this study is that we only used males. Indeed, the appropriate test paradigm and behavioral markers may need to differ for females despite male and female juvenile male rats using the same RTP behavioral patterns (Pellis, Pellis, Burke, et al., 2022). Female rats are more sensitive to the familiarity of their partner when playing in dyads, playing more with familiar animals than strangers (Argue & McCarthy, 2015a), and, similarly, they may be more sensitive to the strain of their play partners. As such, replicating this study with females may further refine the conclusions of this study.

## 5.5 Conclusion

The use of rat RTP has become increasingly popular as a tool for studying social regulation, communication, and development given that it is a naturally occurring behavior in which rats are highly motivated to engage (Pellis, Pellis, Ham, et al., 2022). While it is a powerful tool, our results highlight that the testing paradigm used can greatly affect the behavioral outcomes. For example, when LE rats are given the choice, they prefer to play with same-strain partners. However, this preference is no longer present when the testing paradigm was changed. When tested using the dyadic paradigm (Pellis, Pellis, Burke, et al., 2022), the rats did not play more with same-strain partners. Similarly, focal LE rats did not appear to prefer contexts that were paired with a same-strain partner after 8 days of conditioning using a modified sCPP paradigm (Achterberg et al., 2012, 2019; Trezza et al., 2009, 2011). Instead, the LE rats preferred the context in which they played more, regardless of the identity of the partner. In other words, the environmental or social context in which rats find themselves in may trump partner preferences. In addition to changes in partner preferences, we also found that when playing in groups, the rats played 3-4 times more, suggesting there is a group play contagion affect in rats. Our results underline the need to keep the natural ecology and ethology of the animal in mind when designing studies of play behavior in rats given that the testing paradigm, environment, and social partners used can greatly influence the behavioral outcome.

## PART II: PLAY! (WHAT IS IT GOOD FOR?)

"*Some children never incorporate into games. They run to a teacher for refuge or sit off to the side. Some children seem permanently unable to join in and take the rough and tumble 'in fun.' What are these individuals like as adults? … is there a critical period for developing the ability to rough and tumble, or are these children unusual in some way other than deprivation of the change to play at the right age?*"

— Nicholas Blurton Jones, *Play*, 1976

"*Numerous beneficial effects hypothesized for play…give this behavior the status of a wonder-working elixir. Apparently, play can do almost anything! … Why should play, rather than some other behavior having the same effect, be designed by natural selection to achieve this goal? … This habit results from approaching the search for function as if it were the search for the Philosopher's Stone.*"

— Robert Fagen, *Animal Play Behavior*, 1981

# CHAPTER 6: QUALITY NOT QUANITITY: DEFICIENT JUVENILE PLAY EXPERIENCES LEAD TO ALTERED MEDIAL PREFRONTAL CORTEX NEURONS AND SOCIO-COGNTIVE SKILL DEFICITS*

## 6.1 Introduction

Developing in social isolation during the juvenile period—from weaning to sexual maturity (Pagel & Harvey, 1993)—leads to adults with a wide range of behavioral, emotional, cognitive and neural abnormalities (e.g., Arakawa, 2018; Baarendse et al., 2013; Da Silva et al., 1996; Fone & Porkess, 2008; Hall, 1998; Potegal & Einon, 1989; Von Frijtag et al., 2002). Social play peaks during the juvenile period (Thor & Holloway, 1984b), so one implication of social isolation is that many of these deficits arise because of the lack of opportunity to engage peers in play (e.g., Einon & Morgan, 1977; van den Berg et al., 1999). However, as being reared in social isolation results in more than just being deprived of play (Bekoff, 1976), other ways to limit play without total social isolation have been developed (Pellis, Pellis, Ham, et al., 2023). For example, rearing rats (*Rattus norvegicus*) in the same cage but separated by a perforated partition that allows them to see, smell, hear and, to a limited degree, touch one another, provides some socialization, but prevents social play. This paradigm leads to neural, cognitive, emotional and behavioral deficits (Bell, 2014; Bijlsma et al., 2022, 2023; Marquardt et al., 2023; Pellis et al., 1999) in a similar fashion to total isolation, indicating that social play in the juvenile period is an essential behavior for proper neurobehavioral development. Rearing a juvenile rat or hamster (*Mesocricetus auratus*) with an adult, which allows for a wider range of social

behaviors, including some limited play (Pellis et al., 2017), also leads to neural and socio-cognitive abnormalities (e.g., Bell et al., 2010; Burleson et al., 2016; B. T. Himmler, Pellis, & Kolb, 2013). This finding further emphasizes the importance of peer-peer social play during the juvenile period, but may be confounded by stress effects; being reared with an adult can be stressful (A. R. Burke et al., 2010, 2013), which may contribute to the neural and socio-cognitive abnormalities (Stark et al., 2023). To determine more precisely how the quality and quantity of social play during the juvenile period affects neurobehavioral development therefore requires a slightly more refined approach to limit potentially confounding effects.

Rat strains differ from one another behaviorally in several dimensions, including the amount that they play and the preferred tactics used during play (B. T. Himmler, Stryjek, Modlinska, et al., 2013a; S. M. Himmler, Modlinska, et al., 2014; Keeley et al., 2015). One approach that has been used successfully to probe the effects of the quantity and quality of play on neurobehavioral development has been to rear rat strains that are more playful with strains that are less playful. Schneider and colleagues (Schneider, Bindila, et al., 2016; Schneider et al., 2014; Schneider, Pätz, et al., 2016) reared Wistar rats, a highly playful strain (S. M. Himmler, Modlinska, et al., 2014), with Fischer 344 (F344) partners, a low playing strain (Siviy et al., 1997, 2003). The Wistar rats reared with F344 partners experienced less and atypical play as juveniles and exhibited neural and pain threshold changes, as well as reduced social skills (Schneider, Bindila, et al., 2016; Schneider et al., 2014; Schneider, Pätz, et al., 2016). Following this paradigm, we reared Long Evans rats (LE), another highly playful strain (B. T. Himmler, Stryjek, Modlinska, et al., 2013a; S. M. Himmler, Modlinska, et al., 2014) with F344 peers, with similar results: LE rats reared with F344 peers experienced atypical play as juveniles (Stark et al., 2021),

as well as atypical neural architecture and deficient social skills as adults (Stark et al., 2023; Stark & Pellis, 2020, 2021).

Absent, reduced, or atypical play experience in the juvenile period leads to the development of impoverished socio-cognitive skills and to abnormalities in the physiology and anatomy of the neurons of the medial prefrontal cortex (e.g., Baarendse et al., 2013; Bell et al., 2010; Bijlsma et al., 2022, 2023; Burleson et al., 2016; B. T. Himmler, Pellis, & Kolb, 2013; Schneider, Bindila, et al., 2016; Stark et al., 2023; Stark & Pellis, 2020, 2021). This answers the question as to whether the deficits arising from being reared in social isolation (e.g., Fone & Porkess, 2008; Hall, 1998) result from the lack of play—at least some of the deficits can be attributed to the absence of social play. But if reducing play leads to impoverished development, can increasing play lead to improved outcomes?

Rearing Wistar and LE rats with F344 partners has the effect of significantly reducing the socio-cognitive skills of those rats relative to their peers raised with Wistar and LE partners, respectively (Schneider, Bindila, et al., 2016; Stark & Pellis, 2020, 2021). The logic behind this rearing paradigm was based on the premise that F344 rats are a low playing strain (Siviy et al., 1997, 2003), and indeed, juvenile Wistar and LE rats reared with F344 partners play less and/or atypically compared to peers reared with members of the same strain (Schneider, Bindila, et al., 2016; Stark et al., 2021). But it would be equally likely that F344 rats reared with Wistar or LE rats would experience more and/or qualitatively better play than F344 rats reared with F344 partners. If this were the case, it would be predicted that F344 rats reared with LE peers should have more refined socio-cognitive skills and neural anatomy than F344 rats reared with F344 peers. However, there is an alternative possibility.

The social play of rats mostly involves play fighting, and play fighting involves competing for an advantage, such as biting or otherwise contacting a particular location of the partner's body (Aldis, 1975; Pellis, 1988; Symons, 1978). In the case of rats, the body target is the nape of the neck which is nuzzled with the snout if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). However, unlike serious fighting, which also involves competing for an advantage (R. J. Blanchard & Blanchard, 1994; Geist, 1978; Pellis, 1997) and in rats this involves biting the opponent's rump and lower dorsum (R. J. Blanchard & Blanchard, 1977; Pellis & Pellis, 1987), play fighting involves an element of cooperation (Pellis & Pellis, 1998) which ensures turn-taking, making the interactions reciprocal (Palagi, Cordoni, et al., 2016; Pellis & Pellis, 2017). Being paired with a mismatched partner, be it rats of different ages or different strains, leads to less turn taking and so a reduction in reciprocity (Pellis et al., 2017, 2018; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Moreover, most of the role reversals in pairs of LE-F344 rats are produced by the LE partner (Stark et al., 2021).

Given that role reversals seem to be particularly important experiences derived from play fighting that contribute to facilitating the development of socio-cognitive skills (Pellis et al., 2019), even if the amount of play of F344 rats were increased by interacting with LE peers, the quality of the play may not. Rat strains such as LE, Wistar and Sprague-Dawley seem to be particularly averse to interacting with F344 rats (Kiyokawa et al., 2022; Kogo et al., 2021). Perhaps, as the former three strains are more closely related to each other than any are to F344 (Lindsey & Baker, 2006), they may not be perceived as compatible partners. Indeed, LE and Wistar rats play more similarly to each other than they do to Sprague Dawley rats (S. M. Himmler, Modlinska, et al., 2014), and when reared with Sprague-Dawley peers, LE rats modify how they play (S. M. Himmler, Lewis, et al., 2014),

and Wistar rats reared with Sprague Dawley peers show no long-term effects on socio-cognitive skills as they do when reared with F344 peers (Schneider, Bindila, et al., 2016). That is, the differences between the play of F344 rats and other strains, such as LE rats, may create a chasm in the quality of the exchanges that cannot be overcome by any increase in the amount of play. If so, whatever the intrinsic deficiencies in the play of F344 rats (Siviy, 2020), when reared with LE peers they should have worse developmental outcomes than when reared with F344 peers.

In the present paper, male juvenile F344 rats were reared with either a same sex F344 or LE peer and then compared as adults for socio-cognitive skills and for the development of the neurons of the mPFC. Social competency was evaluated with the stranger test, in which two adult rats unfamiliar with one another are placed together in a neutral enclosure (L. K. Smith et al., 1999). If being reared with a higher playing peer improves socio-cognitive skill development, then F344 rats reared with LE peers should perform better, that is, more likely to play and less likely to escalate to aggression when tested with F344 strangers than those reared with F344 peers. In contrast, if the problem is in reduced coordination of play between peers of different strains, then the reverse should be the case.

As well as play deprivation resulting in pyramidal neurons of the mPFC with altered physiology (Baarendse et al., 2013; Bijlsma et al., 2022, 2023), LE rats reared with adult partners or with F344 peers have adult neurons that have a larger dendritic arbor than do LE rats reared with LE peers (Bell et al., 2010; B. T. Himmler, Pellis, & Kolb, 2013; Stark et al., 2023). Play in the juvenile period prunes the dendritic arbor of mPFC pyramidal neurons and this effect is present in both sexes (Stark et al., 2023). If being reared with a higher playing peer increases dendritic pruning, then F344 rats reared with LE peers should

have a smaller dendritic arbor than those reared with F344 peers. In contrast, if the problem is in reduced coordination of play between peers of different strains, then the reverse should be the case.

## 6.2 Materials and methods

### 6.2.1 Subjects

Twenty-six Fischer-344 (F344) male rats and 10 Long Evans (LE) male rats were purchased from Charles River Laboratories (Kingston, New York) and arrived at the Canadian Centre for Behavioural Neuroscience (CCBN) at 24 days of age. At 26 days of age, the rats were housed in dyads composed of F344-F344 ($n$ = 8) or F344-LE ($n$ = 10) pairs. Eighteen additional F344 male rats were purchased at 75 days of age from Charles River Laboratories (Kingston, New York). These rats were housed in pairs and were used as the unfamiliar partners for the stranger test (see below) when all animals were 80 days of age. All animals were housed in polyethylene cages (46 cm x 25 cm x 20 cm) with corncob bedding. Food and water were available *ad libitum***.** The rats were housed on a 12-hour light-dark cycle (lights on between 0730 and 1930) in a room maintained at a constant temperature of 21°C-23°C. All care and testing procedures were approved by the University of Lethbridge Animal Welfare Committee (Protocol #1809) in compliance with guidelines from the Canadian Council for Animal Care.

### 6.2.2. Apparatus

Social play was tested when the rats were juveniles and again as adults in a sound-attenuating chamber with a clear Plexiglas® enclosure (50 cm x 50 cm x 50 cm) placed inside. The floor of the Plexiglas® enclosure was covered in 2-3 cm of CareFresh® bedding. To dampen external noise, the chamber was lined with sound-attenuating foam (Primeacoustic, Port Coquitlam, British Columbia). Interactions were filmed using an

ExmourRS 4K Sony Handycam with night-shot capability, which was placed in a small window of the chamber at a 45° angle. Using only IR lights, which were placed inside the chamber, the recordings were made using the night-shot capabilities of the camera.

### 6.2.3 Procedure

For juveniles, play was tested between 33 and 38 days of age, the peak play period for rats (Thor & Holloway, 1984), and between 80-90 days, which corresponds to young adulthood after sexual maturity (Pellis & Pellis, 1990). Before their play was tested at both ages, the animals were habituated to the testing enclosure with their cage mates for 15 min a day, for four consecutive days. The only difference was that, as juveniles, they were tested with their cage mates for the play trials, but as adults they were tested with an unfamiliar same strain, same age, and same sex partner (Figure 6.1). Both the play trials and habituation sessions took place in the dark, during the day, with only the infrared lights on. Prior to the play trials, the rats were socially isolated for a 24 h period to increase playfulness (Panksepp & Beatty, 1980; Pellis, Pellis, Burke, et al., 2022; Pellis & Pellis, 1990). For the play trials, the pairs were placed into the test enclosure simultaneously, and left there for 10 min, before being retrieved and placed back in their home cages. Fresh bedding was replaced and the Plexiglas® box was cleaned with Virkon® after each trial to reduce any odors left from the previous rats.

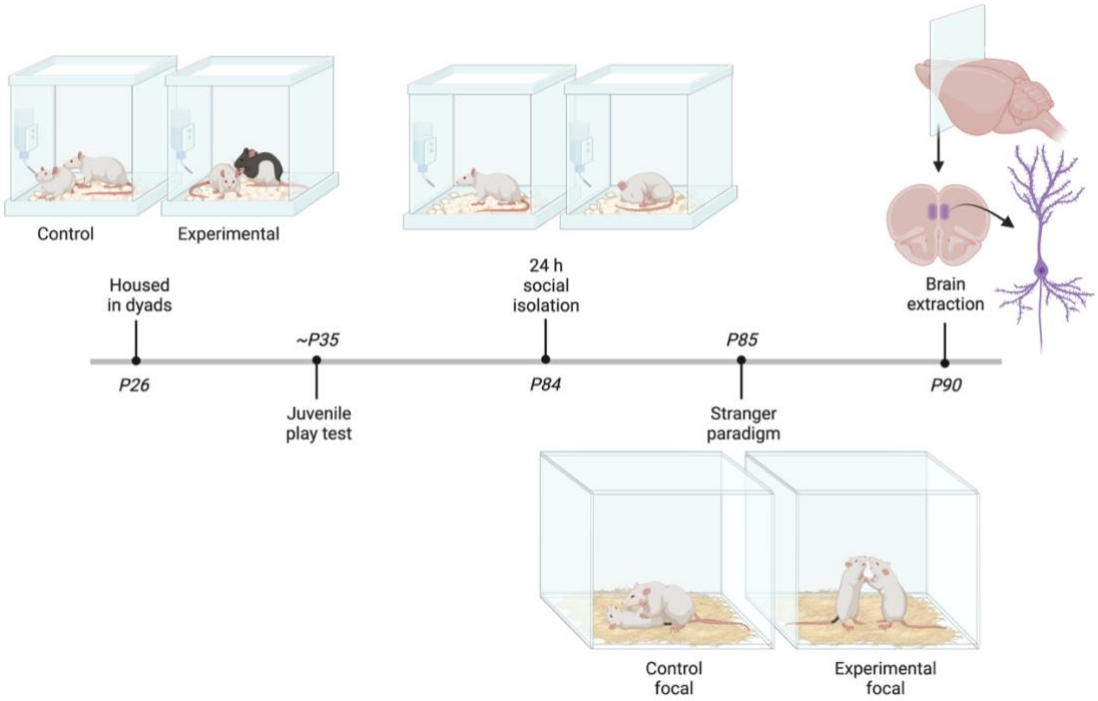


**Figure 6.1** The diagram illustrates how the animals were housed in same and mixed strain pairings (P26), when juvenile play testing occurred (~P35), how animals were socially isolated and then tested with an unfamiliar animal (P84, P85), and when the animals were sacrificed (P90). Created with BioRender.com.

### 6.2.4 Behavioral analysis

Following both juvenile and adult play trials, the video files were analyzed using a combination of both normal speed and frame-by-frame analysis to score play behavior (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022), and for the adult encounters, agonistic behavior (Kisko, Euston, et al., 2015; L. K. Smith et al., 1999; Stark & Pellis, 2021). All videos were scored twice, once noting the actions initiated by the focal animal and directed towards the partner, and the second time noting the actions initiated by the partner and directed towards the focal animal. Playful attacks were scored when the snout of one rat was in contact with, or moved towards, the nape of the other rat (Pellis, Pellis, Burke, et al., 2022). Once an attack was initiated, the recipient either continued with

its ongoing behavior or defended itself, from which the probability of defense was calculated. If the recipient defends itself, it could do so by either evading (running away from the attacker) or by turning to face the attacker. There are several types of facing defense, and these typically lead to particular inter-animal postural configurations (Pellis & Pellis, 1987). Two common configurations include the pin, in which the defender lies on its back, and the other stands on top, and the mutual upright position, in which the two partners rear on their hindfeet and grapple with their forepaws (Pellis, Pellis, Burke, et al., 2022). Some defensive tactics, such as rotating cephalocaudally to supine, are likely to lead to pinning and some, such as rotating vertically around the pelvis to face an attacker approaching from the rear, are likely to lead to mutual uprights (for detailed descriptions, see B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022). Given that previous studies have reported that the distinguishing features of playful defense by F344 rates are low rates of pinning and high rates of evasion (Siviy et al., 1997, 2003), for the present study, it was this initiation of attacks and resulting defensive action that were scored for statistical analysis.

As noted above, an important feature of play fighting is that animals engage in actions which allow partners to reciprocate roles, or take turns in attacking and defending (Palagi et al., 2016; Pellis & Pellis, 2017). In rats, this often involves cooperative actions that ensure the partner can gain the advantage (Pellis et al., 2005). To assess and compare this cooperative aspect of play, the proportion of play fights that lead to a role reversal was scored (S. M. Himmler, Himmler, Pellis, et al., 2016). Another good marker of reciprocity is the degree of symmetry in the playful actions of the partners in a pair (Stark et al., 2021). To assess the degree of symmetry in play, intra-pair differences in launching nape attacks were scored. The number of attacks by one partner was subtracted from the other, and the

absolute difference was divided by the total play score of the pair, resulting in values between 0 and 1. Values close to zero indicate the play was symmetrical whereas values close to 1 indicate a high degree of asymmetry in the initiation of playful attacks between the partners (Pellis, Pellis, Burke, et al., 2022).

The mean number of nape attacks per 10 min, the proportion of nape attacks being defended, and the proportion of nape attacks leading to evasion and pins were scored and calculated for both partners from each pair. As the subjects of this study were the F344 rats, in F344-LE pairs, it was the F344 partner that was selected as the focal animal, and for the F344-F344 pairs, one subject from each pair was randomly assigned as the focal animal for comparison between rearing conditions. Because the behavior of one partner can influence behavior of the other partner (Pellis, Pellis, Burke, et al., 2022), not only the focal rats from the two conditions were compared, so were their partners. Whereas attack, defense, type of defense and pins were scored for each pair mate, the likelihood of role reversals and the degree of symmetry were scored as pair measures and so it is the pair scores that were compared between the F344-F344 and F344-LE pairs.

When unfamiliar male adult rats play, it is rougher than the play in juveniles and among familiar adult males (Pellis & Pellis, 1987, 1992). Because of this increased roughness, the risk of escalating to serious aggression is greater (L. K. Smith et al., 1999). Indications of escalation can be identified by the rats using agonistic threat signals, such as piloerection, tail rattling and the lateral posture and if the encounter escalates further, with bites, typically directed to a lower flanks or rump (Blanchard & Blanchard, 1977; Takahashi & Lore, 1983). Male rats with defective communication and poor social skills are less able to prevent playful encounters with strangers from escalating (C. J. Burke, Kisko, Pellis, et al., 2017; Kisko, Euston, et al., 2015; Stark & Pellis, 2020). Therefore, as

well as the playful actions performed (see above), the occurrence and mean number of threat signals and bites per 10 min between pairs were scored for comparison between F344 rats reared with a F344 peer versus an LE peer when interacting with an unfamiliar adult male F344.

In previous studies, focusing on LE rats reared with F344 partners, both males and females were analyzed, revealing no sex differences (Stark et al., 2021, 2023; Stark & Pellis, 2020, 2021). Here, only males were used as male rats form more clearly delineated, dominance hierarches (Barnett, 1975; Ziporyn & McClinktock, 1991), and as a consequence playful interactions among unfamiliar adult males Smith eta al., 1999) more readily escalate to aggression if there is a social deficiency (Kisko et al., 2015; Stark & Pellis, 2020). In contrast, female dominance relationships involve more subtle gestures (Ziporyn & McClinktock, 1991), making the stranger test less clear-cut to assess social deficiencies in females (Stark & Pellis, 2021). As shown in the present paper, adult male F344 rats are not as assertive as LE rats, making the stranger test still useful but weaker in this strain. Therefore, we concentrated our efforts on males, leaving the effects of cross-strain rearing on female F344 rats to be dealt with in a future study using tests of adult social cognition to which females are more sensitive (e.g., Schneider, Bindila et al., 2016).

### 6.2.5 Histology

Animals were anesthetized at 90 days of age with 300 mg/kg of sodium pentobarbital and perfused, intracardially, with 0.9% saline. The brains from 8 control animals and 10 experimental animals were extracted and weighed. Following a standard Golgi staining protocol (Gibb & Kolb, 1998), the brains were placed in Golgi-Cox solution for 14 days after which they were placed into a 30% sucrose solution for at least 5 days before sectioning. Brains were sectioned using a vibratome at a thickness of 200 μm.

Sections were mounted onto slides coated in 3% gelatin and stored in a light-proof box, wrapped in a damp paper towel as to prevent drying. After 1-2 days, the slides were rinsed in distilled water and ammonium hydroxide and then placed in a 1:1 solution of Kodak Rapid Fix (Kodak, Rochester, NY) and distilled water. To dehydrate the slides, the brain tissue was moved through a graded ethanol series, cleared with a 1:1:1 solution of chloroform, Hemo De (Electron Microscopy Sciences catalog No. 23410-04), and 100% ethanol. Slides were placed in Hemo De before they were cover-slipped with Permount (Fisher Scientific, Catalog No. SP15-500). Slides were left to dry for at least l month or until they were dry to the touch.

### 6.2.6 Anatomical analysis

We selected neurons from area Cg3 (prelimbic cortex) of the mPFC as defined by Zilles (1985), for imaging (Figure 6.2). Neurons selected were between Bregma 4.2 mm and 2.77 mm from the rostral-caudal plane (Zilles, 1985). Using an Olympus VS120 digital slide scanner, with a 40x oil objective (UPlanFL N, 40x/1.30 oil, ∞/0.17/FN26.5) and Olympus VS-ASE FL software, virtual slides of Cg3 were created (Brinkman et al., 2022; Stark et al., 2023). The virtual slides consisted of 147 z-stacked images spaced 0.68 μm apart throughout the section, yielding 99.96 μm of working distance. To reconstruct the neurons, the images of the slides were uploaded into Neurolucida 360® (MicroBrightfield, Williston, VT, USA). The apical and basilar dendrites from pyramidal neurons from layer III were then traced. Cells were only selected if: (1) the cell was fully impregnated and not obscured by large truncations of debris, stain precipitation, or blood vessels; and (2) the cell had to lie centered within the plane z-stack, ensuring that all dendritic branching could be traced. A minimum of three cells were traced from each hemisphere. A total of 172 neurons were traced across all animals.

Using Neurolucida Explorer®, replicating the measurements taken of LE rats by Stark et al. (2023), the following measurements were extracted for statistical analysis: (1) the convex hull volume for both apical and basal projections; (2) the convex hull surface area for both apical and basal projections; (3) the sum of the total length of the apical and basal projections and the length of the cell body, which together obtained the total cell length; (4) the sum of the volume of the apical and basal projections and the volume of the cell body, which together provided a measure of the total volume of the cell; (5) the branch number for both apical and basal projections, which was summed for the total branch number; and finally, (6) the branch order for both apical and basal projections, which was summed for a total branch order.

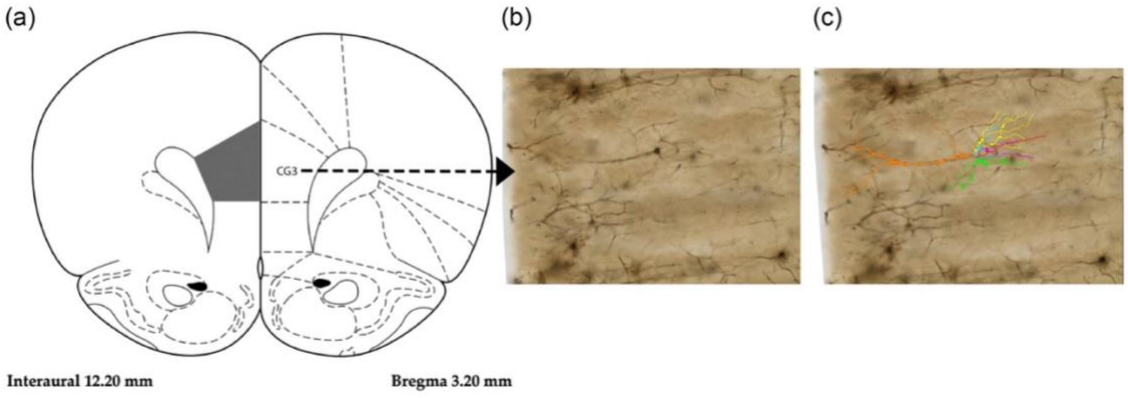


**Figure 6.2** A diagram of a rat brain section through the medial prefrontal cortex (a) (from Paxinos & Watson, 1986). After digitally scanning the slides (b), neurons were traced digitally (c).

### 6.2.7 Statistical analyses

We compared the behavior and outcomes of the focal F344 rats reared in F344-F344 pairs with the focal F344 rats reared in F344-LE pairs. The data for juvenile play of the F344 focal rats from the F344-LE pairs are from a previous publication (Stark et al., 2021), and were scored by the same observers. All the analyzed data on adult stranger interactions and neuroanatomy are new for the present study.

As the behavioral measures involved specific items to be compared between two groups, a Welch t-test was used in each case. The play of focal rats paired from the two conditions were compared. The partners of the focal rats were similarly compared. Measures of Cg3 cortical neurons were assessed with a linear mixed model, using the *lme4* package (Bates et al., 2015), to compare the effects of rearing condition. Rearing condition was set as the independent fixed effect, and a random error term was assigned to account for the repeated measures of individual animals, with hemisphere nested within every subject. The significance level for all tests was set at $p < .05$. Differences were considered significant if $p \leq 0.05$ using the Satterthwaite's approximation ANOVA. All statistics were performed using R Studio (R Core Team, 2020) using the *ggplot2* package (Wickham, 2016) to create graphs.

## 6.3 Results

### 6.3.1 Juvenile behavior

F344 rats launched significantly more playful attacks toward F344 partners than toward LE partners ($t(9.57) = 14.21$, $p < .00001$) (Figure 6.3a), and were more likely to defend themselves when attacked by F344 partners ($0.95 \pm 0.01$) than LE partners ($0.72 \pm 0.07$) ($t(15.39) = 8.48$, $p > .0001$). Additionally, the proportion of playful attacks that resulted in pins was significantly greater for focal rats playing with F344 partners ($0.35 \pm$

0.02) than with LE partners (0.09 ± 0.04) ($t$(13.74) = 5.22, $p$ = .0001). With regard to type of defence, focal F344 rats were just as likely to evade attacks by F344 rats (0.48 ± 0.03) or LE rats (0.52 ± 0.16) ($t$(9.54) = -0.23, $p$ = .82).

The F344 partners launched significantly more nape attacks than did LE partners ($t$(12.61) = 4.16, $p$ = .001, Figure 6.3b) and were more likely to defend themselves (0.95 ± 0.008) than were LE partners (0.86 ± 0.04) ($t$(13.19) = 4.48, $p$ = .0006). Additionally, the proportion of playful attacks that resulted in pins was greater for F344 partners (0.41 ± 0.04) than LE partners (0.29 ± 0.02) ($t$(9.06) = 2.86, $p$ = .02). With regard to type of defence, the proportion of playful attacks that resulted in evasions was significantly greater for F344 partners (0.46 ± 0.03) than LE partners (0.05 ± 0.02) ($t$(12.94) = 12.81, $p$ < .00001).

Play between F334-F334 pairs was significantly more likely to lead to role reversals than F344-LE pairs ($t$(9.54) = 5.79, $p$ = .0002, Figure 6.3c), and playful nape attacks were significantly more symmetrical in F344-F344 pairs than F344-LE pairs ($t$(13.11) = -8.13, $p$ < .00001, Figure 6.3d).

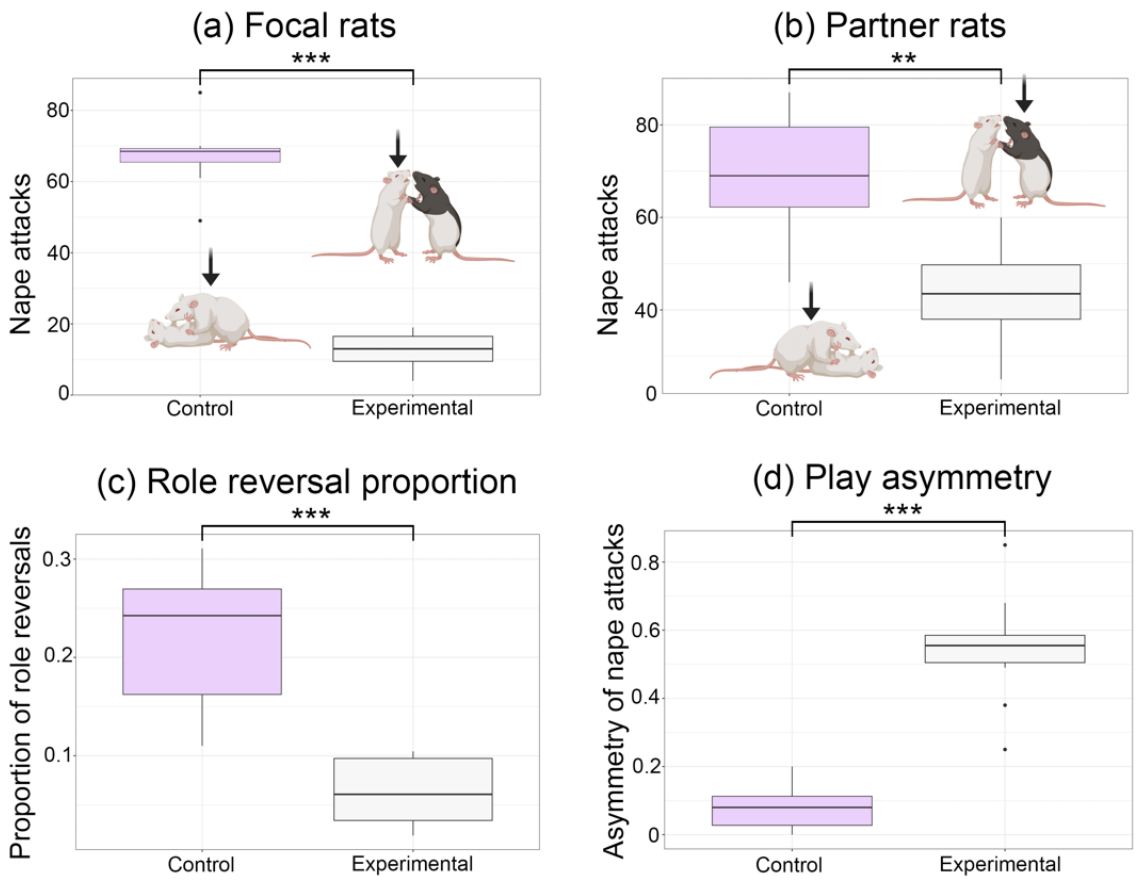


**Figure 6.3** The number of nape attacks initiated by the focal rats in control (F344-F344 pairs) and experimental (F344-LE pairs) rearing conditions was significantly different (a). The number of nape attacks initiated by the focal animal's partner was also significantly different (b). The proportion of playful attacks between pairs that led to a role reversal was significantly different when comparing the two rearing conditions (c). As a symmetry score closer to zero indicates a more symmetrical relationship, panel (d) illustrates that control partners had significantly more symmetrical play relationships compared to experimental pairs. Rat images were made with BioRender.com.

### 6.3.2 Adult behavior

Focal rats reared with a F344 partner launched significantly more playful nape attacks against the F344 'stranger' than did those reared with a LE partner ($t(8.01) = 3.77$, $p = .005$) (Figure 6.4a). Focal rats from both rearing conditions were equally likely to defend themselves when playfully attacked by the stranger ($p > .05$). F344 rats from both rearing conditions were equally likely to be pinned ($p > .05$), but focal rats reared with a

F344 rat were more likely to evade playful attacks compared to focal rats reared with a LE rat (0.85 ± 2.21 versus 0.33 ± 2.21, respectively) ($t(14.24) = 2.50$, $p = .03$).

The 'stranger' partners of the focal rats launched significantly more playful nape attacks towards focal rats that had been reared with F344 than with LE partners ($t(14.96) = 2.84$, $p = .01$) (Figure 6.4b). When stranger rats were playfully attacked by a focal rat, they were equally likely to defend themselves, regardless of their attackers rearing condition ($p > .05$). Additionally, regardless of the focal rats' rearing condition, when defending themselves, stranger partner rats were equally likely to be pinned ($p > .05$) or use an evasion tactic ($p > .05$).

Role reversals were significantly more likely in pairs in which the focal rat had been reared with a F344 partner (Figure 6.4c), and although not significantly different ($p > .05$), aggressive behaviors were only observed in pairs of strangers in which the focal rat had been reared with LE partners (Figure 6.4d). Moreover, the escalation that did occur in these pairs was equally likely to be initiated by the focal rat (1.8 ± 1.01) or the partner (1.4 ± 0.73) ($p > .05$).

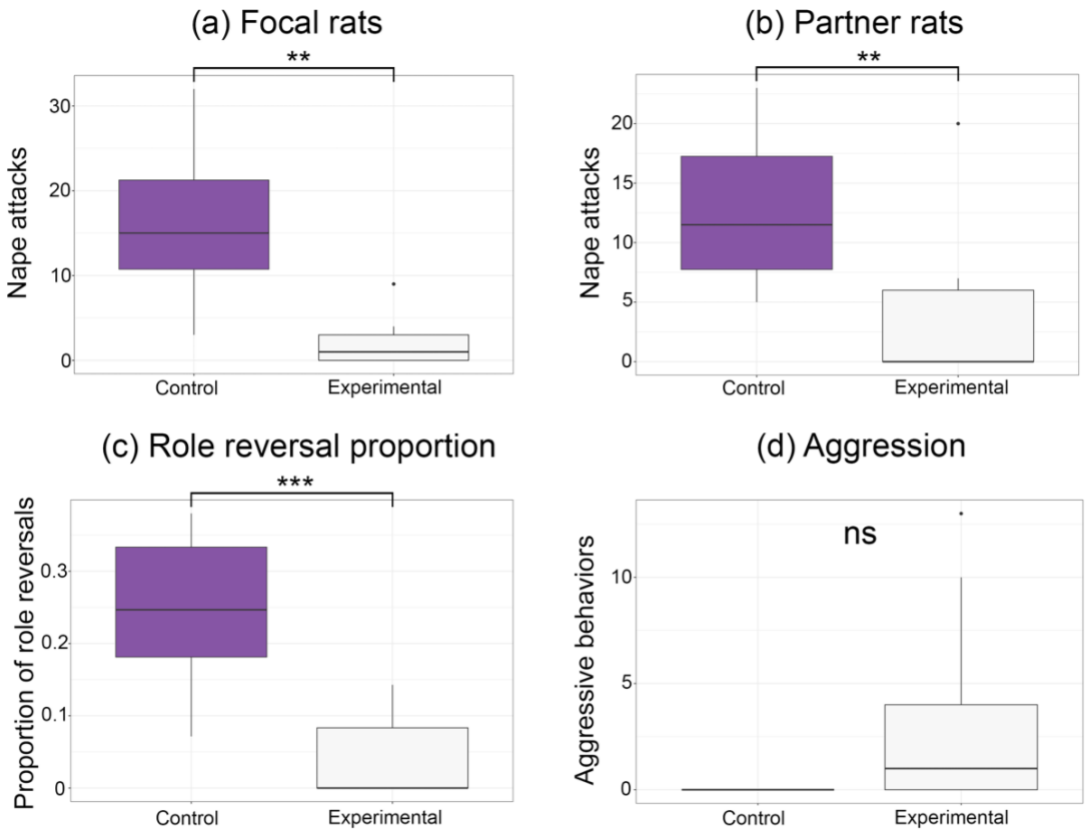


**Figure 6.4** The number of nape attacks initiated by the focal rats interacting with an unfamiliar animal was significantly different when comparing animals reared in the two conditions (a). The number of nape attacks initiated by the focal animal's unfamiliar partner was also significantly different (b). The proportion of playful attacks between the focal rats and unfamiliar rats from that led to a role reversal was significantly different when comparing the two rearing conditions (c). Although not significant, while some pairs of rats escalated to aggression, only experimental animals (i.e., the F344 reared in F344-LE pairs), interacting with a stranger escalated to aggression while control rats (i.e., F344 reared in F344 in F344-F344) never did (d).

### 6.3.3 Neuronal morphology

There were significant effects of rearing condition on both convex hull volume ($F(1,16) = 33.34$, $p < .00001$) and surface area ($F(1,16) = 24.50$, $p = .0001$). This significant effect of rearing condition also extended to the convex hull volumes and surface areas of both the apical ($F(1,16) = 41.08$, $p < .00001$, $F(1,16) = 33.88$, $p < .00001$, respectively) and basilar dendritic projections ($F(1,16) = 17.85$, $p = .0006$, $F(1,16) = 14.24$, $p = .002$, respectively) (Table 1, Figure 6.5a, b). For all convex hull volume and surface area measures, F344 rats reared with F344 partners had neurons with reduced dendritic arbors compared to F344 rats reared with LE partners (Figure 6.5c).

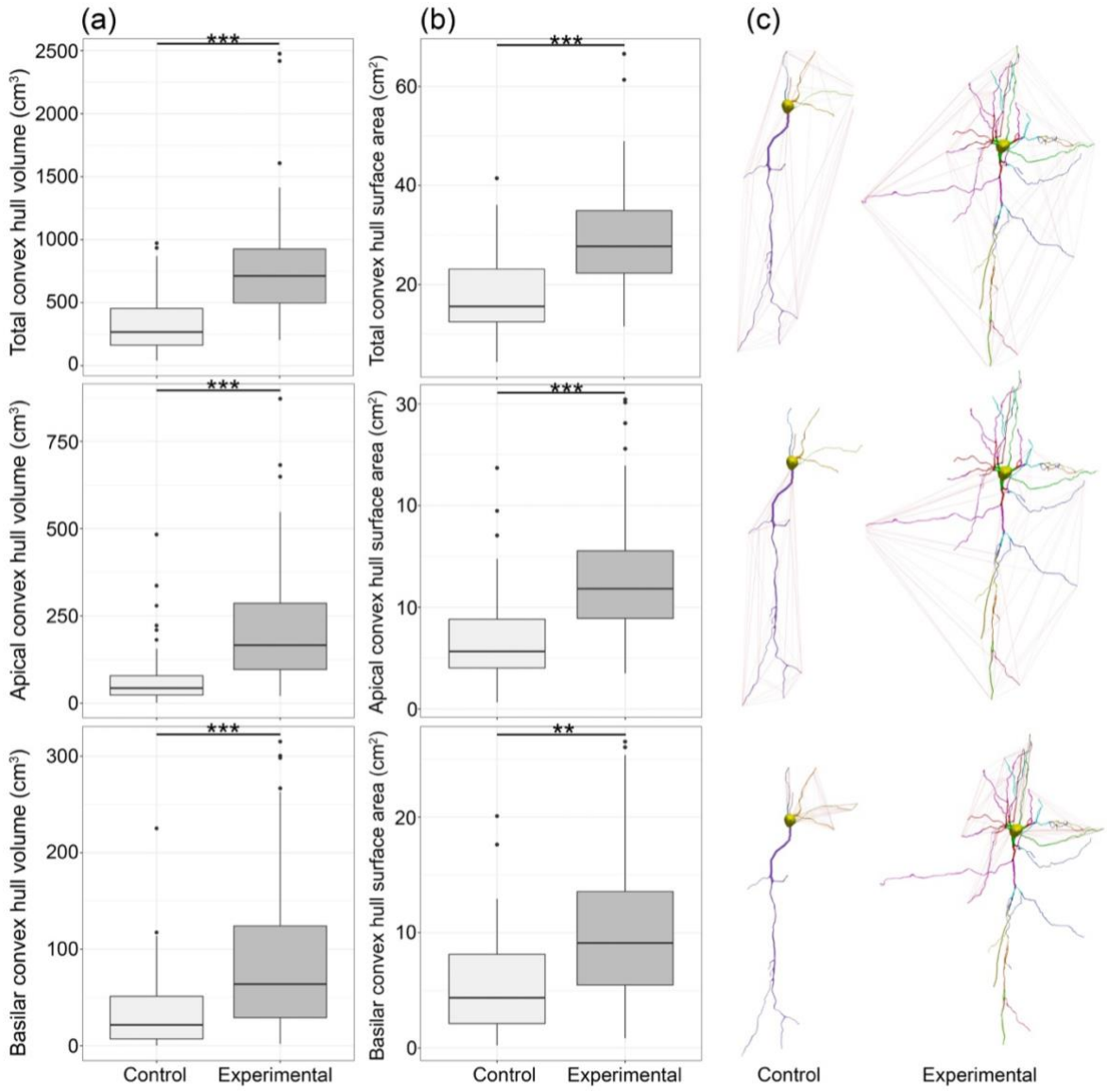


**Figure 6.5** The boxplots illustrate the results for the total convex hull, apical convex hull, and basilar convex hull analyses, for each neuron measured, showing both the volume (a) and surface area measures (b). Digital reconstructions illustrate the size differences of the neurons, with webbing showing the region of the cells being scored (c). Control = F344 from F344-F344 pairs and experimental = F344 from F344-LE pairs.

**Table 6.1** The average measurements (± standard error of the mean) of pyramidal neurons found in Cg3, layer III, of male rats that were either reared with a same-strain Fischer 344 (F344) individual or a mixed-strain Long Evans (LE) individual. The percent difference in the various measures was calculated for cases when significant differences were found.

| Measurement | Control (F334 from F344-F344 pairs) | Experimental (F334 from F344-LE pairs) | % difference |
|---|---|---|---|
| Convex hull volume ($cm^3$) | 342.62 ± 26.70 | 757.55 ± 39.94 | 57.52* |
| Apical convex hull volume ($cm^3$) | 66.77 ± 9.08 | 207.62 ± 16.04 | 82.57* |
| Basilar convex hull volume ($cm^3$) | 33.43 ± 4.30 | 86.69 ± 7.84 | 69.37* |
| Convex hull surface area ($cm^2$) | 18.04 ± 0.94 | 28.85 ± 0.98 | 33.31* |
| Apical convex hull surface area ($cm^2$) | 6.84 ± 0.49 | 12.88 ± 0.61 | 45.45* |
| Basilar convex hull surface area ($cm^2$) | 5.55 ± 0.47 | 10.39 ± 0.64 | 45.05* |
| Length (mm) | 3.37 ± 0.11 | 4.47 ± 0.13 | 22.84 |
| Apical length (mm) | 0.85 ± 0.04 | 1.38 ± 0.05 | 33.81* |
| Basilar length (mm) | 1.39 ± 0.08 | 2.19 ± 0.10 | 32.22* |
| Cell body length (mm) | 1.12 ± 0.03 | 1.10 ± 0.03 | 1.22 |
| Volume ($mm^3$) | 13.25 ± 0.50 | 13.35 ± 0.45 | 0.51 |
| Apical volume ($mm^3$) | 2.60 ± 0.15 | 2.95 ± 0.16 | 8.67 |
| Basilar volume ($mm^3$) | 1.53 ± 0.10 | 2.12 ± 0.15 | 23.00 |
| Cell body volume ($mm^3$) | 9.13 ± 0.40 | 8.28 ± 0.35 | 6.38 |
| Branch number | 15.62 ± 0.85 | 25.30 ± 1.10 | 34.25* |
| Apical branch number | 5.92 ± 0.45 | 11.04 ± 0.55 | 44.75* |
| Basilar branch number | 9.70 ± 0.63 | 14.26 ± 0.72 | 27.12* |
| Branch order | 23.89 ± 0.99 | 35.06 ± 1.23 | 26.96* |
| Apical branch order | 6.95 ± 0.45 | 12.25 ± 0.56 | 40.56* |
| Basilar branch order | 16.95 ± 0.78 | 22.81 ± 0.87 | 20.69* |

*Denotes a significant difference ($p < 0.05$), actual $p$ values are reported in text

Similarly, there was a significant effect of rearing condition on total branch number ($F(1,16) = 8.90$, $p = .009$), apical branch number ($F(1,16) = 11.75$, $p = .003$), and basilar branch number ($F(1,16) = 5.81$, $p = .03$). Additionally, there were significant effects on the total branch order ($F(1,16) = 9.84$, $p = .006$), apical branch order ($F(1,16) = 12.45$, $p = .003$) and basilar branch order ($F(1,16) = 7.02$, $p = .02$) (Table 6.1). For all three measurements, the controls had fewer branches than the rats reared with LE partners.

Although there was no significant effect of rearing condition on the total length of neurons ($F(1,16) = 18.80$, $p = .29$), there was a significant effect on both the apical length ($F(1,16) = 17.64$, $p = .0007$) and basilar length ($F(1,16) = 13.55$, $p = .002$). No significant effect was found for the length of the cell body ($F(1,16) = 0.04$, $p = .84$). For both the length of the apical and basilar projections, F344 rats reared with F344 partners had shorter projections compared to the F344 rats reared with LE partners (Table 6.1).

In contrast to the convex hull volumes and areas, branch numbers and apical and basilar dendrite lengths, no significant effect of rearing condition were detected for the total volume of the cells ($F(1,16) = 0.01$, $p = .91$), apical projection volumes ($F(1,16) = 0.76$, $p = .40$), basilar projection volumes ($F(1,16) = 3.60$, $p = .08$), or cell body volumes ($F(1,16) = 0.76$, $p = .40$) (Table 6.1).

## 6.4 Discussion

When juveniles of high playing strains of rats are reared over the juvenile period with peers from the low playing F344 strain, the play they experience is deficient, and the developmental consequences include poorer social skills and mPFC neurons with a dendritic arbor that is less pruned than when such rats are reared with same strain partners or partners from other highly playful strains (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016; Stark et al., 2021, 2023; Stark & Pellis, 2020, 2021). The present study

evaluated the effects of this cross-rearing on the F344 partners. Two hypotheses with opposing predictions were tested. The first hypothesis was that being reared with a peer from the more playful LE strain, F344 rats should experience more play and that this should improve developmental outcomes (e.g., greater social competence, mPFC neurons with greater dendritic pruning). In contrast, the second hypothesis posited that it is the quality, not the quantity of play that is important, and that the play of F344 rats is so discordant with that of LE rats, and other high playing strains, that the play experiences of F344 rats reared with LE peers would be more impoverished than those reared with F344 peers, and this in turn would lead to worse developmental outcomes. Our findings were consistent with the predictions of the second hypothesis.

### 6.4.1 Differences in play behavior

Juvenile F344 rats reared with LE peers not only engaged in less play, but that play was also qualitatively different. Both the F344 rats themselves launched fewer playful attacks against LE rats than against F344 peers, and their LE partners launched fewer attacks than did F344 partners (Figure 6.3a, b). The style of juvenile play was also different, with mixed-strain pairs engaging in less pinning, so less close-quarter wrestling, and their play led to fewer role reversals and was more asymmetrical (Figure 6.3c, d). Given that it is the ability to sustain reciprocal interactions during play fighting that refines social skills during juvenile play (Pellis, Pellis, Ham, et al., 2023; Pellis & Pellis, 2017), the opportunity for such training is reduced, not improved, by rearing F344 rats with LE rats.

As adults, the F344 rats reared in mixed strain pairs launched significantly fewer playful attacks, and received significantly fewer attacks, when interacting with an unfamiliar same strain partner (Figure 6.4a, b). The interactions with strangers also led to significantly fewer role reversals in pairs with F344 rats reared with LE peers. Although

not significant, given the low rate of escalation to aggression by F344 rats, it is noteworthy that only interactions involving mixed-strain reared rats led to aggression. The reduction in initiating play and inability for at least some of the mixed-strain reared rats to mitigate aggression suggests their socio-cognitive skills were impaired relative to F344 rats reared with a F344 peer. In some strains, like LE rats (Kisko, Euston, et al., 2015; L. K. Smith et al., 1999), strangers interacting jockey for dominance. However, the pairs of unfamiliar F344 rats encountering one another did not. Nonetheless, the diminished levels of play between partners where one animal was derived from mixed-strain rearing, indicate that their conspecifics recognized them as less attractive partners. This is consistent with other findings that rats can detect something odd about potential partners, that may be outside the capacity of humans to detect and measure (Pellis, Pellis, Burke, et al., 2022), and reflect this detection by avoiding playing with them (e.g., Holloway & Suter, 2004; Pellis et al., 2006; Stark & Pellis, 2021).

### 6.4.2 Differences in dendritic pruning

Using different manipulations and species, it has been repeatedly shown that play experience in the juvenile period has a pruning effect on the dendritic arbor of the pyramidal neurons of the mPFC (Bell et al., 2010; Burleson et al., 2016; B. T. Himmler, Pellis, & Kolb, 2013; Stark et al., 2023). A possible explanation for the importance of such pruning was originally proposed by Cajal (1899). The idea is that neurons will be arranged and connected to minimize cost: the 'wiring economy principle.' That is, fewer connections and smaller neurons should be favored over larger, more expansive neurons if they are capable of performing the same task. Indeed, too many connections may lead to less precise signalling and control of action potentials (Stark et al., 2023). If this is true, pruning of the

mPFC, influenced by social play behavior, may create more stream-lined and efficient circuits, ultimately improving control over behavior (Pellis, Pellis, Ham, et al., 2023).

The dendritic arbors of pyramidal neurons in Cg3 (prelimbic cortex) of the mPFC in rats reared in mixed strain pairs were significantly larger in the current study. That is, the male rats in mixed-strain rearing conditions did not go through the same pruning process of the apical and basilar neuronal arbors as did the F344 rats reared in the same-strain pairs. These differences were greatest in the convex hull volumes/areas but were also present in dendritic lengths and branching. The reduced pruning is not only consistent with LE rats and hamsters reared with low playing adults (Bell et al., 2010; Burleson et al., 2016; B. T. Himmler, Pellis, & Kolb, 2013), but also with LE rats reared with low playing F344 peers (Stark et al., 2023). Indeed, the magnitude of the effect is greater in F344 than in LE rats. For example, the convex hull volume measure was 52.5% larger and the convex hull surface area measure was 33.3% larger in F344 reared in mixed strain pairs compared to F344 rats reared in same strain pairs (Table 6.1), whereas for LE rats reared with F344 peers compared to same strain peers was 22.7% and 15.2%, respectively (Stark et al., 2023). Moreover, while for F344 rats reared with LE peers the pruning affected both the apical and basilar dendrites (Table 6.1), for LE rats reared with F344 rats, only the apical dendrites were significantly affected (Stark et al., 2023).

While the dendritic arbour of the mPFC neurons of both F344 rats (present study) and LE rats (Stark et al., 2023) was affected by cross strain rearing, there were several, notable strain differences. First, the magnitude of the effects of being reared with a same strain versus cross strain peer was greater in F344 rats than LE rats (see above). Second, whereas only the apical dendrites were significantly affected in the LE rats (Stark et al., 2023), both the apical and basilar dendrites of F344 rats were affected (present study). As

changes in the dendritic arbor of mPFC neurons following the manipulation play in the juvenile period has only been studied in two strains of rats, LE (Bell et al., 2010; B. T. Himmler, Pellis, & Kolb, 2013; Stark et al., 2023) and F344 (present study), it is possible that the neural sensitivity to this experience varies across strains. Quite simply, while most or all strains may show an effect in the same direction, the magnitude of that effect may vary (Pellis et al., 2018). A fuller comparative survey across domestic strains of rats is needed to determine the degree of such variation, but that play experience in the juvenile period has the effect of pruning the dendritic arbor of mPFC neurons has now been demonstrated in two strains of rats and in a different species, the Syrian hamster (Burleson et al., 2016), suggest that this causal influence of play on the development the mPFC neurons is likely a general one.

Alternatively, abnormal play experiences may increase dendritic size through dendritic overgrowth that pruning does not reverse. During the juvenile period, neurons are pruned, but dendritic and synaptic growth also occurs. Synapses in the mPFC increase between postnatal day 35 and 45 in rats (Drzewiecki et al., 2016). Indeed, if rats are deprived of play during the juvenile period, they show altered development of inhibitory synapses in the mPFC (Bijlsma et al., 2022, 2023). To determine whether the differences in dendritic arborization are due to overgrowth or less pruning, dendrites need to be sampled at various time points throughout the juvenile period. In addition, such an analysis would help further characterize the mechanism by which abnormal play alters neuronal development, as it would determine the critical period during adolescence when the mPFC neurons are most sensitive to the influence of play experiences. Regardless of the mechanism involved, there is mounting evidence that juvenile play experiences alter the dendritic arbors of the mPFC (Pellis et al., 2023).

Rats have considerable variability in their play behavior, varying the amount of play, the partners with which they play, and the style in which they play (Achterberg et al., 2023; Ham & Pellis, 2023; Lampe et al., 2019; Lesscher et al., 2021; Melotti et al., 2014; Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2022; Poole & Fish, 1976), however, it remains unknown if individual variation in play experience is sufficient to result in differences in socio-cognitive skills and altered dendritic pruning. It may be that, irrespective of individual variation, all rats reared with same strain peers may achieve the threshold level of experience to impact neural and socio-cognitive development equally. Alternatively, while most rats may achieve that threshold, some may not. While we did not test how individual variation interacts with development, animals reared in the experimental condition (i.e., F344-LE pairs) had greater variability in convex hull volume and surface area measurements as adults than did the rats in the control condition (i.e., F344-F344 pairs) (see the error bars in Figure 5). This suggests that F344 partners may provide F344 rats with a relatively narrower range of variation in play experiences than did LE partners. A similar pattern is present for LE rats reared with F344 peers compared to LE peers (Stark et al., 2023). Even so, both rats reared with same strain and mixed strain peers show considerable variation in the amount of pruning present (Stark et al., 2023; present paper), suggesting that the magnitude of pruning may depend on the degree of play reciprocation they experienced as juveniles. Indeed, while the mixed strain reared animals were more likely to escalate encounters with strangers to aggression, not all subjects did so (Stark & Pellis, 2020; present study), again supporting the possibility that rats differ in their social competence and that this difference may be, in part, dependent on their play experiences as juveniles. Preliminary data from a large sample of LE rats, suggest that some individuals fail to gain the requisite play experiences as juveniles even when the

opportunity to do so is present, and this appears to have a negative impact on dendritic pruning of mPFC pyramidal neurons and on the development of socio-cognitive skills (Ham, Iwaniuk, et al., 2023). If supported by more detailed analyses, this would support the view that not all normally reared rats benefit equally from juvenile play.

### 6.4.3 Are F344 rats a high playing strain?

An unexpected finding was that the F344-F344 pairs played more than expected for this strain (Siviy, 2020). Our juvenile males initiated around 70 playful attacks during a 10-min play session, which is around 2.5 times higher than what was expected based on previous studies (Siviy et al., 1997, 2003). However, most analyses of play in F344 rats has involved testing them in dyads with partners from other strains, and even though still relatively low, in the cases in which F344-F344 pairs were tested the number of playful attacks were greater (Siviy et al., 1997, 2003, 2011, 2017, 2023), suggesting that F344 rats play more together than when playing with partners from different strains. Indeed, as adults, LE, Sprague Dawley and Wistar rats prefer their own strain but will readily associate with one of the other strains before settling for a F344 partner if that is the only option available (Kiyokawa et al., 2022; Kogo et al., 2021). These findings suggest that F344 rats are so different from other strains that they are unattractive as play partners, as other strains are to them. Consistent with this is that LE and Sprague Dawley can adapt their play to the idiosyncacies of each others' strain-typical pattern of play (S. M. Himmler, Lewis, et al., 2014), and Wistar rats reared with Sprague Dawley rats do not exhibit the long-term developmental effects that Wistar rats reared with F344 rats do (Schneider, Bindila, et al., 2016).

The reason for why our F344 rats initiated more play than expected still needs to be resolved. One possibility is that the original studies from Siviy's laboratory used F344

rats from a different breeding company than the one from we obtained ours (see Siviy et al., 1997, 2003, Stark et al., 2021; present study). Indeed, when Siviy obtained F344 rats from a different breeder, he found that they were more playful than those previously tested (Siviy, pers. comm. 2021). A detailed comparison of the play of F344 rats obtained from different breeders is needed. Importantly, though, irrespective of their origin and whether tested with partners from the same or different strains, F344 rats are more likely to evade nape attacks and less likely to roll over to supine, and so being pinned, when nape contact is successful (Siviy et al., 1997, 2003, 2011, 2023; Stark et al., 2021; present paper), suggesting that they have a strain-typical style of play that is different to that of many other commonly used laboratory strains of rats (B. T. Himmler, Stryjek, Modlinska, et al., 2013a; S. M. Himmler, Modlinska, et al., 2014). Failure to significantly alter that style even when cross-fostered with another strain (Siviy et al., 2017) is unlike the modification present in juvenile LE and Sprague Dawley rats even if only housed together for a few days shortly after weaning (S. M. Himmler, Lewis, et al., 2014). We intended to pair F344 rats with a high playing partner to boost the play experience but instead we found the LE rats were less playful than F344 rats. Nonetheless, the play styles of F344 and LE rats seem to be so incongruent that both LE and F344 rats reared together in cross strain pairs end up having impoverished socio-cognitive skills and less pruned Cg3 neuronal morphology (Stark & Pellis, 2020, 2021; Stark et al., 2023; present study). Therefore, it is the quality of the experiences derived from playing, not the overall quantity of play, that is critical to gain the socio-cognitive benefits from playing in the juvenile period (Pellis et al., 2023).

### 6.5 Conclusion

Our findings show that, like the case for LE rats (Bell et al., 2010; B. T. Himmler, Pellis, & Kolb, 2013; Stark et al., 2023; Stark & Pellis, 2020, 2021), Lister hooded rats

(Baarendse et al., 2013; Bijlsma et al., 2022, 2023), and Wistar rats (Schneider, Bindila, et al., 2016; Schneider et al., 2014; Schneider, Pätz, et al., 2016), diminished play experience in the juvenile period of F344 rats result in both neuroanatomical changes and reduced socio-cognitive skills in adulthood (present chapter). In addition to rats, hamsters seem to show a similar effect (Burleson et al., 2016; Cooper et al., 2023). Studies of wild ground squirrels (*Urocitellus beldingi*) have also demonstrated that juvenile play experience influences adult behavior, refining the development of motor skills and social behaviors, in addition to temperament (Hurst-Hopf et al., 2023; Marks et al., 2017; Nunes, 2014; Nunes, Muecke, Lancaster, et al., 2004; Nunes, Muecke, Sanchez, et al., 2004; Nunes & Monroy Montemayor, 2023). Relevant to the present findings is that, for the ground squirrels, it is social, not solitary play, that specifically influences the development of social behavior (Marks et al., 2017). What remains unclear, however, is the mechanism by which play can produce these effects. The present findings, along with the results from the LE reared with F344 (Stark et al., 2021, 2023; Stark & Pellis, 2020, 2021), suggest that it is not the amount of play an individual experiences, but the quality of the play, that provides the critical experiences for these developmental effects. That is, it is the ability to negotiate interactions with the partner to make play reciprocal (as measured by role reversals and symmetry) that seems to provide the experiences derived from play critical for refining socio-cognitive skills and their underlying neural substrates (Pellis et al., 2017, 2019; Pellis, Pellis, Ham, et al., 2023; Schneider, Bindila, et al., 2016). Juvenile F344 rats reared with LE partners not only played less, but also less reciprocally than their counterparts reared with F344 partners (Figure 3), and most of the role reversals between mix-strain pairs were initiated by the LE and not the F344 (Stark et al., 2021). Thus, F344 rats reared in LE-F344 pairs may gain fewer of the critical experiences than do the F344 rats reared in such mixed strain

pairs, and this might explain why the magnitude of the effects on dendritic pruning was greater in F344 rats than LE rats in mixed strain pairs.

# CHAPTER 7: JUVENILE SOCIAL PLAY AND THE DEVELOPMENT OF SOCIO-COGNITIVE SKILLS IN MALE RATS: DOES EVERYONE BENEFIT?

## 7.1 Introduction

Play fighting or rough-and-tumble play (RTP), is the most reported and studied form of social play (Burghardt, 2005; Fagen, 1981; Pellis & Pellis, 2009). It involves competing for an advantage, such as biting or otherwise contacting a particular location of the partner's body (Aldis, 1975; Pellis, 1988; Pellis et al., 2024; Symons, 1978), but the competition is moderated by cooperation, ensuring some degree of reciprocity (Palagi, Cordoni, et al., 2016; Pellis & Pellis, 2017). Laboratory rats (*Rattus norvegicus*) have been among the most intensively used species to study RTP, yielding our deepest understanding about its neurobiology (Achterberg & Vanderschuren, 2023; Siviy, 2016; J. W. VanRyzin et al., 2020) and functions (Marquardt et al., 2023; Pellis, Pellis, Ham, et al., 2023; Vanderschuren & Trezza, 2014). When rats play, they target the nape of the neck, which is nuzzled with the snout if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). In contrast, when engaging in serious fighting, the lower flanks and rump are targeted, and bitten if contacted (R. J. Blanchard & Blanchard, 1977; Pellis & Pellis, 1987). Moreover, whereas during serious fighting tactics of attack and defense are combined to limit the risk of retaliation by the opponent (R. J. Blanchard & Blanchard, 1977; Pellis, 1997), during RTP this combination is more relaxed, facilitating role reversals and turn taking (Foroud & Pellis, 2003; Pellis & Pellis, 1998).

RTP in rats begins in the last week before weaning, peaks in the juvenile period (30-40 days post-birth), then declines with the onset of sexual maturity, but continues, albeit at a lower frequency, into adulthood (e.g., Baenninger, 1967; Bolles et al., 1964; Pellis & Pellis, 1990, 1997; Thor & Holloway, 1984). In the juvenile period RTP facilitates

the training of executive functions, which, in turn, improves socio-cognitive skills (Pellis, Pellis, Ham, et al., 2023). In adult rats, RTP is used to navigate social relationships (Pellis & Pellis, 2009), as is the case in many other mammals (Palagi, 2023). At both ages, it is the need to maintain some degree of reciprocity that appears to be the critical feature of RTP that enables social skills (Pellis, Pellis, Ham, et al., 2023; Pellis & Pellis, 2017). The developmental effects of RTP on socio-cognitive skills are associated with the anatomy and physiology of the neurons of the medial prefrontal cortex (mPFC) (e.g., Baarendse et al., 2013; Bell et al., 2009; Bijlsma et al., 2022, 2023; Ham et al., 2024; B. T. Himmler, Pellis, & Kolb, 2013; Stark et al., 2023), a brain region implicated in many aspects of mammalian social behaviour (reference). An association among RTP, the development of social skills, and the mPFC also occurs in Syrian golden hamsters (*Mesocricetus auratus*) (Burleson et al., 2016), suggesting that this function of RTP is not limited to rats. Indeed, evidence is accumulating that social play in children also promotes the development of executive functions, socio-cognitive skills and may do so by modifying the prefrontal cortex (e.g., Diamond et al., 2007; Gibb et al., 2021; Nijhof et al., 2018).

In rodent studies, the opportunity to engage in RTP was either prevented by rearing the juveniles in complete isolation (Baarendse et al., 2013) or physical separation with a clear, perforated divider (Bell, 2014; Bijlsma et al., 2022, 2023; Marquardt et al., 2023; Pellis et al., 1999). RTP has also been impaired in rats by rearing juveniles with less playful adults (Bell et al., 2009; Burleson et al., 2016; B. T. Himmler, Pellis, & Kolb, 2013; Pellis & Pellis, 2017), or peers from a less playful strain (Ham, Szabo, et al., 2024; Schneider, Bindila, et al., 2016; Schneider et al., 2014; Schneider, Pätz, et al., 2016; Stark et al., 2023). In all cases, there was a significant group effect, with the experimental animals that experienced no play, limited play or poor quality play showing social deficiencies and/or

altered mPFC anatomy as adults (Pellis, Pellis, Ham, et al., 2023). However, not all rats are the same; there is a lot of behavioral and neuroanatomical variation within experimental groups in these experiments, with some rats scoring as well or even better than the control rats (e.g., see Figure 2 in Stark et al. [2023], Figure 2 in Stark & Pellis [2020]). This suggests that play may be more effective in shaping the development of some individuals more than others.

In a series of studies of wild Belding's ground squirrels (*Urocitellus beldingi*), the naturally occurring variation in play within litters is associated with the development of several several skills (Nunes & Monroy Montemayor, 2023). Specifically, those who played more had improved social (Marks et al., 2017), motor skills (Nunes, Muecke, Lancaster, et al., 2004; Nunes, Muecke, Sanchez, et al., 2004), and temperament (Hurst-Hopf et al., 2023; Shehan et al., 2023). Although not necessarily true for all species and all types of play (Richter et al., 2016), these data suggest that more play results in positive developmental outcomes. If correct, then juvenile rats that play more or are better able to extract the critical play-derived experiences from their play may benefit more from playing than other rats, which could account for variation in the degree to which individuals benefit from play in experimental studies (Pellis, Pellis, Ham, et al., 2023).

As alluded to previously, there are individual differences among rats in the amount of play initiated (Achterberg et al., 2023; Ham & Pellis, 2023; Lampe et al., 2017, 2019; Lesscher et al., 2021; Melotti et al., 2014; Pellis & McKenna, 1992) and in their style of play (Pellis, Pellis, Burke, et al., 2022; Pellis, Pellis, Ham, et al., 2022). Moreover, wild rats live in colonies (Schweinfurth, 2020), which means that young rats have access to many potential play partners, including both siblings and non-siblings. Indeed, when laboratory rats are tested in groups, juvenile rats not only vary in how much they play, but

also preferentially play with some group members more than with others (Ham & Pellis, 2023, 2024, 2025). In a more naturalistic context in which multiple potential play partners are present, different individuals may therefore experience different amounts and quality of play. Here, we tested this by rearing groups of males together from shortly after weaning to young adulthood, tracked their play experiences in the peak juvenile period, tested their social competency as adults and finally quantified the anatomy of their mPFC neurons (Figure 7.1).

To test this hypothesis, we analyzed the RTP of individuals within socially housed groups and compared the amount and quality of play with their social competence as adults, as tested in a stranger paradigm test (Stark & Pellis, 2020). If social incompetence arises because of inadequate play experiences, then three predictions can be made. First, the incompetent rats will experience less RTP during the peak juvenile period and the RTP that does occur has fewer role reversals (Ham, Szabo, et al., 2024; Stark et al., 2021). Second, the lack of adult social skills is reflected not in engaging in less RTP, but in the escalation of RTP into fighting. That is, interactions should begin as playful but become increasingly likely to escalate to aggression as the trial progresses (Ham, Szabo, et al., 2024; Stark & Pellis, 2020). Finally, the incompetent rats will have larger mPFC dendritic arbors than the competent rats because impoverished juvenile play experience is associated with reduced dendritic pruning (Ham et al., 2024; Stark et al., 2023).

## 7.2 Methods

### 7.2.1 Subjects

One hundred and eight male Long Evans rats were ordered from Charles River Laboratories (Kingston, NY, USA) in three separate cohorts. Each cohort was tested in the same manner. The rats arrived at the Canadian Centre for Behavioural Neuroscience

(CCBN) at around 23 days of age. After arriving, 54 of the rats were housed in groups of six and the other 54 were housed in pairs. All groups were maintained in double decker Tecniplast® GR1800 cages and under a 12 h light-dark cycle (lights on at 7:30 a.m.) and a constant temperature of 21-23ºC. At 28 days of age, the animals were ear notched, and the 54 rats housed in groups of six (i.e., 9 groups) were moved into large custom-made Plexiglas® enclosures (80 × 80 × 100 cm) (Figure 7.1). Once moved into the larger enclosures, the rats were housed under a 12-2-10 h light-red-dark light cycle. All rats were provided with water and food *ad libitum*. Cage floors were lined with 2-3cm of corncob bedding.

Both types of groups were maintained in these cages until behavioral testing was completed in adulthood and the animals were sacrificed at 90 days. The groups of six served as the experimental subjects and the rats in pairs served as the partners for the stranger tests.

All care and testing procedures were approved by the University of Lethbridge Animal Welfare Committee (Protocol #1809) in compliance with guidelines from the Canada Council for Animal Care.

### 7.2.2 Behavioral analysis

#### *7.2.2.1 Juvenile home cage behavior*

Juvenile behavior was recorded for the groups of six in their large custom-made home cages. The floor was covered in around 2-3 cm of corncob bedding and included two metal food hoppers (Tecniplast®). One was filled with food (*ad libitum*) and the other was placed on its side and used as a hide by the rats. Krinkle® bedding and paper towel strips were provided as nesting material. Home cage behavior was filmed with cameras (ExmourRS 4K Sony Handycams & a Canon Vixia HFS21) placed at a 45º angle to the cages. Each day shortly before 7:30 p.m., when the lights turned from white light to red,

the cameras began recording the home cage. The first twenty minutes following the lights changing were scored when the rats were 34, 35 and 36 days old.

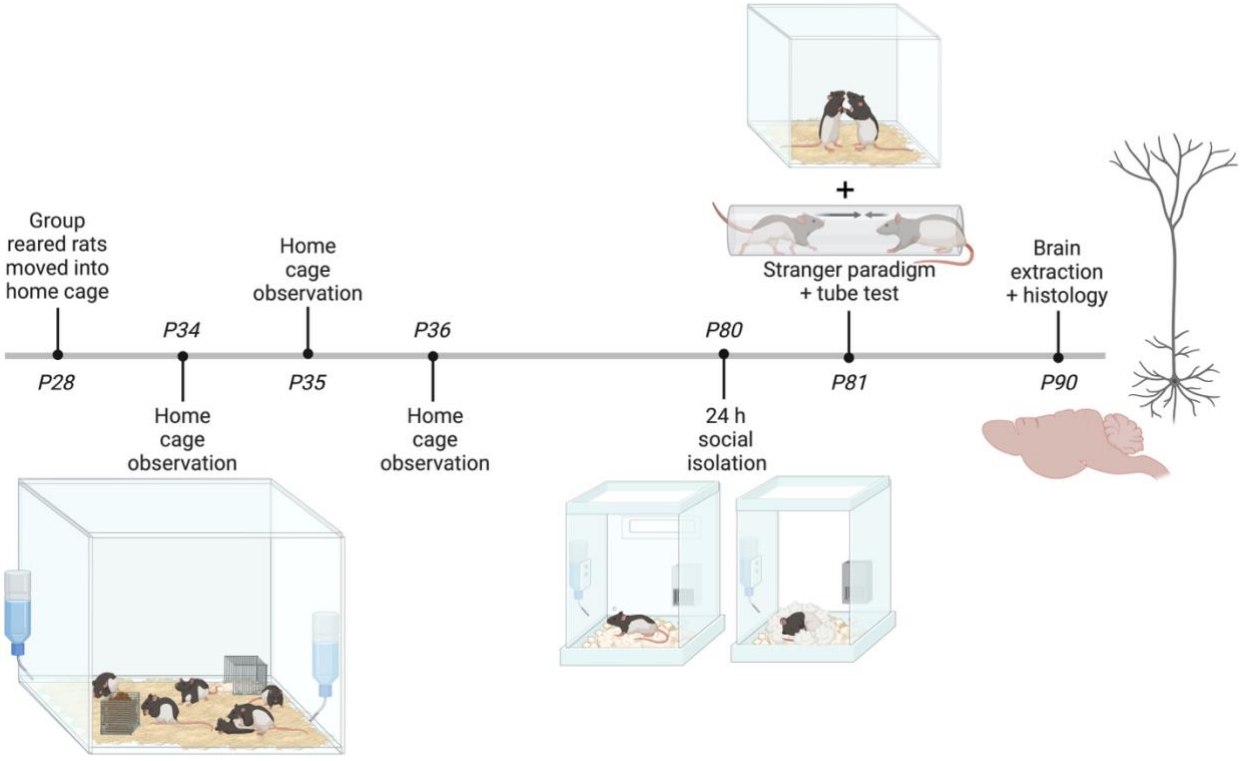


**Figure 7.1** The experimental timeline showing that group reared rats moved into the large enclosures on post-natal day (P) P28 and were observed in the home cage from P34-36. At P80, the rats were socially isolated and tested the following day (P81) with the stranger paradigm. Shortly after the stranger test was completed, the animals were sacrificed (P90).

After identifying the rats that engaged in aggressive behavior (i.e., incompetent rats) during the stranger test as adults (see below), the video files from the home cage recordings were analyzed using a combination of both normal speed and frame-by-frame analysis to score play behavior (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022). Matched individuals from the same groups that did not escalate to aggression in the stranger test (i.e., competent rats) were also scored. Using a focal follow approach (Bateson & Martin, 2021), all attacks initiated by and directed towards both the competent and incompetent rats were scored. To evaluate RTP, the number of nape attacks and how the recipients of attacks defended themselves were scored. Playful attacks were scored when the nose of one rat was in contact with, or directed toward, the nape of the other rat (Pellis, Pellis, Burke, et al., 2022). Once playfully attacked, the recipient either continued with its ongoing behavior (e.g., grooming, eating, drinking) or defended itself, from which the probability of defense was calculated. If the recipient defended itself, it could do so by either running away from the attacker (i.e., evading) or by turning to face its attacker. There are several types of facing defense (Pellis & Pellis, 1987). One of the most common facing defense tactics used by juveniles, especially in Long Evans rats, is to rotate fully to a supine position (B. T. Himmler, Stryjek, Modlinska, et al., 2013b; S. M. Himmler, Modlinska, et al., 2014). The typical outcome is for the defender to lie on its back, with the attacker standing over the defender (i.e., commonly referred to as the pin configuration, Panksepp, 1981; Trezza et al., 2010). Other commonly used defense tactics involve turning to face the attacker by partially rotating around the long axis of the body or by pivoting horizontally around the pelvis. In either case, a frequent outcome is for the two rats to rear up on their hindfeet and grapple with their forepaws (i.e., commonly referred to as the boxing or mutual upright configuration (B. T. Himmler, Pellis, & Pellis, 2013), but rats can also push one

another over from the boxing position to the pin configuration (S. M. Himmler, Himmler, Stryjek, et al., 2016). To assess overall differences in the attack and defense patterns of RTP (Pellis, Pellis, Burke, et al., 2022) we scored: the number of nape attacks were scored, the proportion of those attacks which were defended by the recipient, and of those defended, the proportion that led to evasion and to a pin configuration.

In addition to attack and defense patterns, an important feature of RTP is partner reciprocity in which individuals take turns in being the attacker or the defender (Palagi, Cordoni, et al., 2016; Pellis et al., 2024; Pellis & Pellis, 2017). In rats, this often involves cooperative actions that ensure that the defender can gain an advantage over the attacker (Pellis et al., 2005). To assess this cooperative aspect of play, the number of play fights that lead to role reversal was scored (S. M. Himmler, Himmler, Pellis, et al., 2016). Role reversals are most easily scored from the pin configuration in which the rat lying on its back successfully counterattacks, contacting the on top rat's nape, leading to that rat becoming the defender (Pellis, Pellis, Burke, et al., 2022). The proportion of RTP encounters that resulted in role reversals were compared between the competent and incompetent rats. In addition to the proportion of RTP bouts that led to a role reversal, the frequency of role reversals was also compared. Indeed, it may be that it is the overall frequency of role reversals initiated by the subject rat that provides the key experiences that are critical for juveniles to benefit the gains in socio-cognitive skills from RTP (Pellis et al., 2019) rather than the proportion of role reversals. Consequently, given that small differences in attack and defense frequencies, even when not themselves significantly different (Pellis, Pellis, Burke, et al., 2022) can lead to cumulative differences in the number of role reversals, the absolute frequency of role reversals per trial were also scored and compared.

Last, to determine if rats had partner preferences for either competent or incompetent rats, we scored the percentage of attacks that each focal rat received. This was calculated for each focal rat by dividing the number of attacks directed to a given partner by the total number of attacks launched by that focal rat towards all other rats on that day. If each partner in the group was attacked equally, they would be attacked roughly 20% of the time.

#### *7.2.2.2 Adult stranger test*

Adult social behavior was tested in a sound-attenuated chamber with a clear Plexiglas® enclosure (50 × 50 × 50 cm) placed inside. The floor of the Plexiglas® enclosure was covered in 2-3 cm of CareFresh bedding. Sound-attenuating foam (Primeacoustic) lined the walls of the chamber to dampen external noise. Interactions were filmed through a small window of the chamber with the camera (ExmourRS 4K Sony Handycam) placed at a 45º angle. Using only infrared lights, which were placed inside the chamber, the video recordings were made using the night-shot capabilities of the camera. To record ultrasonic vocalizations, a Pettersson M500-384 ultrasonic microphone (Pettersson Elektronic, AB, Sweden) was placed approximately 35 cm above the center of the enclosure floor.

At 77 days of age, all the rats were habituated to the stranger test enclosure for 10 min for three consecutive days. When the rats were 80 days old, the animals were socially isolated for 24 h with food and water provided *ad libitum* in Tecniplast® GR1800 cages, and then each rat from the groups of six (i.e., the 'focal' subject) was placed simultaneously into the testing enclosure with a rat from the pair housed cohort (i.e., the 'stranger' or 'partner' rat), the 10 min trials were recorded with a camera (ExmourRS 4K Sony Handycam) placed at a 45º angle. Video recordings were made using the night-shot capabilities of the camera, with infrared lights placed inside the chamber to illuminate the

test enclosure. Following testing, the rats were removed from the enclosure, the bedding was changed, and the enclosure was cleaned with Virkon. Once cleaned, the next pair of rats was tested, and the procedures repeated.

The video files were analyzed using a combination of normal speed and frame-by-frame analysis to score play behavior (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022) and agonistic behavior (Kisko, Euston, et al., 2015; L. K. Smith et al., 1999; Stark & Pellis, 2020). All videos were scored twice: first scoring the actions initiated by the focal rat and directed towards the partner; and second scoring the actions initiated by the partner and directed towards the focal rat. As was the case for juveniles (see above), the amount of RTP initiated was measured by scoring playful nape attacks. Agonistic behavior included threat signals, such as piloerection, tail rattling, and lateral posturing, and physical aggression, such as pinning, boxing, and overt bites (R. J. Blanchard & Blanchard, 1977; L. K. Takahashi & Lore, 1983). These were summed into a composite score of aggression (L. K. Smith et al., 1999; Stark & Pellis, 2020), and along with the frequency of playful nape attacks were scored during the 10-minute trials. To assess differences between the competent and incompetent rats both the total play and agonistic scores per trial and their distribution over the course of the trials were compared.

In addition to visual signals, rats emit 22 kHz flat calls during aggressive encounters and other threatening contexts (Barnett, 1975; N. Takahashi et al., 2010; Thomas et al., 1983), we also measured the number of 22 kHz flat calls during the trials. Acoustic recordings from the stranger encounters were analyzed using Raven Pro 1.6 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY), which generates spectrograms with a 256-sample Hann window. The 22 kHz calls that were emitted during the 10 min stranger encounter were manually selected, labelled, and scored.

For comparative purposes, from each group with one or more aggressive rats, we selected an equivalent number of rats that did not escalate encounters to serious fighting, and, to make the difference more extreme, we selected group members that did not even display threat signals. The behavior of both the competent (those that did not escalate) and the incompetent (those that did escalate) rats was then evaluated in the juvenile period (see above), and in detail during the stranger test to ascertain when and how the escalation arose (see below).

To assess the relative dominance between the focal rats and the strangers, we used the tube test (Fan et al., 2019; Fulenwider et al., 2021). We ran the tube test directly following stranger encounters using a Plexiglas® tube that was just large enough so that rats could fit through the tube, but not so large that the rats could turn around in the tube or squeeze by one another. Rats were placed into the tube simultaneously at opposite ends. For each pair, there was a 'winner' and a 'loser' . The loser was the rat that was completely out of the tube (i.e., all four paws were outside the tube) and the winner, the rat remaining in the tube. If both rats remained in the tube for 60 sec, the test was considered a tie. Every pair was tested consecutively five times, alternating which side the focal and stranger rats were placed in the tube. We used the sum of wins from each trial to rank the rats from most dominant to least, with dominant rats having a higher score out of five than subordinate rats. Between testing each pair, the tube was cleaned with Virkon®.

### 7.2.3 Histology

The 54 rats from the groups of six were anesthetized at 90 days of age with 300 mg/kg of sodium pentobarbital and perfused, intracardially, with 0.9% saline. The brains were then extracted and weighed. Following a standard Golgi staining protocol (Gibb & Kolb, 1998), the brains were placed in Golgi-Cox solution for 14 days after which they

were placed into a 30% sucrose solution for at least 7 days before sectioning. The brains were sectioned using a vibratome at a thickness of 200 μm. Sections were mounted onto slides coated in 2% gelatin and stored in a light-proof box and wrapped in a damp paper towel to prevent the tissue from drying. After 3-4 days, the slides were rinsed in distilled water and ammonium hydroxide and then placed in a 1:1 solution of Kodak Rapid Fix (Kodak) and distilled water. To dehydrate the tissue, the slides were moved through a graded ethanol series, cleared with a 1:1:1 solution of chloroform, Hemo De (electron Microscopy Sciences, catalog No. 23410-04), and 100% ethanol. Before cover-slipping with Permount (Fisher Scientific, catalog No. SP15-500), the slides were placed in Hemo De. The slides were left to dry for at least 1 month or until they were dry to the touch.

### 7.2.4 Anatomical analysis

Neurons for reconstruction were selected from area Cg3 (prelimbic cortex) of the mPFC as defined by Zilles (1985), for imaging, between Bregma 4.2 and 2.77 mm in the rostro-caudal plane. An Olympus VS120 digital slide scanner, with a 40× oil object (UPlanFL N, 40×/1.30 oil, ∞/0.17/FN26.5) and Olympus VS-ASE FL software, was used to create virtual slides of Cg3 (Brinkman et al., 2022; Ham, Szabo, et al., 2024; Stark et al., 2023). The virtual slides consisted of 147 *z*-stacked images spaced 0.68 μm apart throughout the section yielding 99.96 μm of working distance. The apical and basilar dendrites and somas from layer III pyramidal neurons were then traced in Neurolucida 360 (MBF Bioscience, Williston, VT, USA). Cell inclusion criteria were as follows: (1) the cell was fully impregnated and not obscured by blood vessels, precipitate, or debris; and (2) the cell was centered within the *z*-stack and not truncated, ensuring that all dendritic branching

could be traced. Five cells from each hemisphere were traced, for a total of 10 neurons traced per animal and 220 neurons across all rats.

Using Neurolucida Explorer, the following measurements were extracted for statistical analysis: (1) convex hull volume for the apical and basilar projections, as well as for the whole cell; (2) the convex hull surface area the apical and basilar projections, as well as for the whole cell; (3) the sum of the total length of the apical and basilar projections and the length of the cell body, which was summed to obtain a total cell length; (4) the sum of the volume of the apical and basilar projections and the volume of the cell body, which was summed to provide a measure of the total volume of the cell; (5) the branch number for both apical and basilar projections, which was summed for the total branch number.

In addition to neuron morphology, we used Neurolucida 360 measure spine density. To do so, four distal sections in the basilar arbors and 4 in the apical arbors, counting the spines in 40 μm length segments, from the end of a dendritic branch. Segments were at least 50 μm distal or 100 μm to the cell body for basilar segments and apical segments, respectively. Proximal sections were also measured, sampling two basilar segments and one apical segment. In both cases, 20 μm were measured from the cell body. Spines were only included if they were longer than the radius of the dendrite to limit the inclusion of artifacts. If the spines bifurcated, they were counted as two separate spines as they have separate points of connection. For each animal, the spines on 4 cells were quantified (2 from each hemisphere), with a total of 88 cells across all rats.

### 7.2.5 Statistical analysis

All statistics were performed using R Studio (R Core Team, 2020) and graphs were created in Prism version 10 (GraphPad Software).

We modelled juvenile play frequency and style with generalized linear mixed models (GLMM) in the "glmer" function of the *lme4* R package (Bates et al., 2015). More specifically, we tested whether the frequency of playful attacks, the number of attacks resulting in a pin, and the number of role reversals initiated by the focal rats predicted adult social competency. To do so, these quantitative measures were set as the predictor variables. The rat's identification number and group number were set as random error terms to account for the repeated measures across the three days and groups. In this way, the measures from each day could be used so that the daily RTP measures were compared. Adult social competency was set as the dependent variable and as the rats could either be competent or incompetent, a binary GLMM was used. The same was repeated for the number of attacks, number of attacks resulting in a pin, and the number of role reversals initiated by the partner of the focal rats. To determine if there were differences in play style, we tested whether differences in the proportion of attacks defended, evaded, and leading to a pin, predicted adult social competency. As before, the proportion measures were set as our predictor variables, rat identification number and group number set as random error terms, and a binary GLMM used. This again was repeated for the partners of the focal rats. Finally, to determine if rats preferred to play with either competent or incompetent rats in the group, the percentage of play directed at each partner was tested with a binary GLMM. Adult social competency was set as the dependent variable and again the rat's identification number and group number were set as random error terms. These GLMMs were run for both competent and incompetent rats. For all GLMMs, Wald's confidence intervals are

reported. In addition, odds ratios are provided to clarify how predictor variables influence the likelihood of the focal rat being classified as either competent or incompetent. Specifically, odds ratios greater than 1 suggest an increased likelihood of being classified as competent, while odds ratios less than indicate a decreased likelihood.

To test the distribution of RTP and serious aggression over the course of the stranger test, the number of playful nape attacks and aggressive behaviors in each one-minute time bin was compared with Freidman's tests after finding that the data were not normally distributed (Shapiro Wilks: $p < .05$). Post hoc pairwise comparisons were made with Nemenyi tests. Using *t*-tests, we compared the number of flat calls emitted, the frequency of play initiated by both the focal rats and the strangers, and the proportion of the total play between the pair that the focal rats initiated. A sign test was used to compare the number of flat calls emitted during the first five minutes of the stranger encounter to those in the last five minutes. The results of the tube test were analyzed using Fisher's exact tests to determine if the number of dominant, subordinate, and indeterminate individuals differed between the competent and incompetent groups.

Measures of the neurons were assessed with a linear mixed model, using the *lme4* package, to determine if neuronal morphology and spine density differed between competent and incompetent adults. Social competency was set as the independent fixed effect, and the identification number of the rat was set as a random error term to account for the repeated measures of individual animals, with hemisphere nested within every subject. Additionally, the group the individual came from was set as a random error term to account for some individuals originating from the same group. Differences were considered significant if $p < .05$ using the Satterthwaite's approximation ANOVA.

## 7.3 Results

### 7.3.1 Juvenile RTP

#### *7.3.1.1 Behavior of competent versus incompetent rats*

As juveniles, incompetent rats launched just as many playful attacks as competent rats (Figure 7.2a) (Table 7.1). When attacked, the focal rats, whether competent or incompetent, defended the same proportion of attacks and did so using similar defensive tactics (Table 7.2) leading to similar frequencies of pins (Table 7.1). Moreover, while the probability of an attack leading to a role reversal did not differ between competent and incompetent rats (Table 7.2), the absolute number of role reversals did, with competent rats reversing role twice as much as incompetent rats (Figure 7.2b) (Table 7.1). That the incompetent rats were less cooperative play partners was also revealed when all the other members of the group were playing in close quarters of one another; the incompetent rats typically isolated themselves either on the opposite side of the enclosure (Figure 7.2g) or in the food hopper (Figure 7.2h). Even so, there was no overt play partner preferences as both competent (Figure 7.2c) and incompetent (Figure 7.2d) rats were equally likely to play with other competent and incompetent rats (Table 7.3).

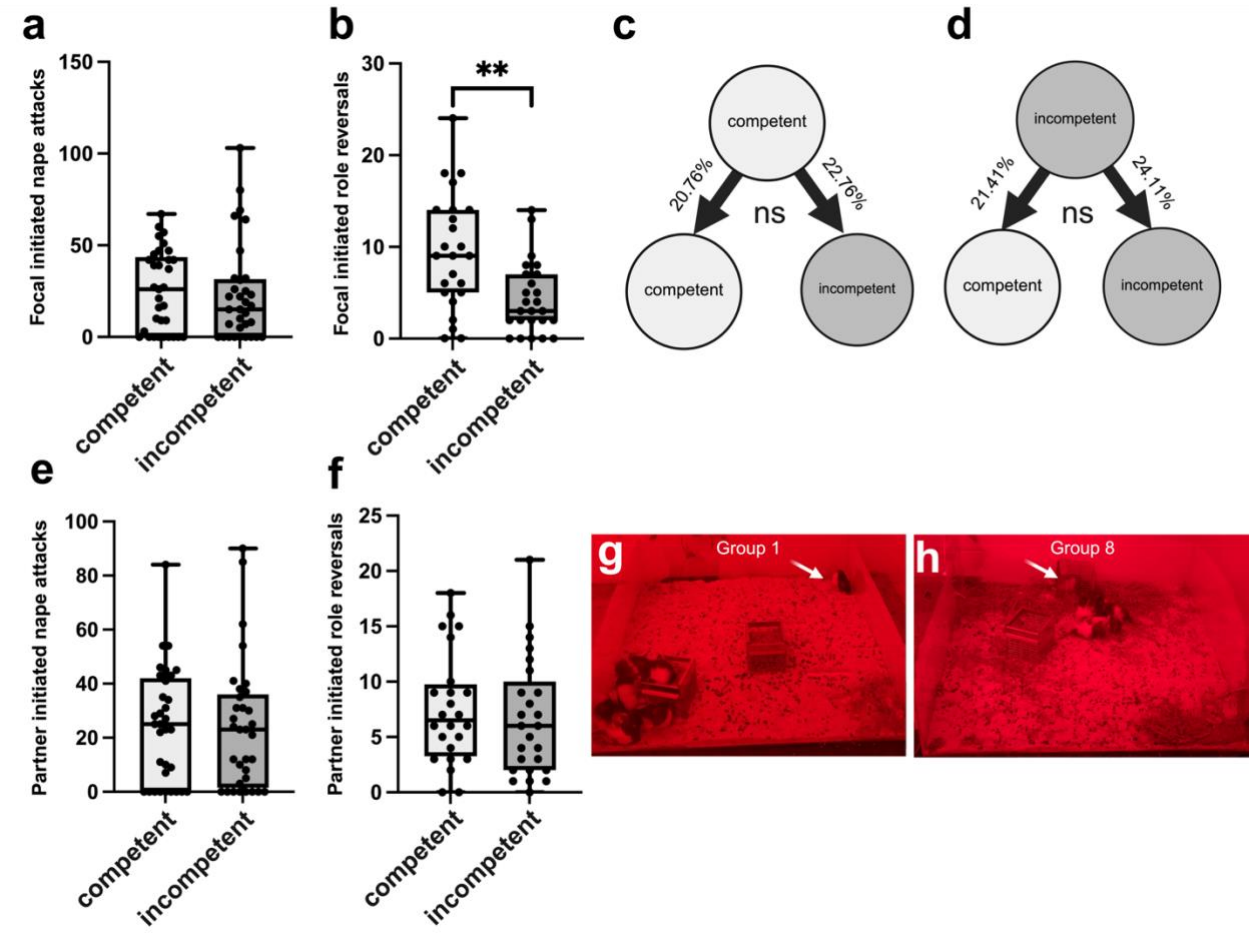


**Figure 7.2** The number of nape attacks initiated by the focal rats (a). The number of roles reversals initiated by the focal rats (b). No significant preference (Table 7.3) was found for competent or incompetent rats by competent rats (c) nor incompetent rats (d). The number of nape attacks initiated by partners and directed towards competent and incompetent rats (e). The number of role reversals initiated by partners when playing with either competent or incompetent rats (f). Finally, two images of the rats in their home cage, show incompetent rats avoiding multi-rat play by either**,** staying at the opposite end of the enclosure (g), or seeking refuge in the rotated food hopper (h). $^{**}p < .01$

**Table 7.1** Comparisons of competent and incompetent focal rats, analysed with a generalized linear mixed model, are presented below for various measures of juvenile play behavior. The average frequency (± standard error of the mean) per day of the various measures are presented, though, the model tested each day.

| Predictor | Competent | Incompetent | Estimate | SE | z | p | OR | CI |
|---|---|---|---|---|---|---|---|---|
| Intercept | | | 15.209 | 5.942 | 2.559 | .010 | $4.028e^{6}$ | 3.562 – 26.856 |
| Focal initiated nape attacks | 25.30 ± 3.82 | 22.97± 4.60 | 0.788 | 0.463 | 1.703 | .088 | 2.199 | -0.0119 – 1.695 |
| Focal attacks leading to a pin | 19.00 ± 2.31 | 16.68 ± 1.48 | 0.092 | 0.403 | 0.228 | .820 | 0.010 | -0.698 – 0.882 |
| **Number of roll reversals** | **9.46 ± 1.28** | **4.36 ± 0.78** | **-4.623** | **1.708** | **-2.707** | **.008** | **1.096** | **-7.969 – -1.276** |

Note. SE = Standard error; OR = Odds ratio; CI = Confidence interval.

**Table 7.2** Comparisons of how the focal rats defended themselves when a partner playfully attacked, analysed with a generalized linear mixed model, are presented below. The average proportion per day (± standard error of the mean) of the various measures are presented, though, the model tested each day.

| Predictor | Competent | Incompetent | Estimate | SE | z | p | OR | CI |
|---|---|---|---|---|---|---|---|---|
| Intercept | | | 13.480 | 16.922 | 0.797 | .426 | $7.149e^{5}$ | -19.686 – 46.646 |
| Proportion defended | 0.82 ± 0.02 | 0.76± 0.04 | -6.854 | 11.030 | -0.621 | .534 | 0.001 | -28.437 – 14.765 |
| Proportion evaded | 0.17 ± 0.02 | 0.14 ± 0.02 | -8.547 | 17.048 | -0.501 | .616 | $1.941e^{-4}$ | -41.962 – 24.867 |
| Proportion leading to a pin | 0.66 ± 0.03 | 0.64 ± 0.04 | -7.246 | 11.005 | -0.658 | .510 | $7.131e^{-4}$ | -28.815 – 14.323 |
| Proportion of role reversals | 0.25 ± 0.03 | 0.14 ± 0.02 | -10.789 | 12.653 | -0.853 | .394 | $2.062e^{-5}$ | -35.589 – 14.010 |

Note. SE = Standard error; OR = Odds ratio; CI = Confidence interval.

**Table 7.3** Comparisons of [1]competent and [2]incompetent rats had partner preferences for either competent or incompetent partners in the group, analysed with a generalized linear mixed model. See also Figure 2c,d, where the average preference for competent and incompetent partners is plotted.

| Predictor | Estimate | SE | z | p | OR | CI |
|---|---|---|---|---|---|---|
| Intercept[1] | 0.847 | 0.465 | 1.820 | .069 | 2.332 | -0.065 – 1.759 |
| % Attacked | -0.010 | 0.015 | -0.661 | .509 | 0.990 | -0.038 – 0.019 |
| Intercept[2] | 3.139 | 1.263 | 2.485 | .013 | 23.084 | 0.664 – 5.615 |
| % Attacked | -0.028 | 0.023 | -1.318 | .188 | 0.972 | -0.071 – 0.014 |

Note. SE = Standard error; OR = Odds ratio; CI = Confidence interval.

#### *7.3.1.2 Behavior of the partners of competent and incompetent rats*

The other rats living in the groups directed similar frequencies of playful nape attacks to both competent and incompetent rats (Figure 7.2e) (Table 7.4). When attacked by either a competent or incompetent rat, the partners defended a similar proportion of attacks, and were just as likely to use the same defensive tactics (Table 7.5). Moreover, partners of both competent and incompetent attackers were just as likely to end in a pin and engage in a role reversal (Table 7.5), leading to comparable frequencies of both pins and role reversals (Table 7.4).

### 7.3.2 Adult Behavior

As noted above, 11 of the 54 pairs of rats tested in the stranger paradigm escalated to physical aggression (Figure 7.3a). Among these 11 pairs, the focal group-reared rats were the aggressors in 5 pairs, the stranger rats in 4 pairs, and in 2 pairs, both the focal and stranger rats displayed aggressive behavior, but the aggression was started by the focal rat with the stranger rat retaliating. Regardless of who was the aggressor, stranger interactions started off with both the focal rats (Figure 7.3b) and the strangers (Figure 7.3c) engaging in more playful behavior at the beginning of the video than at the end (focal rats: $X^2(9) = 37.617$, $p < .00001$; stranger rats: $X^2(9) = 23.177$, $p = .006$; post hoc Nemenyi tests are reported in Fig 3b,c). With time, these playful interactions became rougher (Appendix B, Supplementary Video), eventually escalating to overt aggression around 4 min into the encounter (Figure 7.3b,c). This temporal distribution of aggressive behaviors being significant for the focal rats ($X^2(9) = 17.883$, $p = .037$), but not for the stranger rats ($X^2(9) = 10.973$, $p = .278$). Post hoc pairwise comparisons did not reveal which of the time bins differed significantly from one another (Nemenyi tests $p > .05$), but we note that no aggression was observed in the first four minutes of the trials (Figure 7.3b,c). Biting

occurred in 9 of the 11 pairs, with seven of the focal rats biting the partner. On average, the seven focal rats bit the stranger 3.43 times per video, with one biting the stranger 10 times. Bites were most frequently delivered to the rump and flanks of the recipient, while the aggressor pinned the recipient down. In addition to aggressive behaviors, 22 kHz calls (Figure 7.3d) were only emitted by pairs of rats that escalated to physical aggression (Figure 7.3e) ($t(20) = 2.962$, $p = .008$) and all but two of the 22 kHz calls were emitted in the second half of the encounter (sign test, $n = 403/405$, $p < .0001$). That is, the calls coincided with the advent of overt aggressive behavior, further supporting the view that encounters started off as being playful and then escalated to aggression.

Despite escalating play fighting to serious fighting, the competent focal rats did not engage in more play behavior than the incompetent rats (Figure 7.3f) ($t(20) = 1.077$, $p = .294$). Similarly, the strangers, did not initiate more play with the incompetent than the competent rats (Figure 7.3g) ($t(20) = 1.713$, $p = .102$). Moreover, regardless of whether the focal rats escalated to overt aggression or not, the proportion of the total play per pair initiated by the competent and incompetent rats was not significantly different (Figure 7.3h) ($t(20) = 0.274$, $p = .787$). Based on winning all five trials, the tube test showed that 3 of the competent rats and 4 of the incompetent rats were dominant over the stranger with which they were matched. Similarly, 5 of the competent and 2 of the incompetent rats were subordinate to their matched strangers. The number of dominant and subordinate individuals did not differ significantly between the incompetent and competent rats (Fisher's exact tests, $p > .05$). Given the comparable numbers of dominant and subordinate or indeterminate rats relative to their stranger partners, dominance differences between the pairs in the stranger test, were unlikely to account for the big difference in likelihood of escalation from RTP to aggression between the competent and incompetent rats.

**Table 7.4** Comparisons of how the partners of competent and incompetent rats played, analysed with a generalized linear mixed model, are presented below. The average frequency per day (± standard error of the mean) of the various measures are presented, though, the model tested each day.

| Predictor | Competent | Incompetent | Estimate | SE | z | p | OR | CI |
|---|---|---|---|---|---|---|---|---|
| Intercept | | | 10.960 | 3.662 | 2.993 | .003 | $5.752e^4$ | 3.783– 18.136 |
| Partner initiated nape attacks | 24.06 ± 3.66 | 23.61 ± 4.12 | -0.017 | 0.210 | -0.080 | .936 | 0.983 | -0.429 – 0.395 |
| Partner attacks leading to a pin | 18.36 ± 2.21 | 19.04 ± 3.04 | 0.034 | 0.274 | 0.124 | .902 | 0.979 | -0.503 – 0.571 |
| Number of roll reversals | 7.50 ± 1.03 | 6.64 ± 1.06 | -0.021 | 0.451 | -0.047 | .962 | 1.035 | -0.905 – 0.863 |

Note. SE = Standard error; OR = Odds ratio; CI = Confidence interval.

**Table 7.5** Comparisons of how partners of the focal competent and incompetent rats defend themselves, analysed with a generalized linear mixed model, are presented below. The average proportion per day (± standard error of the mean) of the various measures are presented, though, the model tested each day.

| Predictor | Competent | Incompetent | Estimate | SE | z | p | OR | CI |
|---|---|---|---|---|---|---|---|---|
| Intercept | | | -8.236 | 15.042 | -0.548 | .584 | $2.649e^{-4}$ | -37.718 – 21.245 |
| Proportion defended | 0.81 ± 0.03 | 0.84 ± 0.03 | 4.093 | 8.029 | 0.510 | .610 | 59.923 | -11.643 – 19.829 |
| Proportion evaded | 0.14 ± 0.02 | 0.14 ± 0.03 | 8.331 | 13.128 | 0.635 | .526 | $4.152e^{3}$ | -17.400 – 34.062 |
| Proportion leading to a pin | 0.68 ± 0.03 | 0.73 ± 0.03 | 6.013 | 10.727 | 0.560 | .575 | $4.851e^{2}$ | -15.012 – 27.038 |
| Proportion of role reversals | 0.22 ± 0.02 | 0.20 ± 0.02 | -2.488 | 10.885 | -0.229 | .819 | 0.083 | -23.822 – 18.846 |

Note. SE = Standard error; OR = Odds ratio; CI = Confidence interval.

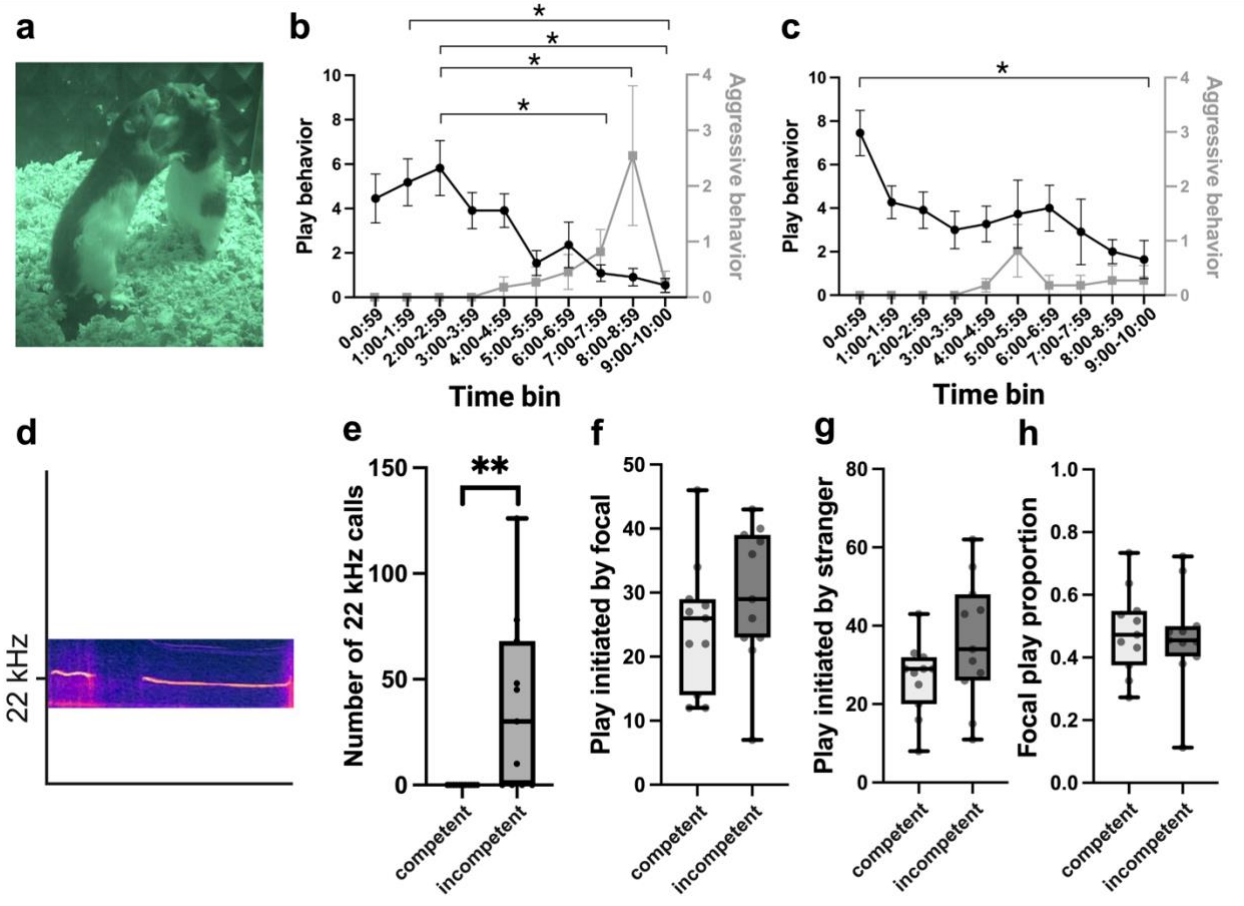


**Figure 7.3** When rats are paired in a neutral arena, some escalate play fighting into serious fighting engaging in aggressive behaviors (a). Though all the incompetent rats start off by engaging in play fighting, some escalate to serious fighting around halfway through the 10 min encounter (c). Results from post hoc Nemenyi tests show which pairwise RTP were significantly different. Similarly, the partners of the focal rats, or the 'strangers' start off by engaging in play fighting, some escalate into serious fighting around halfway through the encounter but deescalate over the remainder of the session (c). Results from post hoc Nemenyi tests show which pairwise RTP were significantly different. 22kHz flat vocalizations can be used to determine the affective state of the rats as they are often emitted when the rats are in fearful state (d). 22 kHz calls were only emitted by pairs of rats that included an incompetent rat that escalated to overt aggression (e). No difference was found in the number of playful attacks initiated by competent or incompetent rats (f). Similarly, no difference was found in the number of playful attacks initiated by the stranger rats when with either a competent of incompetent rat (g). Finally, the proportion of play between the pair that competent and incompetent rats engaged in did not differ (h). *$p < .05$

### 7.3.3 Neuronal morphology

We found a significant effect of social competency on both convex hull volume ($F(1,13.998) = 39.022$, $p < .0001$) (Figure 7.4d) and surface area ($F(1,35.972) = 48.434$, $p < .0001$) (Figure 7.4e). Similarly, significant effects of social competency were found for both the convex hull volume ($F(1,35.787) = 16.625$, $p = .0002$) and surface area ($F(1,13.767) = 18.052$, $p = .0008$) of the basilar dendritic projections (Figure 7.4f) and for the convex hull volume ($F(1,36.220) = 40.515$, $p < .0001$) and surface area ($F(1,36.275) = 58.196$, $p < .0001$) of the apical projections. Incompetent rats had larger dendritic arbors for all convex hull volume and surface area measures compared to competent rats (Table 7.6).

As with the convex hull measures, we found a significant effect of social competency on the total length of neurons ($F(1,36.040) = 68.013$, $p < .0001$) and the length of the basilar dendritic projections ($F(1,14.206) = 35.782$, $p < .0001$) (Figure 7.4g) and apical projections ($F(1,36.432) = 68.681$, $p < .0001$) (Figure 7.4j). For the total length, and the length of the basilar and apical dendritic projections, incompetent rats had longer projections compared to competent rats (Table 7.6).

A significant effect of social competency on the total volume of the cells ($F(1,14.306) = 14.659$, $p = .002$), basilar projections ($F(1,14.327) = 16.482$, $p = .001$) and apical projections ($F(1,36.338) = 24.193$, $p < .0001$) was found. No significant effect of social competency was found for the cell body volumes ($F(1,14.166) = 3.861$, $p = .069$). For both the total volume of the cells, and the volume of basilar and apical projections, incompetent rats had more voluminous cells (Table 7.6).

We found a significant effect of social competency on the total branch number ($F(1,14.756) = 55.485$, $p < .0001$), basilar branch number ($F(1,15.517) = 31.842$, $p < .0001$)

(Figure 7.4h), and apical branch number ($F(1,36.646) = 50.906$, $p < .0001$) (Figure 7.4k). Incompetent rats had a more branches than competent rats for all three measures (Table 7.6).

Finally, we found a significant effect of social competency on spine density (Figure 7.4l,m) in the distal segments ($F(1,42) = 14.133$, $p = .0005$) (Figure 7.4n), but not for the proximal segments of the apical arbors ($F(1,86) = 3.356$, $p = .070$) (Figure 7.4o). No effect of social competency was found for the distal segments ($F(1,20) = 3.274$, $p = .085$) (Figure 7.4p) or the proximal segments ($F(1,14.3) = 3.538$, $p = .081$) (Figure 7.4q) of the basilar dendritic arbors.

**Table 7.6** Average measurements (± standard error of the mean) of pyramidal neurons found in Cg3, layer III, of rats.

| Measurement | Competent | Incompetent | Percentage difference (%) |
|---|---|---|---|
| Convex hull volume ($cm^3$) | 631.66 ± 39.15 | 1131.45 ± 57.47 | 44.17 |
| Apical convex hull volume ($cm^3$) | 225.99 ± 23.19 | 494.73 ± 34.17 | 74.58 |
| Basilar convex hull volume ($cm^3$) | 73.35 ± 7.53 | 145.81 ± 10.82 | 66.13 |
| Convex hull surface area ($cm^2$) | 27.47 ± 1.12 | 41.99 ± 1.32 | 41.82 |
| Apical convex hull surface area ($cm^2$) | 14.03 ± 0.88 | 23.85 ± 1.01 | 51.85 |
| Basilar convex hull surface area ($cm^2$) | 10.06 ± 0.67 | 16.09 ± 0.74 | 46.06 |
| Total length (mm) | 4.34 ± 0.15 | 6.38 ± 0.16 | 38.02 |
| Apical length (mm) | 1.46 ± 0.07 | 2.47 ± 0.09 | 51.24 |
| Basilar length (mm) | 2.00 ± 0.08 | 2.98 ± 0.09 | 39.27 |
| Cell body length (mm) | 0.87 ± 0.08 | 0.92 ± 0.01 | 5.42† |
| Total volume ($mm^3$) | 10.67 ± 0.52 | 14.38 ± 0.40 | 29.67 |
| Apical volume ($mm^3$) | 2.94 ± 0.19 | 4.39 ± 0.20 | 39.45 |
| Basilar volume ($mm^3$) | 1.92 ± 0.12 | 3.04 ± 0.12 | 45.27 |
| Cell body volume ($mm^3$) | 5.82 ± 0.29 | 6.96 ± 0.21 | 17.93† |
| Total branch number | 25.55 ± 0.88 | 42.65 ± 1.62 | 50.12 |
| Apical branch number | 11.50 ± 0.59 | 21.09 ± 1.07 | 58.86 |
| Basilar branch number | 14.05 ± 0.58 | 21.55 ± 0.77 | 42.12 |
| Apical distal spine density (#/40µm) | 10.94 ± 0.61 | 15.22 ± 0.88 | 32.70 |
| Apical proximal spine density (#/20µm) | 0.27 ± 0.10 | 1.05 ± 0.42 | 117.24† |
| Basilar distal spine density (#/40µm) | 12.56 ± 0.93 | 15.52 ± 0.99 | 21.08† |
| Basilar proximal spine density (#/20µm) | 0.78 ± 0.14 | 1.48 ± 0.24 | 61.31† |

†Denotes a non-significant difference ($p > .05$); actual $p$ values are reported in text.

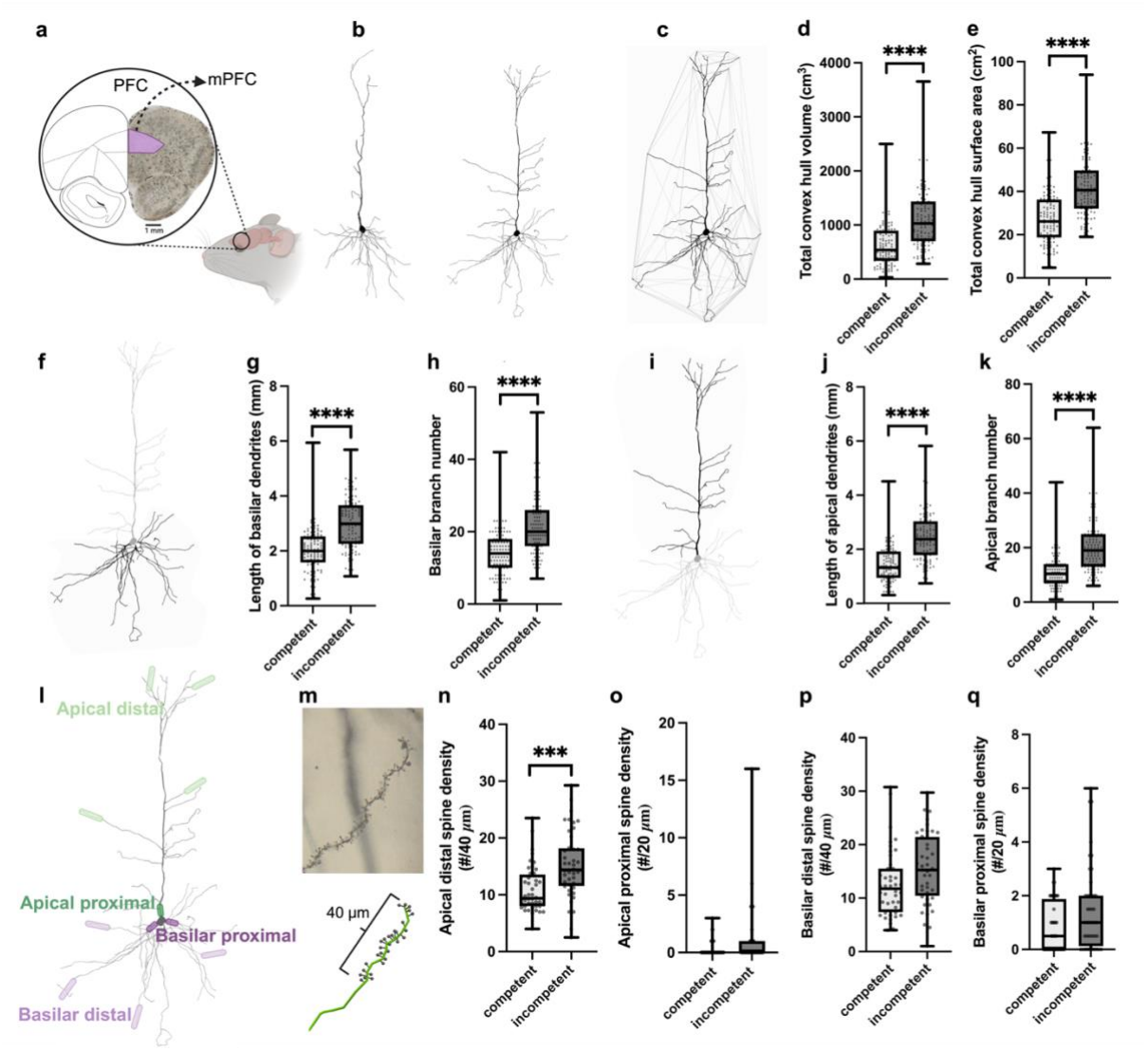


**Figure 7.4** A diagram of the rat brain section and corresponding Golgi stained brain tissue through the medial prefrontal cortex (from Paxinos & Watson, 1986) (a). Once the neurons were traced, various morphological features were compared between competent rats (left) and incompetent rats (right) (b). One such comparison is comparing the total convex hull volume (c). When compared, both the total convex hull volume (d) and total convex hull surface area (e) are significantly larger for incompetent rats. When the basilar components of the cell are compared (f), we find that both the length of the basilar arbor (g) and the basilar branch number (h) are significantly greater in incompetent rats. Similarly, when the apical arbors are compared (i), both the length (j) and the branch number (k) are greater for incompetent rats. Various aspects of the cell were measured to determine spine density (l). Four distal locations on both the apical and basilar branches, one proximal location on the apical branch, two locations on the proximal segments of the basilar branches. For distal segments, 40 µm sections were measured while 20 µm were measured for proximal segments (m). Spine density was significantly greater in incompetent rats on the distal apical segments (n) but not for the proximal apical (o), distal basilar (p), or proximal basilar segments (q). ***$p < .001$; ****$p < .0001$

## 7.4 Discussion

Even though reared in groups of six, so having plenty of opportunity to play, when tested in the stranger paradigm as adults, 20.4% escalated the initially playful encounters into aggressive ones. Our hypothesis was that if such naturally occurring variation was present it would be associated with differential play experiences in the juvenile period. As experiments that manipulate the quantity and quality of RTP experienced in the juvenile period have shown that as adults, such rats and hamsters have reduced social competency (Burleson et al., 2016; Schneider, Bindila, et al., 2016), including escalation to aggression or other evidence of poor social performance in the stranger test (Ham, Szabo, et al., 2024; Stark & Pellis, 2020, 2021), we predicted that the rats performing poorly in the stranger test, should have impoverished juvenile RTP compared to their better performing peers. This prediction was partially supported in that role reversals were significantly fewer in the juvenile RTP of the less competent rats, although neither the proportion of attacks that lead to role reversals nor the quantity and quality RTP experienced differed (Figure 7.2). But given the growing evidence that the critical play-derived experience important for social skill development is negotiating role reversals (Ham, Szabo, et al., 2024; Pellis et al., 2017, 2019; Stark et al., 2021), the support may be stronger than it first seems. In addition, since rats and hamsters reared in experimental contexts with reduced quantity and quality of play have greater dendritic proliferation of the mPFC neurons than their peers with more typical RTP experience (Bell et al., 2010; Burleson et al., 2016; Ham, Szabo, et al., 2024; B. T. Himmler, Pellis, & Kolb, 2013; Stark et al., 2023), we predicted that the rats performing badly in the stranger test should have less dendritic pruning than there better performing peers. This prediction was strongly supported (Figure 7.4; Table 7.6). Finally, based on previous studies with the stranger test, we predicted that initially the animals would engage

in RTP, but then as the encounter continued, the animals would gradually escalate to serious fighting (Kisko et al., 2015; Smith et al., 1999, Stark & Pellis, 2020). The prediction was supported (Figures 7.3b, c), suggesting that the incompetent rats had greater difficulty in sustaining a playful mood or coordinate their behavior effectively with the strangers.

Together, these findings suggest that despite having an equal opportunity to play, not all rats benefit equally from playing and this may account for some of the variance of the effects of RTP on socio-cognitive skills and mPFC anatomy revealed in previous experiments (Bell et al., 2010; Ham et al., 2024; Himmler, Pellis & Kolb, 2013; Stark & Pellis, 2020; Stark et al., 2023). Moreover, it is not the quantity of play, but the quality of the playful interaction—and specifically turn taking—that contributes to improved social behavior and brain development. Whereas the studies with Belding's ground squirrels show a positive correlation between the amount of play and psycho-motor development (Nunes & Monroy Montemayor, 2023), our present findings (Figure 7.2) are consistent with our previous studies in rats (Ham, Szabo, et al., 2024; Pellis et al., 2019; Stark et al., 2021); it is not simply the quantity of RTP that matters, but the quality of interactions, especially those that involve turn taking. If so, this may explain why even limited opportunity for RTP experience may be sufficient to mitigate the negative effects of social isolation (Einon et al., 1978; Whitten et al., 2025). These qualitative experiences during RTP may be essential for developing appropriate social behaviors and the neural correlates for processing social behavior, emotional regulation, and executive functions. It is turn taking that has been suggested to be the mechanism by which behavioral flexibility in children improves (Pellegrini, 1988).

That the mPFC is involved in turn taking has been shown in recent studies involving recording brain activity during a playful game. Juvenile and adult rats were trained to play

hide-and-seek with an experimenter (Bagi et al., 2022; Reinhold et al., 2019). When the rats were hiders, they sought out opaque barriers or boxes in which to hide and they remained silent (i.e., did not emit ultrasonic calls). When seekers, the rats engaged in systematic searchers of the room, checked locations behind which the experimenter had hidden before, and were not silent while searching. Critically, the electrophysiological pattern of mPFC activity was different between the two roles. These findings support the likelihood that the mPFC is differentially active depending on the role taken during RTP, and if so, RTP could provide a means of refining mPFC function. Given our present findings, this hypothesis deserves to be tested experimentally.

While all rats in our present study were provided with similar opportunities to engage in RTP, some individuals engaged in lower levels of turn taking as juveniles (Figure 7.2), and those same individuals exhibited reduced social competence (Figure 7.3) and less pruned dendrites of the mPFC neurons (Figure 7.4) as adults. While experimental manipulations of play experience clearly implicate a causal role for juvenile experience with RTP in both refining social competence and pruning the dendrites of mPFC neurons (Burleson et al., 2016; Ham, Szabo, et al., 2024; Stark et al., 2023; Stark & Pellis, 2020), why RTP failed to do this in about 20% of the rats, despite all rats having the opportunity to play, remains to be explained. We consider two plausible hypotheses.

First, it is possible that the rats could have benefitted from RTP, but that other members of the group failed to adjust their play to meet the needs of the rats that grew up to be socially incompetent, although our data did not identify any obvious ways in which this may have occurred, as they engaged the rats later categorized as incompetent as much as the rats later categorized as competent (Tables 7.2, 7.3-5). Indeed, none of the play measures (e.g., the number of attacks, number of attacks defended, how attacks were

defended), differed significantly between competent and incompetent rats. Yet, for all measures, the incompetent rats had lower scores, the cumulative effect of which could not only explain the significant difference in the quantity of role reversals, but also highlight that these rats did not play in a typical manner. Because of these subtle differences in their play, within their respective groups, they may not have been the preferred partners. Detailed within group play partner preferences (Ham & Pellis, 2025) and more nuanced measures of play (Pellis, Pellis, Burke et al., 2022) are needed to fully assess whether failure to acquire the necessary RTP-derived experiences from peers accounts for the later deficiencies in social behavior.

Second, the deficiency is in the rats themselves, so that rats that grew up to be socially incompetent were already socially incompetent before the juvenile period and so failed to be able to benefit from the RTP in which they engaged. This inability to benefit from the positive effects of RTP in the juvenile period could be linked to genetic factors (e.g., Sgro & Mychasiuk, 2020) and/or early life experiences (e.g., Kentrop et al., 2018; van den Berg et al., 1999; Veenema & Neumann, 2009). For example, research on rhesus macaques (*Macaca mulatta*) has shown that individual differences in social behavior are established early in life, with some individuals exhibiting more aggression and a disruptive temperament (Suomi, 1991). In both captive and wild populations, around 5-10% of rhesus macaques respond to mildly stressful experiences with inappropriate aggression levels (Suomi, 2003). This atypical emotional regulation can have a negative impact on affiliative relationships, as young monkeys prefer to play with individuals that have a similar temperament to themselves (Weinstein & Capitanio, 2008), consequently, these high strung individuals tend to be shunned as play partners (Suomi, 2005).

Early life experiences, particularly maternal care, may play a role in the development of a less cooperative phenotype. Indeed, the quality of maternal care can either exacerbate or attenuate the aggressivity and impulsiveness of serotonin deficient rhesus monkeys (Suomi, 2005). While the maternal care of our study subjects received is unknown, rats that receive less licking and grooming by their mothers engage in more RTP than those that receive more (Parent & Meaney, 2008). If our incompetent rats developed the way they did because of a genetic quirk or a pre-juvenile experiential deficiency, this would suggest that certain individuals would never benefit from RTP, unless intervention occurred before the juvenile period. This is an area of research that warrants further investigation, as distinguishing the effects of early social experience from the later consequences of juvenile play is critical to understanding the development of social behaviors and its underlying neural mechanisms.

In humans, children who engage in more RTP tend to have better social skills and greater behavioral flexibility (Pellegrini, 1988, 1993). Similar to the rats in this study which avoid play when multiple individuals are involved, some children fail to integrate into social groups and instead avoid play, often seeking comfort from adults (Blurton Jones, 1976), or when they do play they escalate the encounters into serious fighting (Willner, 1991). The social deficits observed in these children mirror aspects of the non-cooperative phenotype we identified in the rats.

When unfamiliar rats interact with one another, adults use RTP to establish social relationships and navigate differences in relative dominance (L. K. Smith et al., 1999). However, when two dominant male rats interact, the RTP tends to become rougher and is more likely to escalate into serious fighting than when two subordinate rats, or a dominant and a subordinate interact (L. K. Smith et al., 1999). However, the tube test data suggest

that this is unlikely; the number of competent and incompetent rats that were dominant over the stranger or had indeterminate dominance relationships with the stranger were similar. This suggests that the greater likelihood of escalation to aggression by the incompetent rats cannot be explained by inadvertently having placed two home cage dominants together. Still, a possible contribution of dominance cannot be discounted.

To gain a deeper understanding of the extent of social dysfunction that arises from deficient juvenile RTP experiences, future studies should use additional test paradigms to assess social competency, such as ones that evaluate socio-sexual behavior (Marquardt et al., 2023) and inter-animal coordination (B. T. Himmler et al., 2014). Nonetheless, even with the data available our study supports the conclusion that not all rats benefit equally from RTP.

## 7.5 Conclusion

The present study found that naturally occurring variation in juvenile RTP is consistent with experimental studies that have linked role reversals in juvenile RTP to the development of social competency and dendritic pruning of mPFC neurons (Ham, Szabo, et al., 2024; Stark et al., 2023; Stark & Pellis, 2021). Individuals that engage in less role reversals have atypical social skills and pyramidal neurons in the mPFC. To determine whether individuals who are naturally low-playing or engage in poorer-quality play mirror the outcomes of experimental play deprivation (Ham, Szabo, et al., 2024; Stark et al., 2023; Stark & Pellis, 2020), we used the stranger test to assess social competence and conducted Golgi staining to analyze neurons in the mPFC—the same measures used in prior studies involving play-deficient rats. Our anatomical measures assume that there are benefits to having increased pruning of the cells in the mPFC. First proposed by Cajal (1899), he suggested that neurons are organized and connected in the most economical way, so as to

minimize cost. In this way, fewer connections and smaller neurons should be favored over more complex and large neurons if both can perform the same task. While Golgi staining is effective for detecting group-level differences, the unpredictable variability as to which neurons are stained, combined with the fact that only a small percentage of cells are labeled using this technique (Ramón-Moliner, 1970), makes comparing the severity of manipulations across individuals challenging.

Alternative techniques have shown that rats deprived of juvenile RTP have altered functionality of the mPFC neurons, with fewer inhibitory synapses in pyramidal cells of Layer V (Bijlsma, 2022, 2023). Such a measurement may be more effective for correlating individual differences in the quality of of play with neuronal changes. Even more effective could be using electrophysiological techniques that can measure the activity of hundreds of cells simultaneously *in vivo* while rats are engaging in RTP (Bagi et al., 2022; Concha-Miranda et al., 2020; Reinhold et al., 2019). To move beyond group-level effects and broad correlations within populations, studies investigating electrophysiological activity during role reversals are essential to link the causal relationship between juvenile RTP experience and the development of socio-cognitive skills at the level of the individual animal. Such data are a necessary first step to explain why some rats benefit more than others from their juvenile RTP experiences.

# CHAPTER 8: EARLY-LIFE ADVERSITY CAUSES SOCIAL BUT NOT GENERAL ANXIETY, LEARNING AND MEMORY DEFICITS, AND CHANGES IN NEURON MORPHOLOGY AND METABOLOME OF RATS OF BOTH SEXES

## 8.1 Introduction

Mood disorders, such as depression and anxiety, affect millions, including adults and juveniles (James et al., 2018). Anxiety, the most common psychiatric disorder, is characterized by altered productivity, persistent and generalized fear and anxiety, overactivity and/or inability to relax, difficulty handling uncertainty, indecisiveness, and fear of making the wrong decision (American Psychiatric Association, 2013). With such high prevalence and widespread effects on society, rodent models have been developed to assess the neurological changes associated with the symptomology of anxiety disorders and develop treatments (Harro, 2018).

Many pre-clinical rodent models of mood disorders exist, including developmental, strain, genetic, and chronic stress induction models (Nestler & Hyman, 2010; Xiong et al., 2023). However, these methods of induction frequently lead to contradictory results. This may in part be due to the assays used to assess rodent anxiety-like and depressive-like behavioral phenotypes. With a recent push to use more naturalistic and ethological relevant pre-clinical rodent models and assays (C. J. Burke et al., 2021; d'Isa & Gerlai, 2023), this may limit the inconsistencies among studies (Lehmann & Feldon, 2000).

One of the most frequently used induction method is a developmental model whereby animals are subjected to early life (postnatal) stress. This stress, often achieved by separating rodent pups from their mother for a few hours to a day, impacts brain organization and development resulting in adults with poorer cognitive ability and quality

of life (Kentrop et al., 2018; Sailer et al., 2022). More specifically, rodents have altered learning and cognition, are aggressive, and exhibit anxiety-typical behaviors and/or depressive-typical behaviors (Kambali et al., 2019; Lippmann et al., 2007; Veenema et al., 2006).

Despite anxiety being the most common childhood and adolescent psychiatric disorder (Bosquet & Egeland, 2006), few animal models investigate mood disorders in immature individuals. Most studies focus on neonatal stress to investigate the deficits in adults. However, clinical studies of anxiety and mood disorders in humans have shown that these disorders can be present in children as young as 12-18 months old (Beesdo et al., 2009). Children with anxiety and depression are withdrawn socially, have a hard time focussing academically, and often have a history of other mental health problems and substance-use disorder (Kessler et al., 2001). Given that child neglect is the most prevalent form of child maltreatment (Strathearn, 2011), it is important to develop not only animal models of both early life adversity and anxiety, but also assays that can assess the behavior of both juvenile and adult animals.

Here, we used rough-and-tumble play (RTP) behavior, a naturally occurring form of social behavior that is present in both immature and mature rats (Pellis, Pellis, Ham, et al., 2022), to assess the outcome of early-life stress. In addition, we used a variety of traditional rat behavioral assays (e.g., the elevated plus maze [EPM], Morris water task [MWT]) to determine impacts of early-life stress on anxiety, learning, memory, and cognition (Antoniadis & McDonald, 2000; McDonald et al., 2008; McDonald & White, 1994). These traditional assays were used as the brain regions required for the underlying functions supporting performance on these tasks have been repeatedly implicated in mood disorders (Harro, 2018; Xiong et al., 2023). This knowledge will allow a deeper

understanding of the patterns of RTP behaviour exhibited by the subjects that experience early life stress and what altered brain functions might be mediating them.

As a first step, analyses of the neuronal morphology of the prefrontal cortex and septum were conducted to determine if neural changes resulted. These regions have been consistently implicated in play and sociality (Menon et al., 2022; Pellis, Pellis, Ham, et al., 2023). Serum corticosterone and metabolic profiles were also conducted. The former to determine the potential contribution of elevated stress levels on brain and behaviour and the latter to get a preliminary look at any differences in the metabolome of control and early life stress exposed subjects. By combining new and traditional tests, with known robust neural network correlates, we can assess brain networks that have been previously implicated with mood disorders, social behavior, and with learning and memory.

Like previous studies of early-life stress (Kentrop et al., 2018; Muhammad & Kolb, 2011; Tao et al., 2017; Veenema & Neumann, 2009), we predicted that RTP behavior would likely increase in frequency as both juveniles and adults. When juvenile RTP experiences are deprived, effects on the development of socio-cognitive skills and impulse control are observed, and rats can have an in increase in anxiety- and depressive-like behaviors (Arakawa, 2018; Bijlsma et al., 2024; Einon & Morgan, 1977; Ham, Szabo, et al., 2024; Pellis, Pellis, Ham, et al., 2023; Schneider, Bindila, et al., 2016). Therefore, disruption in juvenile RTP can have long-lasting consequences. Along with the predicted changes in juvenile RTP behavior, we expect deficits would be observed in adult social cognition, as well as with learning and memory. A series of well-established tests were used to assess adult cognition, learning, and memory. Using the EPM we predicted that isolated rats would show increased anxiety-like behavior. We predicted that isolated rats would have compromised generalized fear discrimination and would have difficulties

associating a fearful stimulus with a context during discriminative fear condition to context (DFCTC) test. If no preference was shown during DFCTC, this would indicate disfunction in fear discrimination and orbitofrontal cortex function (Trow et al., 2017; Zelinski et al., 2010). Emotional learning was predicted to be intact, along with the basolateral amygdala and the neural circuitry involved in this form of emotional learning (McDonald et al., 2010) as the animals were not predicted to be anhedonic. This was tested using the conditioned place preference (CPP) test. Two variants of the MWT were used. Cue-place MWT, where rats swim to either a visible or invisible platform, assesses the dorsolateral striatum and the hippocampal networks, respectively (McDonald & White, 1994). We predicted that isolated rats would express difficulties navigating towards the invisible platform, as they would have memory deficits, while they would not be impaired navigating towards the visible platform. Like the invisible platform tests, one-trial MWT assays spatial learning and memory neural networks and places a higher demand on hippocampal processing versus other versions of this task (McDonald et al., 2010; Sutherland et al., 1997, 2000). We predicted isolated rats would show deficits when navigating towards the platform due to deficits in learning and memory and the dorsal hippocampus. Finally, with the disruption of play and social systems we predicted that the morphology of neurons in key social areas would be altered (Hodges et al., 2019), and that early life adversity animals would have higher corticosterone concentrations and a disrupted metabolome (Shi et al., 2013).

In this study, we found that early life adversity, in the form of neonatal isolation, makes both male and female rats socially anxious, engaging in less RTP with unfamiliar individuals but playing typically with cage mates. When given a choice between control or isolated individuals, isolated rats choose to RTP with other isolated rats over controls. As adults, males continue to be socially anxious while both male and females engage in less

cooperative behavior. Only rats that were isolated as neonates were aggressive, escalating to overt fighting. Control and isolated rats avoided contexts paired with foot shocks, and the EPM revealed no difference between control and isolated rats, suggesting that anxiety-like behavior was not generalized and instead more consistent with a social-anxiety phenotype. Isolated rats did not prefer contexts in which they were provided a food reward suggesting an impairment in appetitive associative conditioning. In one-trial place learning of MWT, when compared to controls, female and male isolated rats took longer to find the invisible platform on their second swim, only reaching the platform more efficiently on the second swim after a week of training. Only isolated females had difficulty navigating to invisible and visible platforms in the cue-place variant of the MWT. In many of our paradigms, the isolated rats attempted to escape, including during the adult social testing and CPP, and navigated towards the edge of the pools during both MWT and towards the ends of the arms during the EPM. Neurons in the medial prefrontal cortex (mPFC) of isolated rats were less spiny. Isolated females had longer neurons and somas in the dorsal lateral septum (dLS) compared to control females, while no difference was found between males. Finally, we found that certain metabolites (e.g., glucose, valine) were up- or downregulated in isolate females and males compared to control rats. Our results reinforce the strength of testing animals in ethologically relevant contexts (d'Isa & Gerlai, 2023) along with assays that require different cognitive processes and brain networks, as the effects of early life stress may not affect both sexes equally nor will the stress be generalized to all rodent behavioral tests.

## 8.2 Methods

### 8.2.1 Ethics statement

All care and testing procedures were reviewed and approved by the University of Lethbridge Animal Welfare Committee (breeding protocol: #2307; testing protocol: #2306) in compliance with guidelines from the Canadian Council for Animal Care.

### 8.2.2 Housing environment

All rats were housed on a 12-h light-dark cycle and maintained at a constant temperature of 21°C-23°C at 35% humidity. Food and water were available *ad libitum.*

### 8.2.3 Breeding animals

Twelve adult Long Evans male and female rats were purchased from Charles Rivers (Kingston, NY, USA). After arriving at the Canadian Centre for Behavioural Neuroscience, they were given at least two weeks to acclimate to their new environment before breeding pairs were formed. Males and females were placed in pairs for seven days. After the males were removed, females were pair-housed with their previous cage mate. During the last week of gestation, females were singly housed and recorded 24 h a day in the home cage. Only 8 of the 12 females gave birth.

### 8.2.4 Focal animals

Breeding resulted in 70 pups, 37 males and 33 females from 8 dams. Each litter was randomly assigned a condition (i.e., control or neonatal social isolation). This resulted in 4 control litters and 4 experimental litters, with a total of 17 males and 17 females in the control groups and 20 males and 16 females in the isolated groups. At post-natal day (PND) 21, animals were weaned and placed into pairs or trios.

### 8.2.5 Adult social interaction partner animals

Additional Long Evans male and female rats were purchased from Charles Rivers (Kingston, NY, USA) to serve as the unfamiliar, neutral, partners in the stranger paradigm. These animals were ordered at around 60 days of age giving them time to acclimate to our facilities.

### 8.2.6 Neonatal isolation

Neonates were isolated in small Ziploc® containers (15.875 cm × 15.875 cm × 14.021 cm). Nests were made from Krinkle® and cotton bedding and placed on the floor of the container. Holes were added to the lids; however, the lids were only used during the transportation of the pups to the isolation room. The isolation containers were placed in a Tecniplast heating cabinet, which was maintained at 25°C.

Before isolating the pups, dams were removed from their cage and placed into a clean holding cage. The pups were weighed, and a small number was written on their back so they could be identified the following day. They were then placed into their isolation container and moved to the isolation cabinet. Once the pups were removed from the cage, the dams were placed back in the home cage where they remained while their pups were in isolation. Pups were isolated for 3 h a day, after which they were moved back to the housing room. The dam was removed from the home cage and placed into a clean holding cage while the pups were placed back into the cage. Once the pups were placed back in the cage, the dam was placed back in the cage. Animals were isolated for 12 consecutive days, from PND3 to PND14.

### 8.2.7 Maternal care

Video recordings were made of each dam in their home cage three to five days before their expected due date. Recordings continued until PND20, when the pups were weaned. An experimenter scored the dam's behavior starting on the day the pups were born until PND20 following a standard protocol (Capone et al., 2005). The videos were scored at 8:30, 10:30, 12:30, 14:30, 16:30, 17:30 each day for 20 s. The presence of the following aspects of maternal care and home cage behaviors were scored: (1) *Licking*, where the dam uses her tongue to clean her pups. This was differentiated from *anogenital licking* whereby the dam licks the underside of the pup, and more specifically the genitals and anus. (2) *Nursing* was differentiated into three categories. First, (2a) *arched-back nursing* where the dam is still and in a high upright position and head depressed. As this allows most, if not all, pups to feed, this is an ideal feeding position. Second, (2b) *blanket nursing*, where the dam is relatively still but the head is not depressed, and the body is not arched. The dam lies flat on the pups with little to no support from her fore and hind limbs. Finally, (2c) *passive nursing*, was defined by the dam lying down on her side and not actively facilitating nursing. (3) *Stepping,* where the dam is mobile but gently steps over the pups' bodies, stimulating them. (4) *Digging,* the dam digs in the home cage bedding, moving it with its nose or paws. (5) *Rearing,* the dam on its' hindlimbs, often sniffing the area. (6) *Moving* around the cage. (7) *Eating* pellets either out of the food hopper or the pellets found on the floor of the cage. (8) *Drinking* from the water bottle. (9) *Self-grooming* of the body, including licking, combing, or scratching. (10) *Resting* in a sleeping position while remaining motionless. (11) *Out of nest* to *eat* or *drink* was scored. This can also include *moving* to explore or perform non-maternal activities. Dams could also be *resting* outside of the nest away from the pups. (12) *Nest building,* gathering supplies to improve nest or

actively adding bedding to the nest. The behavior of all experimental dams was compared against half of the control dams.

### 8.2.8 Juvenile play testing

Both conditions and cohorts were tested as juveniles. Animals were PND 30 at the time of testing. The rats were tested in a variety of social situations, both in dyads and in groups. Animals always played with the same sex.

*Apparatus.* For dyadic testing, juveniles were tested in a clear Plexiglass® enclosure measuring 50 cm × 50 cm × 50 cm. For group testing, animals played in a clear tested in a clear Plexiglass® enclosure measuring 80 cm × 80 cm × 50 cm. The floor of both enclosures was covered in approximately 3 cm of corncob bedding. Both dyadic and group interactions were tested in red light. All interactions were video recorded and scored afterwards.

*Procedure.* At PND28, rats from the same cage were habituated to the dyadic test enclosure for 10 min in red light for two consecutive days. At PND 30, the rats were socially isolated for 2.5 h, daily, before RTP testing with food and water provided *ad libitum*. Brief isolation was done to increase playfulness (Pellis, Pellis, Burke, et al., 2022). Following isolation, rats were transferred to the play test enclosure. Two animals were placed into the enclosure simultaneously and left to play freely for 10 min. Dyadic RTP testing occurred between PND30-35, however, the partners varied. For the first two days, animals participated in the 'standard' juvenile RTP test whereby two unfamiliar animals of the same condition interact (Pellis, Pellis, Burke, et al., 2022). In the following two days, animals played with unfamiliar individuals of the opposite condition (i.e., control animals played with isolated animals). Finally, during the last two days of dyadic testing, same condition familiar animals (i.e., cage mates) played with one another. After the dyadic test period, daily isolation ceased, and the rats started the group play habituation period. During this

period, animals were placed in the group play enclosure with their cage mates, in red light, and left to freely explore for 10 min. Rats were habituated to the enclosure for two consecutive days. Group play testing and daily 2.5 h social isolated started at PND38. In the large enclosures, groups of three consisted of either two isolated rats and one control or two control rats and one isolated rat. All individuals were unfamiliar. Group play trials lasted 10 min.

*Behavioral analysis.* Following testing, the video files were analyzed using both normal speed and frame-by-frame analysis to score RTP (Pellis, Pellis, Burke, et al., 2022). For each video, the playful actions initiated by each animal and the defense tactics used by the recipient were noted. Playful attacks were scored when the snout of one rat was in contact with the partners nape (Pellis, Pellis, Burke, et al., 2022). In addition, unsuccessful attacks, where the snout is directed towards the nape of their partner, but the partner evades before contact is made, were considered to be playful attacks. As noted above, the defensive tactics used were scored. There are several ways rats defence themselves. First, a rat can evade (i.e., run away) or can engage in a facing defense. There are various ways in which a rat can engage in a facing defense. Second, the animal can role onto its dorsum, ending in a 'pin' configuration. Third, a rat can stand in an upright position with its partner, both standing in a mutual upright position, also known as a 'boxing' configuration (Pellis, Pellis, Burke, et al., 2022). Finally, the rat could not respond to a playful attack. In this case, the response is categorized as 'none.'

One important feature of RTP is that animals engage in certain actions that keep the play *fun* (Pellis et al., 2024). To do so, animals engage in turn taking or role reversals, so that there is reciprocity in attacking and defending (Palagi, Cordoni, et al., 2016). To assess this cooperative aspect of play, the proportion of role reversals was measured.

In addition to role reversals, we used symmetry to assess the quality of play. To calculate symmetry in play, intrapair differences in launching nape attacks were scored. To do so, the number of attacks by one partner was subtracted from the other, and the absolute difference was divided by the total play of the pair. This value was subtracted from one, giving a score between 0 and 1. Values closer to 1 indicate a high degree of symmetry while values closer to 0 indicate a high degree of asymmetry. Given that for play to remain playful, play relationships need to be reciprocal and have some degree of symmetry (Aldis, 1975; Pellis et al., 2024), symmetry scores closer to 1 indicate higher quality play relationships while those closer to zero indicate lower quality play relationships.

As playfulness varies among rats, with some being high players and others low (Achterberg et al., 2023; Lesscher et al., 2021), we used the percentage of play directed towards each play partner when determining if partner preferences were exhibited during group play.

**8.2.9 Social interaction test**

When the animals were PND60 (adolescents), they were tested in the social interaction test (File & Seth, 2003),used to test anxiety. Group scores were used to assess if animals that underwent neonatal separation were anxious.

*Apparatus and procedure.* We placed two rats of the same condition into a Plexiglass® enclosure measuring 80 cm × 80 cm × 50 cm when the lights were red. The floor of the enclosure was covered in approximately 3 cm of corncob bedding. Animals were placed into the enclosure in opposing corners and allowed to freely interact for 5 min. Latency to interact, the time spent in social contact, and the number of play behaviors and agonistic behaviors (see Adult stranger test) was calculated for each pair.

### 8.2.10 Adult stranger test

Both conditions and cohorts were tested in the stranger paradigm. Animals were around PND90 at the time of testing.

*Apparatus.* Adult social behavior was tested in a clear Plexiglass® enclosure measuring 50 cm × 50 cm × 50 cm. The floor of the enclosure was covered in approximately 3 cm of corncob bedding. The interactions were video recorded in red light.

*Procedure.* Rats were habituated to the enclosure for 10 min for three consecutive days starting when the animals were PND86. When the animals were PND89, the animals were socially isolated for 24 h with food and water provided *ad libitum*. After being isolated for 24 h, a 'focal' animal (either a control or experimental animal) was paired with a 'partner' (an animal not included in the study but ordered specifically to serve as a partner in the stranger paradigm). Focal and partner animals were placed in the enclosure simultaneously and allowed to interact for 10 min. Interactions occurred in red light. Following testing, the animals were removed from the enclosure, the bedding mixed around, and the next pairing was placed into the enclosure.

*Behavioral analysis.* Social behaviors were manually scored using a combination of normal speed and frame-by-frame analysis (Pellis, Pellis, Burke, et al., 2022). Like juvenile interactions, RTP behavior was scored similar to juvenile interactions. In addition, agonistic behaviors were scored. When adult rats play, the play is rougher than those of juveniles (Pellis & Pellis, 1987). This increased roughness in turn increases the risk of escalating to serious aggression (L. K. Smith et al., 1999). Hallmark behaviors of aggression were scored, such as piloerection, tail rattling, and lateral posturing. Escalation to overt aggression, such as biting, was also scored.

### 8.2.11 Discriminative fear conditioning to context (DFCTC)

To test the function of the amygdala, animals were tested on the DFCTC paradigm at around PND105. White lights were used during testing and testing occurred during their light phase. Only animals from Cohort 1 were tested (control females $n$ = 11; isolated females $n$ = 8; control males $n$ = 11; isolated males $n$ = 9).

*Apparatus*. Two context chambers were used that differed on three dimensions: shape, odor, and color. One context was a black triangle-shaped chamber measuring 61 × 61 cm at the base and a depth of 30 cm. The other chamber was a white square-shaped enclosure measuring 41 × 41 cm at the base and a depth of 20 cm. The floors of both chambers were constructed with stainless-steel bars that were spaced 1.5 cm apart. Distinct odors were provided in each chamber using a small plastic cylindrical container mounted to walls of their respective chambers. Each day a drop of the odorant was placed on a cotton ball and was inserted into the 'odor container.' In the black chamber, Iso-amyl-acetate was provided as the olfactory cue. In the white chamber, eucalyptus was provided. During pre-exposure and preference testing, the two chambers were connected by a gray alley (16.5 cm × 11 cm × 11 cm). The chambers and connecting alley were placed on a clear Plexiglass® table with a height of 100 cm. To view the animals on the inside of the chambers, a mirror (91 cm × 61 cm), at a 45º angle, was placed below the table. A video camera was placed in front of the mirror to record the testing and preferences phases of the experiment. The apparatus was cleaned after each rat's trial.

*Pre-exposure*. Rats were placed in the middle alley and allowed to freely explore the two chambers and alley for 10 min. Dwell time, or the time spent in a chamber, was accumulated when the forepaws and shoulders were past the doorway into one of the chambers and ended when both forepaws and shoulders were past the doorway into the

alley. Rats were counterbalanced so that any innate preferences for either context were balanced. For each group and sex, half of the rats experienced their paired context as the white square and the other half the black triangle. Further subdivision occurred so that half of each group experienced their paired context on the odd number days (1, 3, 5, and 7) and the unpaired on the opposing days (2, 4, 6, and 8). The other half experienced the opposite. Pre-exposure occurred in Room A.

*Training.* Training began around 24 h after pre-exposure. All unpaired days occurred in Room A, while paired days occurred in Room B. During the training period, Plexiglass® panels were inserted into the chamber doorway to block access to the middle alley. In the unpaired condition, each rat was placed in its assigned context individually and remained there for 5 min. For the paired foot shock condition, 0.6 mA of current (scrambled shock) was delivered through the floor at min 2, 3, and 4. Rats alternated between experiencing their paired foot-shock context and their unpaired neutral context on opposing days for eight training days.

*Testing.* To determine if the groups learned which chamber predicted the aversive event, testing was conducted around 24 h after the final training session. During testing, no shocks were administered, and all testing sessions occurred in Room A. On each testing day, the rats were placed within the apparatus for 5 min and the session was recorded. Time spent freezing was scored by an observer blind to the experimental conditions. Freezing was defined as total immobility of the rat's body, including whiskers, other than the movement required for breathing. The testing consisted of two consecutive days during which all the rats spent one of the days confined within their respective paired context, and the other day confined within their unpaired context, counterbalanced for each condition and sex.

*Preference test.* Approximately 24 h after the final testing day, rats were tested in the apparatus to determine if they showed an avoidance to the context previously paired with a shock. The gray alley was replaced so that the two contexts were connected, as in *pre-exposure*, allowing the rats to move freely between the chambers. The rats were left in the apparatus for 10 min. Dwell time in each context was recorded by a blind observer. The same scoring procedure for dwell time was used as in pre-exposure. The preference test occurred in Room A.

### 8.2.12 Conditioned place preference (CPP)

Rats were tested on the CPP at around PND110. Only animals from Cohort 2 were tested (control females $n = 6$; isolated females $n = 8$; control males $n = 6$; isolated males $n = 11$).

*Apparatus.* Like DFCTC, two context chambers were used that differed on three dimensions: shape, odor, and color. The same boxes described above for DFCTC were used.

*Pre-exposure*. Rats were placed in the middle alley and allowed to freely explore the two chambers and alley for 10 min. Dwell time, or the time spent in a chamber, was accumulated when the forepaws and shoulders were past the doorway into one of the chambers and ended when both forepaws and shoulders were past the doorway into the alley.

*Training.* Training began around 24 h after pre-exposure. During the training period, Plexiglass® panels were inserted into the chamber walls to block access to the middle alley. Rats were counterbalanced so that any innate preferences for either context were balanced. For each group and sex, half of the rats experienced their paired context as the white square and the other half the black triangle. Further subdivision of these rats was

performed so that each group experienced their paired context on the odd number days (1, 3, 5, 7, and 9) and the unpaired on the opposing days (2, 4, 6, 8, and 10). The other half experienced the opposite. In the unpaired condition, each rat was placed in its assigned context individually and remained there for 30 min. In the paired condition, males received 10 g and females were given 7 g of chocolate chip cookies (No Name brand broken in to pieces) that were placed in the corner farthest away from the doorway of the chamber. Like the unpaired condition, each rat was placed in the context and remained there for 30 min.

*Preference test*. Within 24 h of the last day of training, the rats were placed individually in the middle alley and allowed to access both chambers for a total of 20 min. Dwell time was scored as in pre-exposure. Time spent in the paired context served as a measure of appetitive context memory.

**8.2.13 Elevated plus maze (EPM)**

To measure anxiety-like behaviors, rats were tested on the EPM for 5 min at around PND120. Both cohorts were tested. This task assesses anxiety-like behavior. Constructed from black Plexiglass®, the apparatus consisted of two open and two closed arms, each 50 cm × 10 cm. The apparatus was elevated 100 cm above the floor. Open arms had no side or end walls while the closed arms had side and end walls that were 40 cm tall. The experimenter placed the rat in the center of the plus maze, with the animal facing an open arm. The animal was left in the plus maze for 5 min. Each session was recorded, and a blind observer scored the amount of time each animal spent in the closed and open arms. In addition, the percentage of time the rat spent assessing risk was calculated. Rats were determined to be in the closed or open arm when its forepaws and half of the body were in the arm. The total amount of time at the edge of a closed arm with its nose towards the center of the platform was calculated and used as a measure of risk assessment.

### 8.2.14 Cue-place Morris water task (cue-place MWT)

Rats were tested on the cue-place version of the MWT at around PND95. Only animals from Cohort 2 were tested (control females $n = 6$; isolated females $n = 8$; control males $n = 6$; isolated males $n = 11$). This task assesses both dorsolateral striatum (during the cue-visible platform test) and hippocampal (during the place-invisible platform) network functions.

*Apparatus*. A white plastic pool (154 cm in diameter and 50 cm in height) was filled with water (20-21ºC) to a level of 31 cm. White, non-toxic, paint (Tempera) was added to make the water opaque. A Plexiglass® platform, 28 cm tall, with a 12 cm × 12 cm top, was submerged 2-3 cm below the water surface on the invisible days. A black visible platform was placed on top of the Plexiglas® platform, protruding 3 cm above the water surface on the visible days. The testing room was 310 cm × 610 cm. The pool was raised 48 cm above the ground in the center of the room. Cues in the room were left unchanged throughout all trials, and included a computer and desk, posters of varying size and color, chair, the experimenter, a sink and cabinet, and the door. Using EthoVision (Noldus, Leesburg, USA), animals were tracked with an overhead video camera. The tracking software recorded the latency and path length to the platform, and dwell time in each quadrant.

*Procedure*. The rats were trained for 12 consecutive days with the platform remaining in the same placed for all days. A 3:1 cue:place schedule was used. For the first three days, the platform was visible (training), but on the fourth day, an invisible platform was placed in the location of the previously visible platform. This was repeated three times so that rats received a total of 9 days of training with the visible platform and 3 days with the invisible platform. Each day, each subject received 4 trials per day with 4 pre-determined starting points. The circular pool was conceptually divided into four quadrants

of equal size. The starting points were chosen to be 45°from cardinal compass points and the perimeter of the pool. Rats were run in a distributed manner and were allowed a maximum search time of 30 s in the pool. A trial ended when the subject located the platform, or after 30 s had elapsed. In the latter event the rat was guided to the platform by the experimenter. The subject was required to remain on the platform for 10 s before being returned to its holding cage.

### 8.2.15 Morris water task (MWT) – one trial place learning

Both conditions were tested using the MWT at around PND100. Only animals from Cohort 1 were tested (control females $n$ = 11; isolated females $n$ = 8; control males $n$ = 11; isolated males $n$ = 9).

*Apparatus.* A white plastic pool (154 cm in diameter and 50 cm in height) was filled with water (20-21ºC) to a level of 40 cm. White, non-toxic, paint (Tempera) was added to make the water opaque. A Plexiglass® platform, 28 cm tall, with a 13 cm × 13 cm top, was submerged 2-3 cm below the water surface. The testing room was 310 cm × 610 cm. The pool was raised 48 cm above the ground in the center of the room. Cues in the room were left unchanged throughout all trials, including a computer and desk, posters of varying size and color, chair, the experimenter, a sink and cabinet, and the door. Using EthoVision (Noldus, Leesburg, USA), animals were tracked with an overhead video camera. The tracking software recorded the latency and path length to the platform, and dwell time in each quadrant.

*Procedure*. Rats were placed in the pool facing the wall and allowed to swim for 60 s to reach the submerged platform. If they did not reach the platform, the experimenter guided them to the platform. After reaching the platform—whether having done so on their own or guided there—rats remained on the platform for 10 s before the experimenter

removed them. Two blocks of four trials starting from each of the cardinal compass points (N, E, S, W) was randomized for each training day, with a rule that a start point could not be used consecutively. For all trials, there was an intertrial interval of at least 8 minutes. On each day, the platform was moved to a new location in the pool.

### 8.2.16 Histology

Rats were anesthetized at around PND130 with sodium pentobarbital (300 mg/kg body weight) and perfused, intracardially, with 0.9% saline. The brains were then extracted and weighed. Following a standard Golgi staining protocol (Gibb & Kolb, 1998), the brains were placed in Golgi-Cox solution for 14 days in amber bottles. After two weeks, the brains were placed into amber bottles containing 30% sucrose solution and were left in this solution for a minimum of 5 days. Brains were sectioned using a vibratome set at a thickness of 200 μm. The sections were mounted onto slides coated in 2% gelatin and stored in a light-proof box, wrapped in a damp paper towel to prevent the tissue from drying out. After a few days, the slides were rinsed with distilled water and ammonium hydroxide and then placed in a 1:1 solution of Kodak Rapid Fix (Kodak) and distilled water. To dehydrate the brain tissues, the slides were moved through a graded series of ethanol. Then, the tissue was cleared with a 1:1:1 solution of chloroform, HemoDe (Electron Microscopy Sciences catalog No. 23410-04) and 100% ethanol. Slides were placed in HemoDe before they were cover-slipped with Permount (Fisher Scientific, Catalog No. SP15-500). Slides were left to dry for at least 1 month or until they were dry to the touch.

### 8.2.17 Anatomical analysis

Neurons were selected from area Cg3 (the prelimbic cortex) of the mPFC (Bregma 4.2-2.77 mm) as defined by Zilles (Zilles, 1985) for imaging. Virtual images Cg3 were created using an Olympus VS120 digital slide scanner, with a 40× oil object lens (UPlanFL

N, 40×/1.30 oil, ∞/0.17/FN26.5) and Olympus VS-ASE FL software(Ham, Szabo, et al., 2024). The virtual slides consisted of 147 z-stacked images spaced 0.68 μm apart throughout the section, yielding 99.96 μm of working distance. Neurons were reconstructed in Neurolucida 360 (MicroBrightfield) using the virtual images of the slides. Pyramidal neurons in layer III were selected. The cell body, apical and basilar dendrites, along with spines, were traced. Cells were only selected if (1) the cell was fully impregnated and not obscured by large truncations of debris, strain precipitation, or blood vessels and (2) the cell had to lie centered within the z-stack plane, ensuring that all dendritic branching could be traced. Neurons were traced from control animals (8 male and 8 female rats) and isolated animals (8 male and 8 female rats). Six neurons, three from each hemisphere, were traced for every rat. A total of 192 neurons were traced across both sexes and conditions.

Neuron measurements included: (1) the total convex hull volume; (2) the convex hull volume of both apical and basilar dendrites; (3) the total convex hull surface area; (4) the convex hull surface area of both apical and basilar dendrites; (5) the sum of the total length of the apical and basilar projections and the length of the cell body, which together obtained the total length of the cell; (6) the sum of the volume of the apical and basilar projections and the volume of the cell body, which together provided a measure of the total volume of the cell; (7) the branch number for both apical and basilar projections, which was summed for the total branch number; and (8) the branch order for both apical and basilar projections, which was summed for a total branch order.

In addition, spines were counted using Neurolucida 360 (MBF Bioscience) to calculate spine density. To do so, four distal sections in the basilar arbors and 4 in the apical arbors, each 40 μm in length, from the end of a dendritic branch were counted. Segments were at least 50 μm distal or 100 μm to the cell body for basilar segments and apical

segments, respectively. Proximal sections were also measured, sampling two basilar segments and one apical segment. In both cases, 20 μm were measured from the cell body. Spines were only included if they were longer than the radius of the dendrite to limit the inclusion of artifact. If the spines bifurcated, they were counted as two separate spines as they have separate points of connections. For each animal, 4 cells were quantified (2 from each hemisphere), with a total of 128 cells being counted for spine density.

Neurons were also traced from the dorsolateral septum (dLS) as defined by Zilles (1985) for imaging. Neurons were located between Bregma 1.44 and 0.12 mm the rostral-caudal plane. Images of the tissues were created using the methodology described above. Stellate neurons were selected using the selection criteria described above. Neurons were traced from control animals (8 male and 8 female rats) and isolated animals (8 male and 8 female rats). Six neurons, three from each hemisphere, were traced for each rat A total of 192 neurons were traced across both sexes and conditions. Neuron measurements included: 1) the total convex hull volume; (2) the convex hull volume of all dendrites; (3) the total convex hull surface area; (4) the convex hull surface area of all dendrites; (5) the sum of the total length of the dendritic projections and the length of the cell body, which together obtained the total length of the cell; (6) the sum of the volume of the dendritic projections and the volume of the cell body, which together provided a measure of the total volume of the cell; (7) the branch number for the dendritic projections, which was summed for the total branch number; and (8) the branch order for dendritic projections, which was summed for a total branch order. Spine density was not determined for these stellate neurons in dLS because they were primarily aspiny.

### 8.2.18 Serum corticosterone

To get a baseline level of serum corticosterone concentrations, at around PND65, roughly 0.7 ml of blood was collected from the tail vein while the rats were under anaesthesia. Blood was drawn from animals in both cohorts. The blood was centrifuged at 4ºC at 3000× g for 10 min and then the serum was removed and stored at -80ºC. Using 0.2 µL of the serum, the levels of corticosterone in the samples were estimated using a cortisol enzyme linked immuno-sorbent assay (ELISA) kit (Abcam, product # AB108821). All samples and standards were run in duplicate and their resultant data averaged. Data were quantified by comparing light absorption readings of our samples against known standards that were run in parallel.

### 8.2.19 Metabolomics sample preparation and data collection

Serum obtained from the same blood draws described above was processed for water soluble metabolite extraction and analysis in the Magnetic Resonance Facility at the University of Lethbridge (Lethbridge, AB, Canada). Briefly, 22-150 µL of serum, based on sample availability, and methanol were pipetted into a 2mL centrifuge tube in a 1:2 ratio, respectively. The volume of serum was recorded for each sample. The sample mixture was then vortexed for 5 seconds and subsequently stored at -80 ºC for 20 minutes to aid in protein precipitation. The mixture was then centrifuged at 12,000g and 4ºC for 30 minutes and the resulting supernatant was transferred to a separate 2mL centrifuge tube. The sample was then placed in a nitrogen gas drying box and allowed to fully evaporate for 3 to 4 days. After evaporation, each sample was redissolved in a mixture of 560 µL of buffer (4:1 ration of 0.625 M $K_2HPO_4$:$KH_2PO_4$ in $dH_2O$-pH 7.4, 3.75 mM $NaN_3$, and 0.375 M KF) and 140 µL of deuterium oxide (D2O) containing 0.03% w/v 3-(trimethylsilyl)propanoic acid (TSP). The sample was then vortexed for 10 seconds to fully dissolve the pellet and

subsequently centrifuged at 12,000g and 4ºC for 5 minutes. 550 µL of the supernatant was then transferred to a 7" NMR tube for analysis. NMR spectra were obtained on a Bruker 700 MHz Avance III HD spectrometer (Bruker, Milton, ON, Canada) equipped with a TBO probe. The data acquisition parameters are described in detail elsewhere(Heynen et al., 2022). In order to achieve a similar signal to noise ratio, the number of scans acquired was based on the amount of serum available with 512, 1024, 1536, and 2048 scans being utilized when there was 125-150 µL, 100-124 µL, 50-99 µL, and less than 50 µL, respectively.

**8.2.20 Statistical analyses**

To compare maternal behavior, juvenile play frequency, and style we used 2-way repeated measures analysis of variance (ANOVA). To compare if rats had partner preferences when playing as juveniles in groups, paired *t* tests compared the number of napes attacks directed at each play partner. Latency to first interaction, time spent in close social proximity, and the number of nape attacks between pairs of adolescent rats during the social interaction test was tested with two-way repeated ANOVAs. Likewise, the number of nape attacks, play style, and play symmetry were tested with two-way repeated ANOVAs. Repeated measures three-way ANOVAs were used to test latency and distance travelled for both the one trial place learning MWT and cue-place MWT. The visible days and invisible days from the cue-placed MWT were tested separately. Repeated measures three-way ANOVAs with Bonferroni corrections were also used to test the time spent in each chamber during both DFCTC and CPP, as well as the time spent in each location of the EPM. All ANOVAs were performed using SPSS (v.28.0) and figures were created in Prism version 10 (GraphPad software). Measures of neurons were assessed with linear mixed models using the *lme4* package (Bates et al., 2015) in R (R Core Team, 2020). The condition × sex was set as the independent fixed effect, and sex was included as a random

error term as well as the individual identity of the to the repeated measure of individuals animals, with hemisphere nested within every subject. When significant, estimated marginal means were calculated with the *emmeans* package (Length, 2025), then a pairwise comparison was performed.

All NMR data was initially processed using TopSpin 3.5pl7 (Bruker, Milton, ON, Canada) with zero-filling to 256k points, automatic phase and baseline correction, line broadening of 0.3 Hz, and referenced to TSP at 0.00ppm. Spectra were then exported as ascii files to MATLAB where they then underwent dynamic adaptive binning (Anderson et al., 2011) followed by manual inspection and correction of the bins. Each spectrum was then normalized to the total area of all spectral bins (excluding the water region), Pareto scaled, and log transformed prior to statistical analysis (Craig et al., 2006). For univariate analysis, a Shapiro-Wilk test for data normality was applied, all data was found to be non-parametric, and the Mann-Whitney U test was performed (Goodpaster et al., 2010). Bonferroni-Holm correction was applied to all univariate tests to correct for multiple comparisons. The percent difference of the metabolites was calculated using the following: [isolated – control / ((isolated + control)/2)] × 100. All multivariate modelling was carried out and visualized using MetaboAnalystR v3.0(Pang et al., 2020) and all supervised testing was validated using double ten-fold cross-validation and 2000 permutations. A multivariate machine learning approach known as Variable Importance Analysis based on random Variable Combination (VIAVC) was utilized to determine which metabolites were most important for determining group classification in a synergistic way (Yun et al., 2015). Significant metabolites were identified using Chenomx NMR Suite v8.31 (Chenomx, Edmonton, AB, Canada).

## 8.3 Results

### 8.3.1 Maternal behavior is unaffected by pup isolation

As separating pups from a dam is stressful to both the mother and her offspring, we examined the quality of maternal care, as well as other dam behaviors (Capone et al., 2005). As a consequence, this could potentially change how the dams cared for their offspring (Bölükbas et al., 2020). We found little difference in how the dams of controls or isolated pups cared for their offspring (Appendix C, Figure C1). Dams of both conditions exhibited high quality feeding and care, spending most of their time in the nest. The only difference found was that control dams engaged in more blanket nursing than dams of isolated pups ($F(1,4) = 1073.00$, $p < 0.0001$). This suggests that differences between conditions cannot be explained by altered maternal care due to increased maternal stress when isolating the pups. See Appendix C, Figure C1, for detailed analysis and post hoc tests.

### 8.3.2 Isolated pups play less and engage in poorer quality play when with strangers

To determine if juvenile RTP was affected by neonatal isolation, we used a standard testing paradigm where two individuals of the same sex and experimental condition, who are unfamiliar with one another interact in a neutral environment (Pellis, Pellis, Burke, et al., 2022).

#### *8.3.2.1 Unfamiliar, same condition pairs*

When tested with unfamiliar, same condition partners, we found that pairs of control rats engaged in more RTP than isolated pairs (Figure 8.1a) and engaged in a higher proportion of role reversals (Figure 8.1b). Comparison of the total frequency of play between pairs of unfamiliar, same condition pairs revealed a significant effect of condition ($F(1,26) = 22.32$, $p < .0001$) and sex ($F(1,26) = 7.429$, $p = .0113$), but no condition × sex

interaction ($F(1,26) = 2.360$, $p = .137$). Control male rats played more than isolated male rats ($p = .0002$), isolated female rats ($p < .0001$), and control female rats ($p = .0057$) (Figure 8.1a). Additionally, control female rats engaged in more RTP than isolated female rats ($p = .028$). In addition to play frequency, the defensive tactics that rats adopt to defend themselves from a playful attack can vary (Pellis, Pellis, Burke, et al., 2022). Despite a reduction in play between isolated and control rats, the strategies they used to defend themselves while playing remained the same (Appendix C, Table C1). One important feature of RTP is that animals engage in certain actions that keep the play fun. To do so, animals engage in turn taking or role reversals, so that there is reciprocity in attacking and defending (Palagi, Cordoni, et al., 2016). A comparison of the proportion of role reversals (Figure 8.1b) revealed a significant effect of condition ($F(1,26) = 28.53$, $p < .0001$), but not a significant effect of sex ($F(1,26) = 1.892$, $p = .181$) nor a condition × sex interaction ($F(1,26) = 0.798$, $p = .380$). This suggests that the quality of play was significantly reduced (Ham, Szabo, et al., 2024). No significant effect was found when the play attack symmetry was compared (Figure 8.1c), however, there was a significant difference in the symmetry of role reversals (i.e., one partner is engaging in more turn taking than the other, see Appendix C, Table C1). We found that isolated females had more symmetrical role reversal relationships than control females ($p = .0180$) and males ($p = .0088$). When playing with unfamiliar, same condition partners, isolated rats RTP less than controls, and this RTP is poor quality with less turn taking occurring.

#### *8.3.2.2 Unfamiliar, opposite condition pairs*

To determine if the condition of the RTP partner could alter how isolated rats played, we paired unfamiliar control rats with isolated rats. Male but not female control

rats engaged in more RTP than their isolated play partners (Figure 8.1d) while isolated males engaged in more role reversals (Figure 8.1e). However, female and male control rats engaged in higher proportions of RTP than their isolated rat partners. When comparing the frequency of RTP initiated by each of the rats in the pair (Figure 8.1d), we found a significant relationship between condition ($F(1,52) = 7.328$, $p = .0092$), but not sex ($F(1,52) = 0.463$, $p = .499$) nor a condition × sex interaction ($F(1,52) = 1.203$, $p = .278$). Control and isolated males responded differently to playful attacks with attacks initiated by isolated rats resulting in more pins ($p = .029$) (Appendix C, Table C2). A comparison of the proportion of role reversals (Figure 8.1e) revealed a non-significant effect of condition ($F(1,52) = 0.008$, $p = .928$), but significant effects of sex ($F(1,52) = 15.60$, $p = .0002$) and condition × sex interaction ($F(1,52) = 4.199$, $p = .0455$). When the symmetry of play was compared (Figure 8.1f), we found a significant effect of condition ($F(1,52) = 34.91$, $p < .0001$), but non-significant effects of sex ($F(1,52) = 0.00$, $p > .999$) and condition × sex interaction ($F(1,52) = 3.062$, $p = .086$). That is, isolated rats played proportionately less than their control partners.

#### *8.3.2.3 Familiar, same condition pairs*

As the novelty of a play partner could influence the frequency of RTP, we also tested rats with familiar partners (i.e., their cage mates). When tested with familiar partners, we found no difference in the frequency of RTP (Figure 8.1g), the proportion of role reversals (Figure 8.1h), nor the symmetry in attacks (Figure 8.1i). Unlike when playing with unfamiliar partners, when we compared the total frequency of RTP between familiar, same condition pairs (Figure 8.1g), we found that there was no significant effect of condition ($F(1,27) = 1.170$, $p = .289$), sex ($F(1,27) = 0.003$, $p = .957$), no condition × sex

interaction ($F(1,27) = 1.054$, $p = .314$). Rats used similar play styles, regardless of condition and sex (Appendix C, Table C3). The quality of RTP was not significantly different as there was no difference in the proportion of role reversals used by either condition ($F(1,27) = 0.007$, $p = .934$), sex ($F(1,27) = 0.001$, $p = .976$), or interaction effect of condition × sex ($F(1,27) = 0.091$, $p = .765$). Similarly, there was no difference in the symmetry of play relationships (Figure 8.1i) when the condition ($F(1,27) = 0.001$, $p = .977$), sex ($F(1,27) = 0.875$, $p = .358$), and interaction between condition × sex ($F(1,27) = 0.124$, $p = .728$) was compared. Despite playing less with unfamiliar partners, when isolated rats RTP with their cage mates, they play just as much as control rats.

#### *8.3.2.4 Group play*

One of the downsides with the 'dyadic test' employed above is that the rats do not have a choice in play partner and thus may mask their true play style and frequency to match their play partner (Achterberg et al., 2023). By testing in a group setting, the rats can choose with whom they wish to play with and how they wish to engage in RTP (Ham & Pellis, 2023, 2024). When rats were grouped with a control and isolated rat, simultaneously (Figure 8.1j), female (Figure 8.1k) and male (Figure 8.1m) control rats did not express preferences based on the condition of their partner ($t(7) = 1.314$, $p = .230$; $t(7) = 0.797$, $p = 0.412$, respectively). However, isolated females (Figure 8.1l) and males (Figure 8.1n) did show preferences, directing a higher percentage of their RTP towards a same-condition partner ($t(7) = 5.309$, $p = .0011$; $t(6) = 4.175$, $p = 0.0058$, respectively). When tested in groups, control rats show no preference for either condition, however, isolated rats prefer to RTP with other isolated rats over control rats.

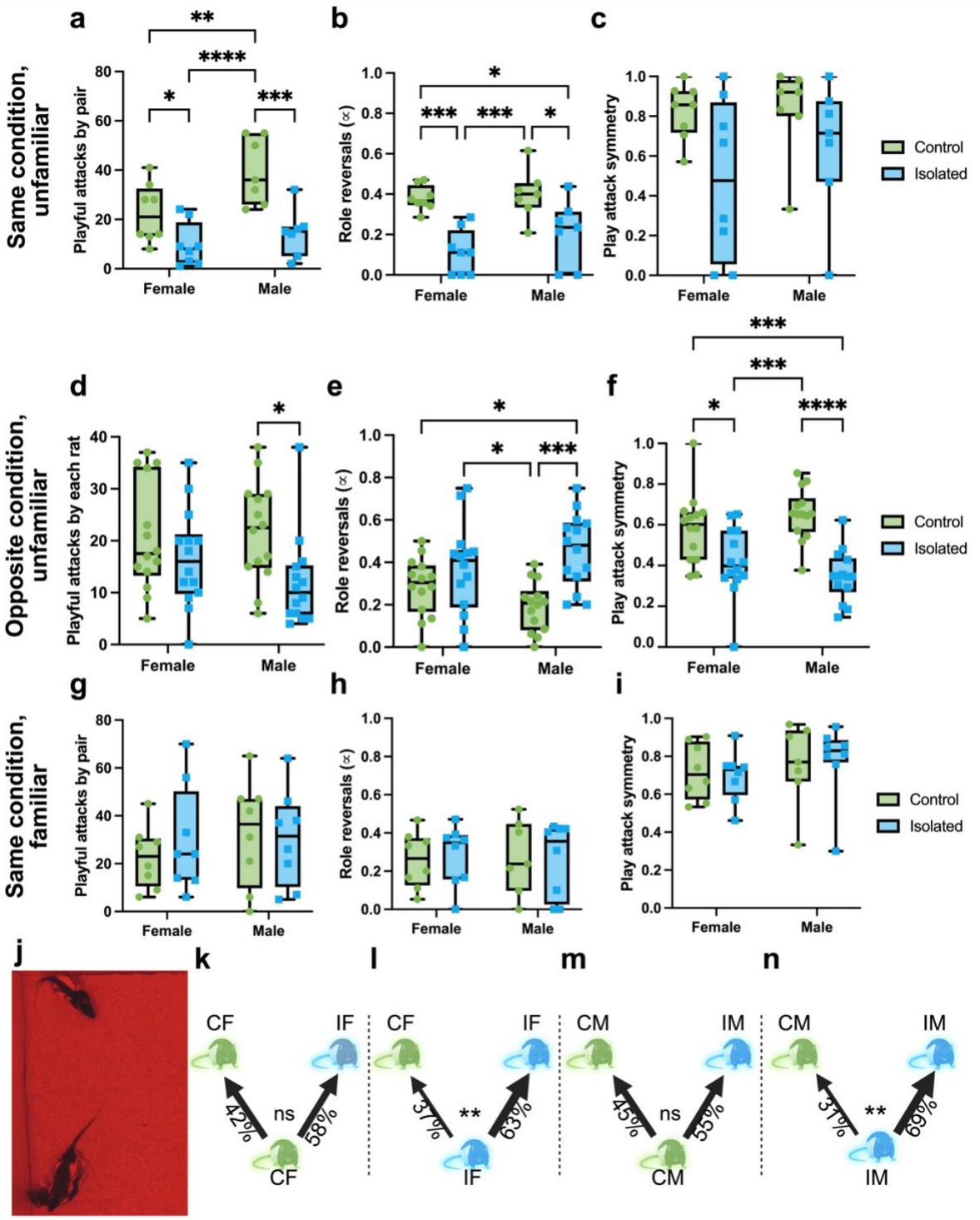


**Figure 8.1 Juvenile social play measures.** When same condition, unfamiliar pairs are tested, the number of nape attacks initiated by the pair are less in pairs of isolated rats (a), isolated rats engage in less role reversals (b). Symmetry in play was not different, though isolated pairs varied more than control pairs (c). When paired with the opposite condition, control male rats initiate more play than their isolated partners (d). Isolated male rats engage in more role reversals (e). Symmetry was significantly different, as control rats of both

sexes initiated more nape attacks than their isolated partners (f). When tested with their cage mates, no difference was found in the number of nape attacks (g), role reversals (h), or symmetry (i) in play among pairs. When tested in groups (j), control females (k) and control males (m) showed non-significant preferences. Isolated females (l) and males (n), however, both preferred to play with same-condition isolated individuals.
*Note*. CF = control female; IF = isolated female; CM = control male; and IM = isolated male. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Created in part with BioRender.com.

### 8.3.3 Isolation does not affect adolescent social behavior

Frequently used to assess anxiety (File & Seth, 2003), we found that rats from both conditions did not differ significantly in the social interaction test in any of the behaviors measured. When pairs of unfamiliar**,** same condition and sex rats interacted in a large arena (File & Seth, 2003) (Appendix C, Figure C2), a two-way ANOVA found that there was no difference in the latency to be within close social proximity between conditions ($F(1,28) = 1.223$, $p = .278$), sex ($F(1,28) = 1.664$, $p = .208$), nor was an interaction effect found between condition × sex ($F(1,28) = 1.585$, $p = .218$). When compared (Appendix C, Figure C2), no significant difference was found in the total duration rats spent in close social proximity between conditions ($F(1,28) = 1.434$, $p = .241$), but a difference was found for sex ($F(1,28) = 5.350$, $p = .0283$). However, a post-hoc Tukey test did not find any significant pairwise differences between sex. No significant interaction effect was found ($F(1,28) = 0.002$, $p = .965$). When the frequency of RTP between pairs was compared, we found that there was a significant effect of condition ($F(1,28) = 6.135$, $p = .0196$), but no significant effects of sex ($F(1,28) = 1.336$, $p = .258$) nor interaction ($F(1,28) = 1.466$, $p = .236$). However, a post-hoc Tukey test did not find any significant pairwise differences between conditions (Appendix C, Figure C2). Aggressive behaviors were not observed

between any of the pairs. Pairs of both control and isolated rats did not differ in social proximity duration and engaged in similar amounts of play.

### 8.3.4 Isolation alters adult social behavior

Using the stranger test (Ham, Szabo, et al., 2024) (Figure 8.2a), a test of social RTP, inter-animal coordination, and emotional control, control and isolated rats were paired with unfamiliar rats that were not a part of the study, but used specifically as neutral, test partners. We found that control males engaged in more RTP than isolated rats (Figure 8.2b), but both male and female control rats engaged in significantly more role reversals in RTP than isolated rats (Figure 8.2d). While not all isolated pairs escalated RTP to serious fighting, only pairs of isolated rats escalated to overt aggression (Figure 8.2f). Comparison of the total amount of play between pairs revealed a significant effect of condition ($F(1,66) = 4.580$, $p = .0360$), but not sex ($F(1,66) = 2.630$, $p = .110$) nor a condition × sex interaction ($F(1,66) = 2.757$, $p = .102$). A post hoc Tukey test revealed that pairs that included male control rats engaged in more RTP than those that had isolated male rats (Figure 8.2b). Because the total RTP score includes the play of the "stranger" animal, we also tested whether the focal animals (i.e., the ones from our study) varied significantly. When compared, there was a significant effect of condition ($F(1,66) = 7.458$, $p = .0081$), no significant effect of sex ($F(1,66) = 1.159$, $p = .286$), and a significant interaction effect of condition × sex ($F(1,66) = 5.676$, $p = .0201$). A post hoc Tukey test revealed that control males engaged in significantly more RTP than isolated males (Figure 8.2c). To determine if the quality of play varied, the proportion of role reversals was measured. We found that there was a significant effect of condition ($F(1,66) = 25.90$, $p < .0001$), but not sex ($F(1,66) = 0.224$, $p = .637$) nor an interaction effect between condition × sex ($F(1,66) = 0.304$, $p =$

.583). A post hoc Tukey test revealed that control females and males engaged in a higher proportion of role reversals than isolated females and males, respectively (Figure 8.2d). Additionally, control females engaged in more role reversals than isolated males, and control males engaged in more role reversals than isolated females. When the symmetry in RTP relationships was tested, we found that there was a significant effect of condition ($F(1,66) = 20.97$, $p < .0001$), but not sex ($F(1,66) = 0.061$, $p = .805$) nor an interaction effect between condition × sex ($F(1,66) = 2.11$, $p = .151$). A post hoc Tukey test revealed that control males had more symmetrical play relationships with their "stranger" partner than isolated males and isolated females did (Figure 8.2e). Additionally, female control rats had more symmetrical play relationships than isolated males. In addition to play, the stranger test can reveal differences in emotional regulation as individuals with deficits will escalate play fighting into serious fighting. Descriptively, only isolated females and isolated males escalated to overt aggression, engaging in biting behaviors (Figure 8.2f). Isolated rats engaged in less role reversals and aggressive behavior, suggesting they have compromised social skills. In addition, isolated male rats engaged in less play than control male rats when with unfamiliar partners.

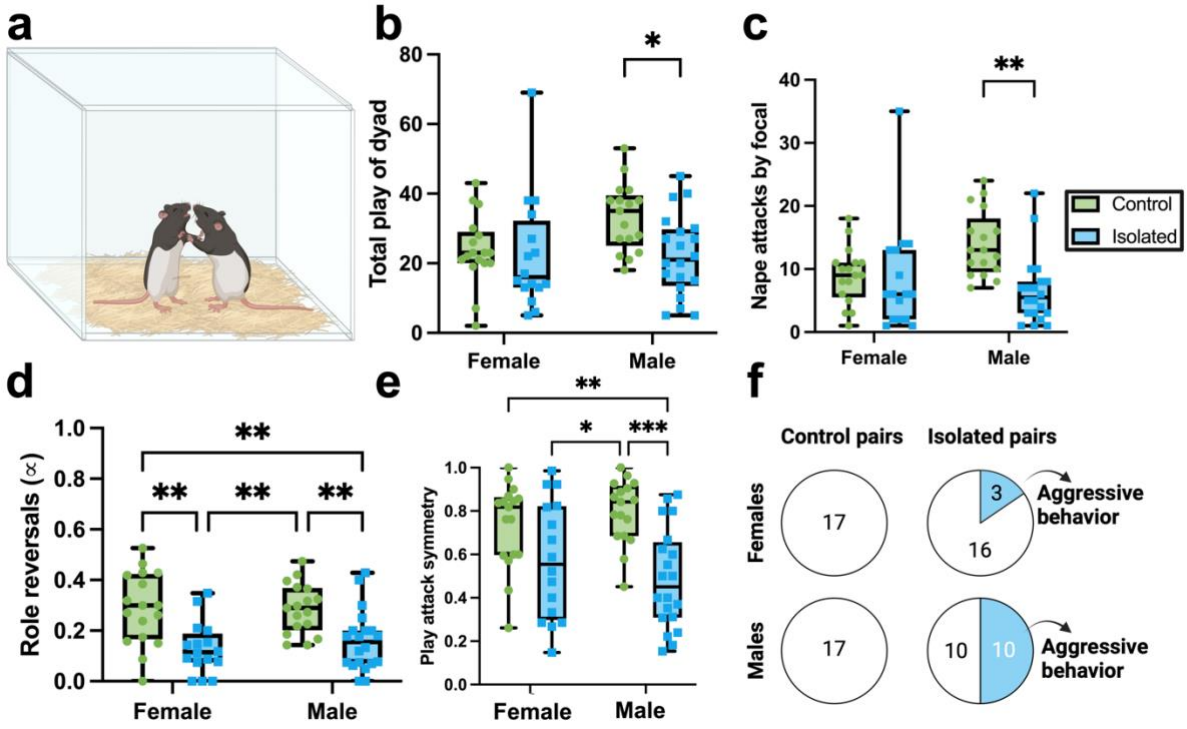


**Figure 8.2 Behavioral measures from the adult stranger paradigm.** When tested in the adult stranger paradigm (a), control males, but not control females, show increased play levels as a pair, compared to pairs with isolated individuals (b). Similarly, when the playful attacks launched by the subjects of this experiment is plotted, and not also included the unfamiliar, stranger animals, a similar result is found with male controls, but not female controls, playing more than isolated rats of the same sex (c). The quality of play is reduced, with less role reversals occurring between pairs that include an isolated rat (d). Control male, but not control females, have more symmetrical play relationships than do isolated animals (e). Only isolated rats escalated to overt aggression, such as biting (f).
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Created in part with BioRender.com.

### 8.3.5 Isolation affects anxiety-like behavior and emotional learning, but not discriminative fear conditioning in adults

Discriminative fear conditioning to context (DFCTC) is used as an assay as different brain networks have been associated with normal aversive conditioning on this task (Antoniadis & McDonald, 2000) and deficits in orbitofrontal cortex function result in generalized fear discrimination (Trow et al., 2017; Zelinski et al., 2010). Accordingly, we believe this is a relevant assay for generalized anxiety. All rats, regardless of condition, developed strong associations for the paired chamber, avoiding the paired chamber during the preference test. To determine whether there was an initial preference for the to-be-paired or to-be-unpaired chamber (Figure 8.3a), a three-way repeated measures ANOVA was performed on the dwell time accumulated in each context (Figure 8.3b) revealing there was no significant difference in context ($F(1,35) = 1.464$, $p = .234$), context × condition ($F(1,35) = 0.000$, $p = .995$), context × sex ($F(1,35) = 0.274$, $p = .604$), nor a context × condition × sex interaction ($F(1,35) = 0.008$, $p = .928$). *Freezing.* After eight days of training, rats were assessed for freezing duration in both contexts (Figure 8.3c). We found that there was a significant effect of context ($F(1,35) = 70.067$, $p < .0001$). No differences in freezing were found between context × condition ($F(1,35) = 0.025$, $p = .876$), context × sex ($F(1,35) = 0.169$, $p = .683$), nor a context × condition × sex interaction ($F(1,35) = 0.444$, $p = .510$). *Preference Test.* Additionally, all rats spent more time in the unpaired context (Figure 8.3d) ($F(1,35) = 104.452$, $p < .0001$). No effect of context × condition ($F(1,35) = 1.620$, $p = .212$), context × sex ($F(1,35) = 0.053$, $p = .819$), nor context × condition × sex interaction ($F(1,35) = 2.482$, $p = .124$) found. Together, this indicates that regardless of sex and condition, all rats showed discriminative fear conditioning.

Using CPP paradigm, we classically conditioned the rats to a context where a food reward was presented. Given that this task relies on the basolateral amygdala, deficits would indicate if there was disruption to the neural circuitry involved in a form of emotional learning (McDonald et al., 2010). Unlike DFCTC, we found that isolated rats did not prefer the context that was paired with a food reward while the control rats did. To assess whether an initial preference was present for the to-be-paired or to-be-unpaired chamber (Figure 8.3e), a repeated measures ANOVA was performed on the dwell time accumulated in each context (Figure 8.3f). No significant effects were found of context ($F(1,27) = 1.385$, $p = .250$), context × condition ($F(1,27) = 1.513$, $p = .229$), context × sex ($F(1,27) = 0.005$, $p = .944$), nor interaction between context × condition × sex ($F(1,27) = 0.018$, $p = .893$) suggesting that the rats did not have an inherent preference for either chamber. *Preference test.* After 12 days of conditioning, a repeated measures ANOVA revealed that preferences for the paired context were formed ($F(1,27) = 11.70$, $p = .0020$). No significant effect of context × condition ($F(1,27) = 3.737$, $p = .064$), context × sex ($F(1,27) = 0.405$, $p = .530$), nor context × condition × sex ($F(1,27) = 0.850$, $p = .365$). Bonferroni post hoc tests revealed that female control rats spent more time in the paired context ($p = .0164$) while isolated females did not (Figure 8.3m). Similarly, control males spent more time in the paired context ($p = .0294$), while isolated males did not express a preference (Figure 8.3g). These results suggest that isolated rats have deficits in emotional reward learning as they did not express a preference for the context in which they received a reward.

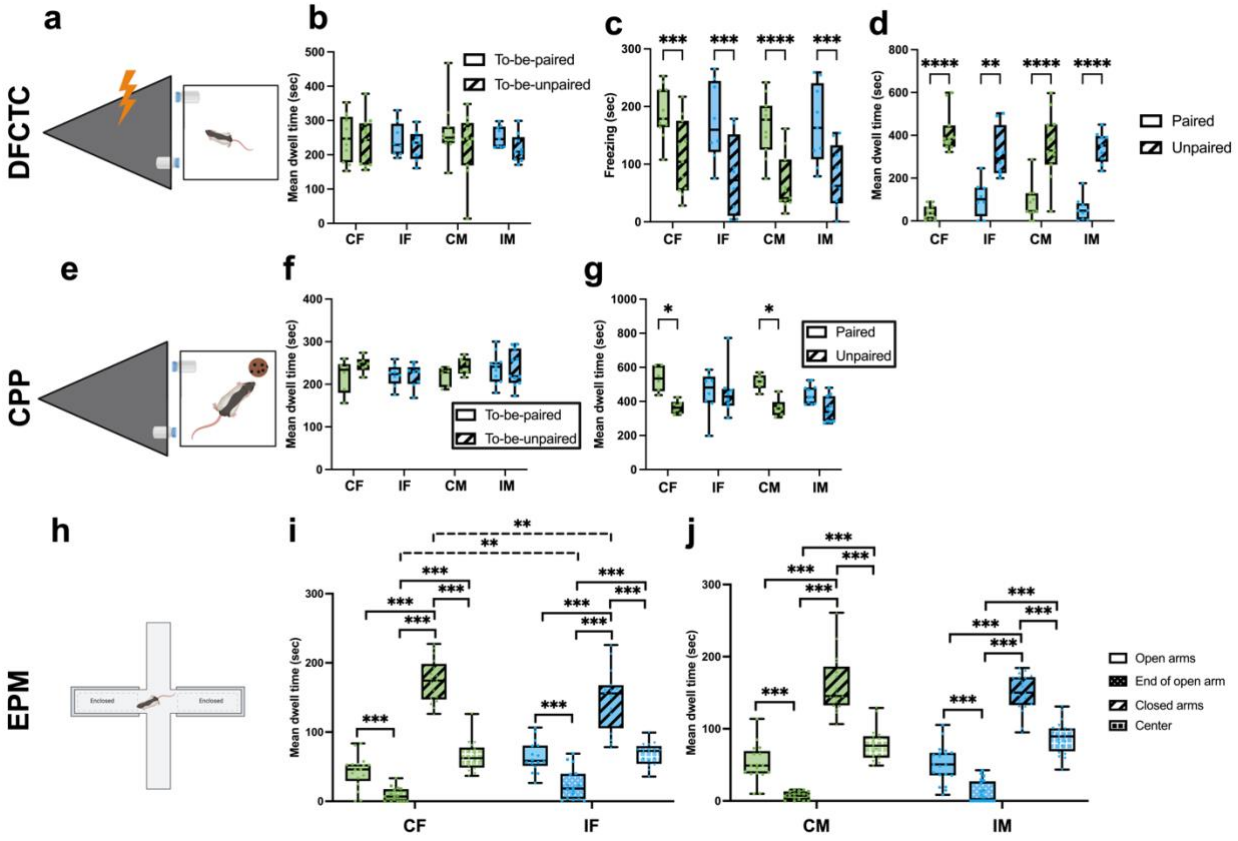


**Figure 8.3 Behavioral measures from DFCTC, CPP, and EPM.** Discriminative fear conditioning to context (DFCTC), where one context was paired with a foot shook, was conducted during adulthood (a). Pre-testing found that there was no preference to either context before conditioning began (b). After repeated conditioning, the rats spent more time freezing in the paired context compared to the unpaired context (c). All rats, regardless of sex and context, discriminated between the paired context (foot shock) and unpaired context (d). Conditioned place preference (CPP), where one context was paired with a food reward, was conducted during adulthood (e). Pre-testing found that there was no preference to either context before conditioning began (f). After repeated conditioning, only the control rats formed preferences for the context that was paired with a food reward (g). During adulthood, the rats were tested on the elevated plus maze (EPM) (h). Female rats spent more time in the closed arms compared to both the open arms and the end of the open arms. Isolated females spent more time at the end of the open arms and less time in the closed arms, when compared to control females (i). Male rats spent more time in the closed arms compared to both the open arms and the end of the open arms (j).

*Note*. CF = control female; IF = isolated female; CM = control male; and IM = isolated male. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Created in part with BioRender.com.

The EPM is a task used extensively in the field and is considered a measure of general anxiety (Figure 8.3h). Rats with anxiety-like behavior should remain in the closed arms while typically behaving rats should explore the open arms. We found that all rats exhibited a similar patter, spending the most time in the closed arms, however, isolated females spent more time at the end of the open arms and less time in the closed arms, when compared to control females. When compared (Figure 8.3i,j), we found that the rats did not spend equal amounts of time in each arm location ($F(3,64) = 327.581$, $p < .001$). Additionally, we found an effect of arm location × condition ($F(3,64) = 4.140$, $p = .007$) but not between arm location × sex ($F(3,64) = 2.578$, $p = .055$) nor arm location × condition × sex ($F(3,64) = 1.710$, $p = .166$). In addition to the variation within condition × sex (Figure 8.3i,j), Bonferroni post hoc tests revealed that isolated females spent more time at the far end of open arm ($p = .007$), and less time in the closed arms ($p = .032$), compared to control females (Figure 8.3i).

*Risk assessment.* The time spent engaging in risk assessment was compared. No effect of condition ($F(1,66) = 0.744$, $p = .392$), sex ($F(1,66) = 0.142$, $p = .708$), nor condition × sex ($F(1,66) = 1.145$, $p = .289$) was found. Our results indicate that isolated females were less anxious than control females as they spent more time at the end of the open arms.

### 8.3.6 Isolation leads to spatial learning and memory deficits in adults

To test the use of a cue and spatial learning, we used the cue-place MWT where the platform switches from being visible to invisible across days. This task assesses both dorsolateral striatum (during the cue-visible platform) and hippocampal (spatial learning during the place-invisible platform) network functions (McDonald & White, 1994). *Visible*

*days.* We found that isolated female rats swam longer distances when navigating to the platform than control female rats on multiple days, while males showed no difference. ANOVAs revealed that latency to reach the platform varied significantly depending on the day ($F(8,20) = 64.097$, $p = .004$), day × sex ($F(8,20) = 3.217$, $p = .002$), and day × condition × sex ($F(8,20) = 2.957$, $p = .004$). We did not, however, find an effect of day × condition ($F(8,20) = 1.970$, $p = .051$). Bonferroni post hoc tests revealed that on multiple days, female isolated rats took longer to reach the platform than female control rats. On days 9 ($p = .036$), 10 ($p < .001$), and 11 ($p < .001$), females swam longer than males. Similarly, we found that when visible, the distance travelled to reach the platform varied significantly depending on the day ($F(8,20) = 64.097$, $p < .001$), but not day × condition ($F(8,20) = 1.067$, $p = .423$), day × sex ($F(8,20) = 1.178$, $p = .359$), nor day × condition × sex interaction ($F(8,20) = 2.452$, $p = .050$). Bonferroni post hoc tests revealed that on multiple days, female isolated rats swam further distances than female control rats (Figure 8.4a). Despite males showing similar patterns, isolated females performed worse throughout testing, swimming further and taking longer to reach the platform suggesting that they may have deficits in the dorsolateral striatum.

*Invisible days*. On invisible days, isolated female rats swam further to reach the platform than control rats on Days 8 and 12, while males showed no difference. ANOVAs revealed that the latency to reach the platform varied significantly depending on the day ($F(2,26) = 17.009$, $p < .001$) and day × condition ($F(2,26) = 3.595$, $p = .034$). We did not, however, find an effect of day × sex ($F(2,26) = 0.139$, $p = .871$) nor day × condition × sex ($F(2,26) = 3.032$, $p = .057$). Bonferroni post hoc tests revealed that on multiple days, female isolated rats took longer to reach the platform than female control rats.

Similarly, we found that when invisible, the distance travelled to reach the platform varied significantly depending on the day ($F(2,26) = 20.067$, $p < .001$), day × condition ($F(2,26) = 4.005$, $p = .024$), but not day × sex ($F(2,26) = 0.024$, $p = .086$) nor day × condition × sex interaction ($F(2,26) = 2.566$, $p = .050$). Bonferroni post hoc tests revealed that on multiple days, female isolated rats swam further distances than female control rats (Figure 8.4a). On each day, females, regardless of condition, swam further than males (Day 4: $p = .020$; Day 8: $p = .004$; Day 12: $p = .026$). Together, this suggests that isolated female rats may also have hippocampal deficits, as they exhibited poor spatial learning.

Heatmaps illustrating the average swim path for each condition × sex show that control females swam in more direct paths to the platform on visible and invisible days (Figure 8.4c and 8.4e, respectively), while isolated females spent more time swimming around the platform on visible days (Figure 8.4d) or around the circumference of the pool on invisible days (Figure 8.4f). Interestingly, isolated females performed like control females on the first three visible days and on the first invisible day, with their performance worsening half-way through the testing period. Heatmaps of the group mean swim path show that control and isolated males swam in similar paths on visible days (Figure 8.4g and 8.4h, respectively), while isolated males on invisible days spent more time swimming around the platform (Figure 8.4j) than control males (Figure 8.4i), though, not significantly so. When the paths are plotted, it suggests that the isolated female rats swim around the perimeter of the pool more than the controls, and thus, are either using different behavioral strategies to navigate to the platform or are looking to escape rather than navigate to the platform. We have reported this effect before in rats showing that when receiving partial reinforcement to the escape platform they increase time spent in the periphery of the pool

as this behaviour results in removal from the pool by the experimenter if they cannot find the platform (Devan et al., 2003; Devan & McDonald, 2001).This pattern of results suggests that the MWT has at least two reinforcement contingencies with finding the escape platform the best predictor of safety and the pool wall a partial predictor of safety.

Unlike CPP, MWT 1-trial place learning relies on the hippocampus, as MWT is a spatial learning and memory task (McDonald et al., 2010). Therefore, deficits in navigating towards the platform would indicate deficits in the hippocampus, and the spatial learning and memory neural networks. We have previously shown that this paradigm places a high demand on hippocampal function so even subtle dysfunction of this network will be revealed with this version of the MWT (Sutherland et al., 2000). While all rats learned to navigate towards the platform, we found that control rats learned the paradigm faster than isolated rats. When comparing the average latency from the first and second swim trial across blocks of three days, a significant effect of day was found ($F(1,35) = 34.554$, $p < .001$). No interaction effect of swim trial × condition ($F(1,35) = 0.586$, $p = .767$), swim trial × sex ($F(1,35) = 1.203$, $p = .302$), nor swim trial × condition × sex ($F(1,35) = 1.263$, $p = .270$). Though no direct differences in latency were observed between control and isolated females, the latency to reach the platform on the second swim was significantly less for control females by the Day 4-6 block, while the latency for isolated females to reach the platform did not decrease until Days 7-9. Like females, control males reached the platform faster on the second swim on days 4-6 while isolated males did not reach the platform faster until days 7-9. When comparing the average distance travelled from the first and second swim across blocks of three days, we found a significant effect of swim trial ($F(1,36) = 33.731$, $p < .001$) and swim trial × sex ($F(1,36) = 2.431$, $p = .022$). We did not find a

significant effect of swim trial × condition ($F(1,36) = 0.562$, $p = .786$) nor swim trial × condition × sex ($F(1,36) = 1.991$, $p = .057$). Control males took shorter paths on their second swim to the platform as early as the first block of days, while isolated males did not take shorter paths on their second swim until days 7-9 (Figure 8.4l). While all rats learned the location of the platform, isolated rats took longer to learn the paradigm than control rats suggesting that the HPC might be compromised leading to these spatial learning and memory deficits.

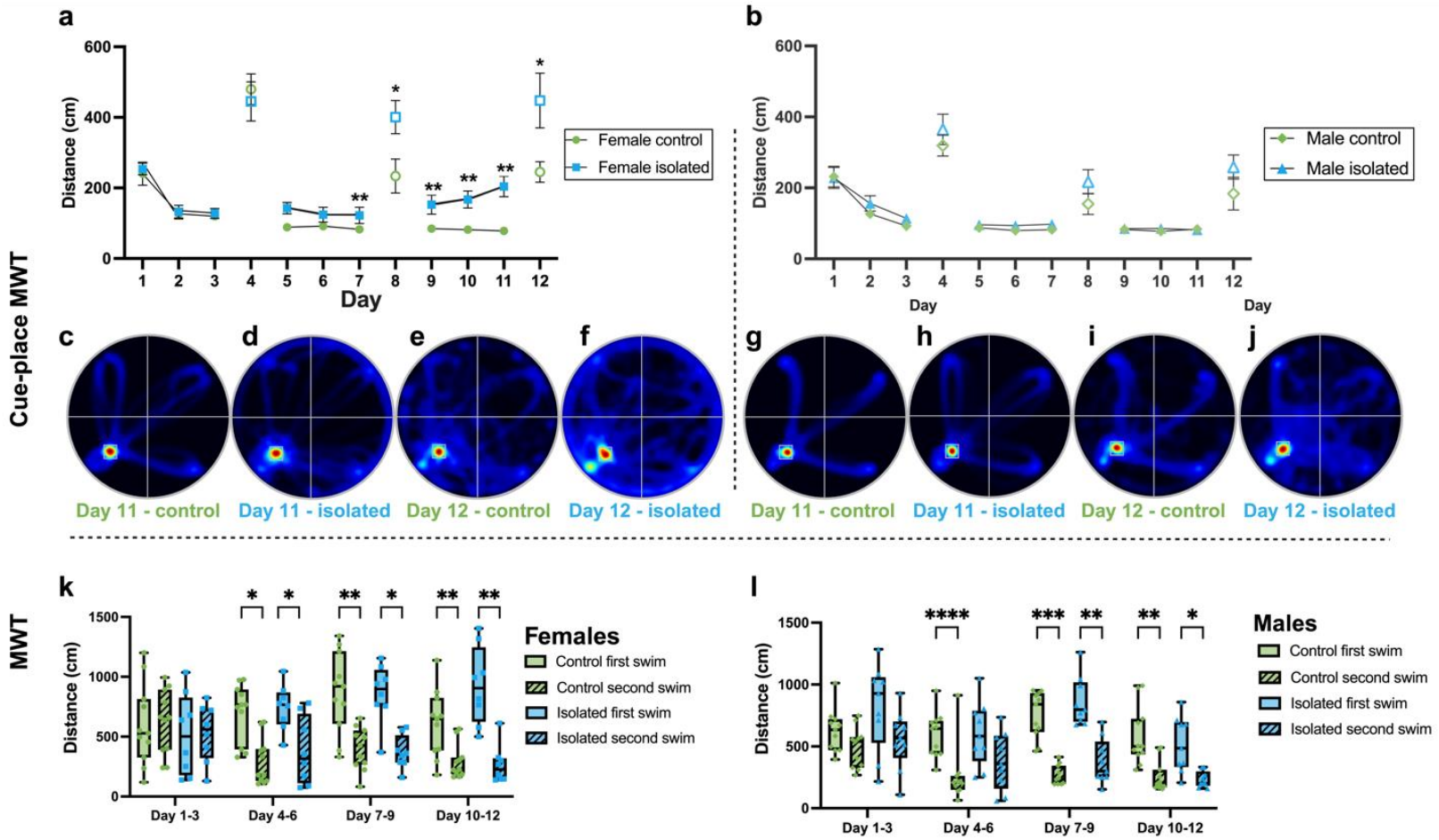


**Figure 8.4 Behavioral measures from two variants of the Morris water task.** The distance swam to reach the visible platform (filled points) and invisible platform (open points) for females in the cue-place Morris water task (MWT) are plotted (a). Isolated females ($n$ = 8) swam a longer distance on four of the visible days and two of the invisible days when compared to control females ($n$ =6). Both control ($n$ = 6) and isolated males ($n$ = 11) swam similar distances to reach both the visible and invisible platforms (b). Mean swim paths,

where red represents the area of the arena the animals spent the most time and black meaning the animals spent little to no time in that area, on a visible day reveal that control females swam directly to the platform (bottom left quadrant) (c) while isolated females swam around the edge of the pool (d). Female control rats swim in a less direct pathway when the platform is invisible (e), however, isolated female rats again spend more time swimming around the circumference of the pool (f). Male control rats swim directly to the visible platform (g), as do isolated male rats (h). Male control rats swim towards the invisible platform (i) while isolated male rats swim towards the platform (j), but spend more time swimming in the correct quadrant, around the platform, but not significantly so. When the first swim was compared to the second swim in four training blocks of MWT one-trial, we found that both female control rats ($n$ = 11) and isolated rats ($n$ = 8) had a shorter swim path on their second swim in the second block of training (k). Control rats ($n$ = 11) had a shorter swim path on the second swim in the first training block while it took isolated males ($n$ = 9) until the third training block to have a reduced path length on the second swim (l). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

### 8.3.7 Spine density and neuron length is affected by isolation in two different neuronal populations

Using digital reconstructions (Figure 8.5a-c,f) of pyramidal neurons found in layer III of the mPFC (Cg3/prelimbic cortex), we compared neuron morphology. Using linear mixed models, we found no significant effect of condition nor sex (Table 8.1; means ± SEM can be found in Appendix C, Table C4) for any of the measures (length, volume, surface area) of soma or dendritic arbors (Figure 8.5d,e,g,h). As well, digital reconstructions of the spines (Figure 8.5i,j) found on the pyramidal neurons were compared. The four different dendritic arbor locations compared were: basilar distal, basilar proximal, apical proximal, and apical distal arbors (Figure 8.5k). Linear mixed models (Table 8.1) revealed that control rats, of both sexes, had a higher density of spines at all four locations surveyed, excluding the apical proximal location were only a difference in control versus isolated females was significant (Figure 8.5l-o). Thus, while the neurons themselves where similar sizes, control rats had a higher density of spines suggesting that the neurons in the mPFC of control rats had more connections to neighboring cells than isolated rats.

Like the mPFC, the dLS has been implicated in anxiety and social behavior (Hodges et al., 2019; Parfitt et al., 2017), as such we measured the stellate-like neurons found in this location (Figure 8.5p,q). When digital reconstructions were compared (Table 8.2; means ± SEM, can be found in Appendix C, Table C5), we found that isolated females had longer neurons (Figure 8.5r) and longer somas than control females (Figure 8.5s). No difference in volume or surface area of the soma or dendritic arbors (Figure 8.5u,v). Spine density was not measured as these cells are generally aspiny.

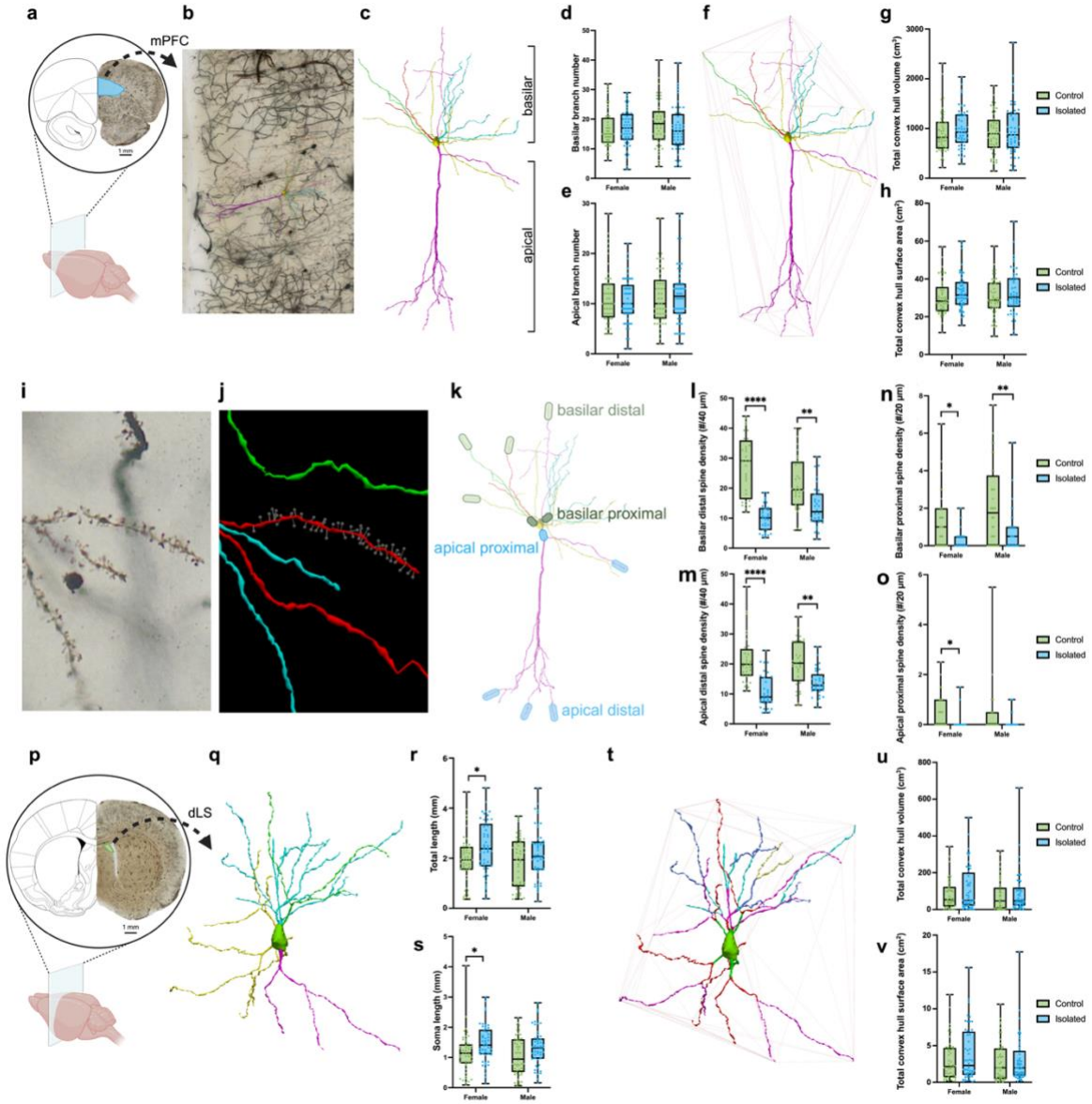


**Figure 8.5 Measures for the neurons in the mPFC and dLS. a**, The medial prefrontal cortex (mPFC), the first region of interest, showing both a brain atlas (from Paxinos & Watson, 1986) and a hemisphere stained with Golgi-Cox. **b**, After digitally scanning the slides, neurons in layer III were traced. **c**, Soma and apical and basilar dendritic arbors were measured from reconstructions of pyramidal neurons. **d**, The number of branches in the basilar arbors did not vary between conditions nor did the branch number vary in the **e**, apical arbors. **f**, Digital reconstructions were used to measure **g**, the convex hull volume, which was not significantly different between conditions, and **h**, the convex hull surface area, was also not significantly different between conditions. **i**, Using the digital scans, **j**, spine densities were measured in four different regions. **k**, Spine densities were measured at tips and base of both the basilar and apical branches with four sites being counted for both distal measures. Two proximal locations were measured on the basilar branches while only one location was measured on the apical branch. **l**, Control rats had a higher spine

density than isolated rats in the both the distal parts of the basilar branches and the **m**, apical branches. **n**, Similarly, controls had a higher spine density in the proximal parts of the basilar branches, **o**, while only control females had a higher density of spines than female isolated rats on the proximal location of the apical dendrites. **p**, The dorsolateral septum (dLS), the second region of interest, showing both a brain atlas (from Paxinos & Watson, 1986) and a hemisphere stained with Golgi-Cox. **q**, Reconstructions of stellate-like neurons were created. **r**, In females, the total length of the dLS neurons were longer in isolated rats **s**, especially the length of the soma. **t**, A digital reconstruction is shown, with the convex hull volume illustrated. **u**, No difference was found between conditions in the total convex hull volume nor **v**, in the total convex hull surface area. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. Created in part with BioRender.com

**Table 8.1** The analysis of pyramidal neurons found in Cg3, layer III, of rats. Significant results are in bold.

| Measurement | Estimate | Std. Error | t | *p* |
|---|---|---|---|---|
| Convex hull volume ($cm^3$) | | | | |
| Intercept | 918.523 | 67.790 | 13.550 | < .00001 |
| Condition | 66.353 | 95.869 | 0.692 | .895 |
| Sex | -12.784 | 95.869 | -0.133 | .895 |
| Condition × sex | 9.766 | 135.579 | 0.072 | .943 |
| Apical convex hull volume ($cm^3$) | | | | |
| Intercept | 321.89 | 42.17 | 7.633 | < .00001 |
| Condition | 69.55 | 59.64 | 1.166 | .253 |
| Sex | -44.99 | 59.64 | -0.754 | .457 |
| Condition × sex | -89.80 | 84.35 | -1.065 | .296 |
| Basilar convex hull volume ($cm^3$) | | | | |
| Intercept | 127.28 | 19.83 | 6.418 | < .00001 |
| Condition | -20.06 | 28.05 | -0.715 | .475 |
| Sex | 33.30 | 28.05 | 1.187 | .237 |
| Condition × sex | 29.95 | 39.67 | 0.755 | .451 |
| Convex hull surface area ($cm^2$) | | | | |
| Intercept | 30.240 | 1.679 | 18.801 | < .00001 |
| Condition | 2.55 | 2.375 | 1.075 | .292 |
| Sex | -0.015 | 2.37 | -0.006 | .995 |
| Condition × sex | 0.36 | 3.358 | 0.107 | .915 |
| Apical convex hull surface area ($cm^2$) | | | | |
| Intercept | 14.70 | 1.398 | 10.51 | < .00001 |
| Condition | 2.49 | 1.978 | 1.262 | .217 |
| Sex | -0.872 | 1.978 | -0.441 | .663 |
| Condition × sex | -2.143 | 2.797 | -0.766 | .450 |
| Basilar convex hull surface area ($cm^2$) | | | | |
| Intercept | 13.523 | 2.767 | 4.887 | < .00001 |
| Condition | 0.336 | 3.914 | 0.086 | .932 |
| Sex | 5.047 | 3.914 | 1.290 | .202 |
| Condition × sex | -5.361 | 5.535 | -0.969 | .337 |
| Total length (mm) | | | | |
| Intercept | 5.133 | 0.228 | 22.506 | < .00001 |
| Condition | 0.079 | 0.322 | 0.246 | .808 |
| Sex | 0.192 | 0.322 | 0.597 | .555 |
| Condition × sex | -0.034 | 0.456 | -0.076 | .940 |
| Apical length (mm) | | | | |
| Intercept | 1.691 | 0.127 | 13.357 | < .00001 |

| | | | | |
|---|---|---|---|---|
| Condition | -0.142 | 0.179 | -0.796 | .433 |
| Sex | -0.055 | 0.179 | -0.309 | .760 |
| Condition × sex | 0.266 | 0.253 | 1.049 | .303 |
| Basilar length (mm) | | | | |
| Intercept | 2.505 | 0.147 | 17.004 | < .00001 |
| Condition | 0.134 | 0.208 | 0.642 | .526 |
| Sex | 0.155 | 0.208 | 0.742 | .464 |
| Condition × sex | -0.221 | 0.295 | -0.751 | .459 |
| Cell body length (mm) | | | | |
| Intercept | 0.937 | 0.090 | 10.424 | .0085 |
| Condition | 0.088 | 0.081 | 1.090 | .285 |
| Sex | 0.093 | 0.081 | 1.156 | .258 |
| Condition × sex | -0.079 | 0.114 | -0.692 | .495 |
| Total volume ($mm^3$) | | | | |
| Intercept | 11.177 | 1.455 | 7.680 | .022 |
| Condition | 0.560 | 1.208 | 0.463 | .647 |
| Sex | 1.696 | 1.208 | 1.403 | .172 |
| Condition × sex | -1.393 | 1.709 | -0.815 | .422 |
| Apical volume ($mm^3$) | | | | |
| Intercept | 2.844 | 0.375 | 7.585 | .0039 |
| Condition | -0.013 | 0.409 | -0.031 | .975 |
| Sex | 0.151 | 0.409 | 0.370 | .714 |
| Condition × sex | 0.178 | 0.578 | 0.308 | .761 |
| Basilar volume ($mm^3$) | | | | |
| Intercept | 2.332 | 0.263 | 8.858 | .0028 |
| Condition | -0.163 | 0.283 | -0.576 | .570 |
| Sex | 0.369 | 0.283 | 1.303 | .204 |
| Condition × sex | -0.243 | 0.400 | -0.607 | .549 |
| Cell body volume ($mm^3$) | | | | |
| Intercept | 6.001 | 0.900 | 6.665 | .022 |
| Condition | 0.725 | 0.794 | 0.925 | .362 |
| Sex | 1.176 | 0.794 | 1.481 | .150 |
| Condition × sex | -1.256 | 1.123 | -1.119 | .273 |
| Total branch number | | | | |
| Intercept | 27.875 | 1.742 | 16.003 | < .00001 |
| Condition | -0.458 | 2.424 | -0.189 | .851 |
| Sex | 1.854 | 2.424 | 0.765 | .451 |
| Condition × sex | -0.813 | 3.428 | -0.237 | .814 |
| Apical branch number | | | | |
| Intercept | 11.438 | 0.957 | 11.949 | < .00001 |
| Condition | -0.896 | 1.354 | -0.662 | .514 |
| Sex | -0.167 | 1.354 | -0.123 | .903 |
| Condition × sex | 1.417 | 1.914 | 0.730 | .465 |
| Basilar branch number | | | | |
| Intercept | 16.438 | 1.316 | 12.487 | < .00001 |
| Condition | 0.438 | 1.862 | 0.235 | .816 |

| | | | | |
|---|---|---|---|---|
| Sex | 2.021 | 1.862 | 1.086 | .287 |
| Condition × sex | -2.229 | 2.633 | -0.847 | .404 |
| Apical distal spine density (#/40µm) | | | | |
| Intercept | 21.469 | 1.165 | 18.430 | < .00001 |
| **Condition** | **-10.625** | **1.647** | **-6.450** | **< .00001** |
| Sex | -1.094 | 1.647 | -0.664 | 0.509 |
| Condition × sex | 4.063 | 2.330 | 1.744 | 0.0865 |
| Apical proximal spine density (#/20µm) | | | | |
| Intercept | 0.484 | 0.156 | 3.097 | 0.059 |
| **Condition** | **-0.406** | **0.162** | **-2.513** | **0.013** |
| Sex | -0.069 | 0.162 | -0.290 | .772 |
| Condition × sex | 0.109 | 0.229 | 0.479 | .633 |
| Basilar distal spine density (#/40µm) | | | | |
| Intercept | 27.011 | 2.034 | 13.278 | .00018 |
| **Condition** | **-16.894** | **2.349** | **-7.192** | **< .00001** |
| **Sex** | **-5.504** | **2.349** | **-2.343** | **.022** |
| **Condition × sex** | **9.230** | **3.315** | **2.784** | **.0072** |
| Basilar proximal spine density (#/20µm) | | | | |
| Intercept | 1.286 | 0.467 | 2.756 | .122 |
| **Condition** | **-0.926** | **0.389** | **-2.384** | **.024** |
| **Sex** | **0.855** | **0.389** | **2.200** | **.037** |
| Condition × sex | -0.355 | 0.549 | -0.646 | .524 |

**Table 8.2** The analysis of stellate-like neurons found in dorsolateral septum of rats. Significant results are in bold.

| Measurement | Estimate | Std. Error | t | *p* |
|---|---|---|---|---|
| Convex hull volume ($cm^3$) | | | | |
| Intercept | 81.75 | 19.59 | 4.172 | .00026 |
| Condition | 31.39 | 27.71 | 1.133 | .267 |
| Sex | -6.20 | 27.71 | -0.224 | .825 |
| Condition × sex | -17.15 | 29.19 | -0.438 | .665 |
| Convex hull surface area ($cm^2$) | | | | |
| Intercept | 54.613 | 8.089 | 6.752 | < .00001 |
| Condition | 7.622 | 11.439 | 0.666 | .511 |
| Sex | -5.869 | 11.439 | -0.513 | .612 |
| Condition × sex | -0.882 | 16.177 | -0.055 | .957 |
| Total length (mm) | | | | |
| Intercept | 1.936 | 0.221 | 8.779 | .0016 |
| **Condition** | **0.517** | **0.249** | **2.074** | **.0477** |
| Sex | -0.096 | 0.249 | -0.384 | .704 |
| Condition × sex | -0.180 | 0.353 | -0.509 | .615 |
| Dendrite length (mm) | | | | |
| Intercept | 0.788 | 0.116 | 6.785 | < .00001 |
| Condition | 0.198 | 0.164 | 1.207 | .238 |
| Sex | -0.037 | 0.164 | -0.223 | .825 |
| Condition × sex | -0.098 | 0.232 | -0.422 | .676 |
| Cell body length (mm) | | | | |
| Intercept | 1.148 | 0.148 | 7.735 | .014 |
| **Condition** | **0.319** | **0.137** | **2.323** | **.028** |
| Sex | -0.061 | 0.138 | -0.444 | .661 |
| Condition × sex | -0.080 | 0.194 | -0.411 | .684 |
| Total volume ($mm^3$) | | | | |
| Intercept | 10.838 | 2.137 | 5.071 | .051 |
| Condition | 2.984 | 1.693 | 1.763 | .083 |
| Sex | -1.569 | 1.699 | -0.923 | .360 |
| Condition × sex | -0.026 | 2.399 | -0.011 | .991 |
| Dendrite volume ($mm^3$) | | | | |

| | | | | |
|---|---|---|---|---|
| Intercept | 2.514 | 0.607 | 4.142 | .064 |
| Condition | 0.551 | 0.503 | 1.096 | .278 |
| Sex | -0.851 | 0.505 | -1.685 | .097 |
| Condition × sex | 0.378 | 0.713 | 0.530 | .598 |
| Cell body volume ($mm^3$) | | | | |
| Intercept | 8.323 | 1.581 | 5.265 | .038 |
| Condition | 2.433 | 1.357 | 1.792 | .078 |
| Sex | -0.729 | 1.362 | -0.525 | .595 |
| Condition × sex | -0.394 | 1.923 | -0.205 | .838 |
| Branch number | | | | |
| Intercept | 5.229 | 0.992 | 5.273 | < .00001 |
| Condition | 2.563 | 1.402 | 1.827 | .078 |
| Sex | -0.396 | 1.402 | -0.282 | .780 |
| Condition × sex | -1.750 | 1.983 | -0.882 | .385 |

### 8.3.8 Isolation does not affect corticosterone in young adults, but does affect blood serum metabolomics in a sex-specific manner

To determine if isolated rats had altered corticosterone concentrations, we analyzed blood samples taken at P65 with an ELISA kit. Blood was taken under anesthesia from the tail vein. A one-way ANOVA revealed significant differences in serum corticosterone concentrations ($F(3,63) = 18.87$, $p = .003$). A post-hoc Tukey test found that serum corticosterone concentrations were higher in female control animals compared to male isolated rats ($p = .04$) and elevated in female isolated rats compared to both male controls ($p = .0002$) and male isolated rats ($p < .0001$) (Figure 8.6a). Females, in general, had higher serum corticosterone concentrations relative to males, with isolated females having higher serum corticosterone than both control and isolated males, but not control females.

Finally, given the power of -omics studies to identify biological markers of stress and anxiety, we examined the metabolome of blood serum from rats at P65 using an

untargeted $^{1}H$ nuclear magnetic resonance (NMR) spectroscopy-based metabolomics approach. A combination of univariate and multivariate testing identified 9 and 14 metabolites as significantly altered ($p < 0.05$) in female (Figure 8.6d) and male (Figure 8.6e) rats, respectively. Succinate, glucose, and valine concentrations were altered for both sexes; however, the direction of regulation for glucose and valine differed across the sexes. The scores plot obtained from orthogonal partial least squares discriminant analysis (OPLS-DA) for females (Figure 8.6b; Q2 = 0.368, $p$ = .002; R2 = 0.702, $p$ = .0005) and males (Figure 8.6c; Q2 = 0.265, $p$ = .0035; R2 = 0.424, $p$ = .047) illustrate the separation between the control and isolated groups. Females show better group classification compared to males, as indicated by a higher quality of the model (Q2) and amount of variance explained by the model (R2). Furthermore, there is less overlap in the 95% confidence interval for female rats, with only one isolated sample being misclassified by the data variance presented on the x-axis.

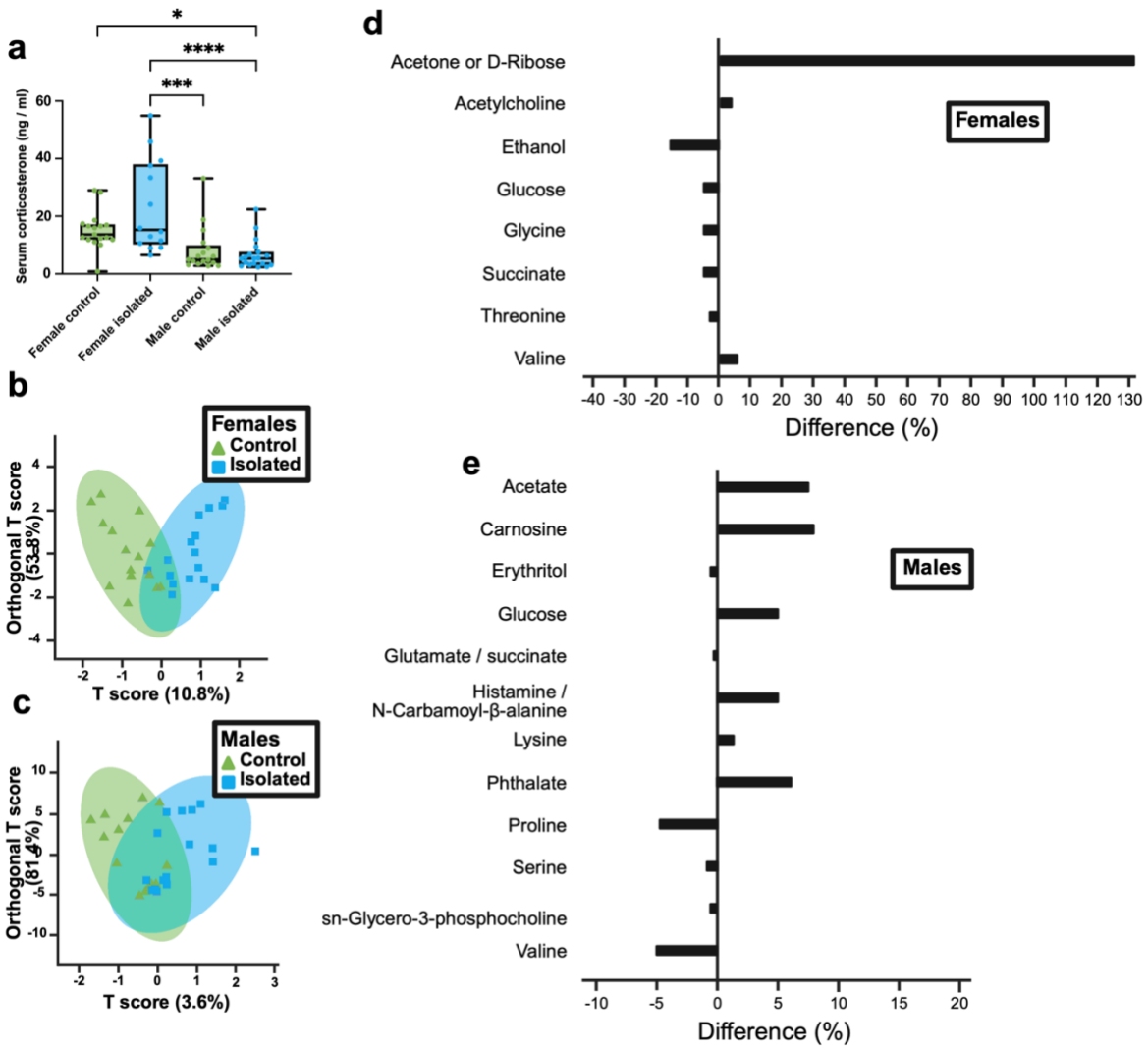


**Figure 8.6. Corticosterone and metabolic measures.** Blood serum corticosterone concentrations in female isolated rats were higher than in male control and male isolated rats (a). Female control rats also had higher levels of blood serum corticosterone than isolated male rats. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) score plots showing the group separation for serum from female (b) and male (c) rats. Multiple metabolites were either upregulated or downregulated in isolated female (d) and male (e) rats when compared to controls. The metabolites shown were also utilized to create the OPLS-DA scores plots. Most of the significantly altered metabolites differed in males and females, with only glucose, succinate, and valine being common to both sexes.

## 8.4 Discussion

Using a model of early life stress, this study found that rats separated from their mother and siblings during the neonatal period, develop social anxiety-like behavior and learning and memory deficits, abnormal mPFC neurons, and altered metabolism. Isolation affected males and females differently, with social deficits being more apparent in males while spatial memory deficits more apparent in females. Overall, isolation in early life has significant and long-term consequences for social behavior, cognition, brain structure, and physiology and these differences can vary by type and severity between the sexes.

Isolated males showed a reduced motivation to engage in RTP, whereas females are just as motivated to play but adopt different RTP tactics. In contrast, when animals are left with their siblings during maternal separation, this reduction in RTP is not observed. Instead, maternally separated rats show either no change in the amount of play initiated or an increase in RTP (Arnold & Siviy, 2002; Veenema & Neumann, 2009). Given that the pups in the maternal separation paradigm are still able to remain in contact with one another, perhaps the social contact provided by siblings is enough to compensate for the lack of touch provided by the mother. It is likely the direct social contact, which is lost during the neonatal isolation paradigm used in the present study, is what is critical to the development of the typical social repertoire, including juvenile play behavior (Imanaka et al., 2008; Muhammad et al., 2011; Siviy & Harrison, 2008). Tactile stimulation, such as skin-to-skin touch in humans, is also important for the development of the mother/father-child bond, improves the parenting process, and has a positive impact on babies, reducing stress and benefitting cognitive and motor development (Cleveland et al., 2017; Feldman et al., 2002; Vittner et al., 2018). Social touch during these early ages likely plays a pivotal role in the development of the social brain for both humans and rats.

### 8.4.1 Combining traditional and new testing paradigms

Social deficits are only revealed under appropriate, ethologically relevant, testing conditions (Winiarski et al., 2022). Similarly, our study reveals that when using play as a behavioral indicator of sociality, the testing conditions must be chosen appropriately. Had we tested our animals exclusively with unfamiliar individuals, we would have concluded our rats were low playing, when, instead, they exhibited social anxiety-like behavior. Indeed, when the rats were tested with familiar partners, in pairs, no difference was found between control and isolated rats. This was unexpected and highlights the need to carefully choose the circumstances in which animals are tested, especially within the social domain (Jabarin et al., 2022). Further, using a more naturalistic group play paradigm (Pellis, Pellis, Ham, et al., 2022), the rats '*tell us*' if they perceive deficits in their partners by either choosing to play with them or avoiding them (Ham & Pellis, 2023, 2024). Our control rats had no preference, but rats that underwent early-life isolation chose to play with one another over those who did not undergo this early-life stress, indicating subtle deficits in social behavior.

One take home message from this study is that using a multifaceted approach helps detect subtle differences that are nevertheless biologically important and with lifelong consequences. Testing social behaviors during both the juvenile period and adult period reveals that social anxiety persists, and that it worsens for some individuals, with the isolated individuals being the only rats that escalated to overt aggression. However, in other tests, the rats do not differ from controls, such as when learning to discriminate between contexts during fear conditioning. In contrast, isolated female rats showed decreased anxiety-like behaviors during the elevated plus maze test, spending more time in the open

arms, contrary to what was predicted based on previous work and assumptions about what this task measures (Rodgers & Dalvi, 1997).

Indeed, we found that when the animals were conditioned to a fearful stimulus, there was no difference between controls and isolated rats. All rats learned to associate the context with a fearful stimulus. However, when conditioned to a food reward, the isolated rats did not exhibit a preference to the rewarded context. This different pattern of effects on two tasks that are identical except for the valence of the reinforcement is revealing. For the fear conditioning task, foot-shock is employed to condition the rats to learn to associate one context with something unpleasant and another context with safety. Alternatively, for the CPP task rats are repeatedly exposed to a highly palatable and rewarding food in one context and no food availability in the other context. The DFCTC task is highly dependent on the basolateral amygdala and associated hypothalamic and brainstem regions with the former being the site of convergence of the sensory information defining the different contexts and unconditioned responses (heart rate changes, respiration, stress hormone release, freezing, etc) elicited by the aversive shock mediated by the latter regions. Accurate performance on the CPP task also requires the basolateral amygdala which is a convergence site for the sensory information defining the different contexts with a representation of the food reward via connectivity with the ventral striatum and nucleus accumbens (McDonald et al., 2017, 2021).

Like the differences in CPP and DFCTC, differences in condition emerge depending on the MWT test. When tested with the cue-place MWT, isolated male rats navigated towards the platform, whether hidden or visible, just as fast, and in as short of distances, as control rats. Isolated males, however, navigating the one-trial place learning MWT did not improve on their second swim time until the third testing block while control

males improved in the second training block. Unlike the males, we did find a difference among females, with isolated females swimming longer distances and taking longer to navigate to both the hidden and visible platforms during cue-place MWT. This difference in performance was an effect that only emerged halfway through testing with isolated females plateauing in performance before control females. Surprisingly, the isolated female's performance worsened over time. Indeed, this is an unusual pattern of results and to our knowledge is the first time this has been shown. Cue-place MWT assess both dorsolateral striatal (during cue-visible) and hippocampal (during place-invisible) network function (Lee et al., 2019; McDonald et al., 2006; McDonald & Hong, 2000). As such, our data suggest that both networks are compromised in isolated females. During one-trial place learning MWT, isolated females, like the isolated males, did not improve on their second swim time until the third block of training while control females improved in the second block.

### 8.4.2 Brain and physiology

We found that while the dendritic arbors of pyramidal neurons in the mPFC did not vary significantly between control and isolated rats in length, volume, or branch number, they were less spiny. This, a relatively consistent finding (Leuner & Shors, 2013) and suggests that the neurons in the mPFC are less connected in the isolated subjects (Shepherd, 1996). Given the mPFCs association with social behavior and emotional regulation (Klune et al., 2021), this could in part explain the aggression observed in some of the adult rats (Ham, Szabo, et al., 2024; Stark & Pellis, 2020). Given the social dysfunction, it was unsurprising that the stellate-like neurons found in the dLS varied in total and soma length, as this brain region is sensitive to social stress (Hodges et al., 2019). The mPFC and dLS are both sensitive to social stress when this stress occurs during the juvenile period (Hodges

et al., 2019). For example, the mPFC is sensitive to play experiences during the juvenile period, and when poor play is experienced (including social isolation), neurons found in the mPFC are large and relatively less pruned (Bell et al., 2009; Ham, Szabo, et al., 2024; Stark et al., 2023). In contrast, when isolated as a neonate, the neurons are relatively more pruned, having less spines (present study; Monroy et al., 2010; Romano-López et al., 2016). Variation in brain and behavior depending is likely linked to variation in adrenocortical stress response sensitivity. Indeed, rats are in a hyposensitive period (Sapolsky & Meaney, 1986) during the neonatal phase and this may in part explain why we did not see similar brain changes as seen with rats isolated as juveniles (Baarendse et al., 2013; Einon, 1980).

The blood serum metabolome of both male and female isolated rats was altered when compared to controls. The blood serum level of succinate was decreased in both male and female isolated rats. Recent studies have shown that succinate has anxiolytic effects when utilized as an anti-anxiety treatment in animal models (Chen et al., 2003; Mia et al., 2023; Volchegorskii et al., 2015, 2016). Therefore, the decreased levels of succinate available in the blood of isolated rats could explain higher levels of anxiety. In addition, the levels of valine, lysine, and glutamate were altered in isolated rats. Two previous studies of early life stress, which utilized shipment stress as its paradigm, found that altered levels of these metabolites in male mice were correlated with anxiety-like behaviour (Poplawski et al., 2020, 2024). Both human and animal studies have indicated that a deficiency in dietary lysine can elevate stress-induced anxiety, with supplementation leading to a reduction in anxiety (Smriga et al., 2002, 2004). Interestingly, lysine levels were elevated in male rats, which could explain the lack of anxiety like behaviors in some of the behavioural tests. Elevated levels of valine, which is one of the branch chain amino acids, is implicated in several stress related pathophysiological conditions such as insulin

resistance, type 2 diabetes, obesity, cancer, and heart failure (Neinast et al., 2019). Glutamate is a key neurotransmitter, and previous studies have shown that inosine, which regulates the release of glutamate, is a potential biomarker for depression (Zhou et al., 2019). Isolated rats also had altered levels of glucose, threonine, and histamine. Previous studies of trans- and multi-generational prenatal stress have found that glucose, threonine, and histamine levels were altered in rats from the F3 and F4 generation who had a history of ancestral stress (Heynen et al., 2022; Kiss et al., 2016), with histamine levels being correlated to anxiety-like behaviours in the open-field locomotor activity test (Kiss et al., 2016). Changes in these metabolites could also be indicative of an increased immune system response, as stress often activates the immune system and both threonine and histamine play a key role in regulating immune response (Dhabhar, 2008; Ruth & Field, 2013; Smolinska et al., 2014). Lastly, blood glucose levels have been included in a panel of physiological markers associated with the rat cumulative allostatic load measure (rCALM), which provides an assessment of the burden of stress (McCreary et al., 2019).

### 8.4.3 Escape tendencies: interpreting some of the behavioural results through a different lens

Anecdotally, we noticed that isolated rats attempted to escape social tests more than their control counterparts. This was especially true of females. Indeed, we had to use a lid during the stranger paradigm to keep the females from jumping out of the test enclosure—something that we have never needed to do in the past, and during CPP the lids needed to be taped down to prevent them from escaping the context. Further, during both the cue-place and one-trial MWT, females swam near the edge of the pool (Figure 8.4f,h) rather than navigating directly to the platform, suggesting they were attempting to escape the pool. During EPM, isolated females did not exhibit typical anxiety-like behavior (Keeley et al.,

2015). Instead, the isolated females spent time more time in the open arms than control females, and closer to the edge of the open arms. Consistent with the observed escape tendencies, the females may have been spending time in the open and near the edge while attempting to find an escape from the EPM testing apparatus. When using the traditional measures of these tasks, we conclude that the rats did not learn to dissociate between CPP contexts as they did not express a preference for either box, and that they had learning and memory impairments when navigating to a platform in both the cue-place or one-trial MWT as they swam longer distances compared to control rats. However, these results may be inadvertently biased by the effort the rats expended on attempting to escape. For example, latency and the distance swam to reach the platform during MWT will be increased if the rats spend time swimming around the perimeter of the pool but does not necessarily indicate that they have a memory deficit. Instead, this could indicate that the rats are severely anxious and attempting to escape whatever environment they are in. Indeed, the escape tendencies reflects the higher serum corticosterone concentrations we found in the isolated females (Berger et al., 1981). Thus, taking the traditional measures at face value may be misleading as they may not represent the impairments accurately. Though we did not test escape directly, our anecdotal observations suggest that perhaps rats with heightened anxiety may attempt to escape novel social situations and environments. This should be tested in future studies.

## 8.5 Conclusion

Though neonatal isolation is a commonly used induction model of mood disorders, it is clear that behavioral results vary from one lab to another (Lehmann & Feldon, 2000). Developing robust models of mood disorders, such as anxiety, that are valid and reliable is crucial to the study of disease etiology and treatment. Beyond the model itself, the way in

which rodent models are tested and phenotyped would also benefit from being improved and more consistent. We found that social behavior is altered following neonatal isolation, but only when interacting with unfamiliar partners. Isolated females show deficits in the cue-place MWT while both isolated female and male rats are slow to learn the one-trial MWT variant. Nonetheless, all rats performed similarly in the DFCTC and the EPM, though, isolated female rats spent more time at the ends of the open arms than control female rats. These effects are at odds with the idea that maternal separation is a model of general anxiety as both tasks are thought to assay this specific mood disorder. We suggest that a multifaceted approach is important to detect subtle behavioral deficits. In conclusion, chronic, short-term social isolation during the neonatal period can induce social anxiety-like behaviors in rats, producing unique patterns of disruption of various brain networks involved in different forms of learning, memory, and cognitive processes linked to sociality, emotions, and decision making.

# CHAPTER 9: GENERAL DISCUSSION

"*...seeing a child play with dice, knucklebones, or counters is not bad, since it is customary for children to be always playing.*"

— Artemidorus, *Oneirocritica*, ca. 150 C.E.

With mounting evidence that playing with peers during the juvenile period is critical for the development of socio-cognitive skills and the underlying neural mechanisms that support these functions, this thesis aimed to address two foundational questions about rat rough-and-tumble play (RTP): (1) Do rats form preferences when playing in groups? (2) Given the degree of individual variation in RTP experiences (Achterberg et al., 2023; Lampe et al., 2017; Lesscher et al., 2021; Melotti et al., 2014; Poole & Fish, 1976), do all rats benefit from play?

Rats are highly social animals (Barnett, 1975; Calhoun, 1963), living in large colonies in the wild, some of which number in the hundreds (Schweinfurth, 2020), and juvenile rats naturally interact with multiple peers. However, laboratory studies of RTP typically test rats in dyads (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022; J. VanRyzin et al., 2020), limiting our understanding of how partner selection and avoidance might shape the development of social skills and the brain. As a first step in addressing this gap, Part I of this thesis explored the social dynamics of rats playing in groups. Part II then explored how individual differences in RTP behavior influence developmental outcomes.

Given that there is a large degree of variation in how much RTP individuals initiate and in their preferred defensive tactics (Achterberg et al., 2023; Lampe et al., 2019; Lesscher et al., 2021; Pellis & Pellis, 1987; Poole & Fish, 1976), it may be that not all rats

benefit equally from playing. Indeed, the variation in the frequency of RTP present in litters of wild Belding's ground squirrels (*Urocitellus beldingi*), has been shown to have developmental consequences. The ground squirrels that engage in more play exhibit improved social and motor skills and temperament (Hurst-Hopf et al., 2023; Marks et al., 2017; Nunes, Muecke, Lancaster, et al., 2004; Nunes, Muecke, Sanchez, et al., 2004; Shehan et al., 2023). To determine whether similar patterns hold true for the development of rat social skills and the medial prefrontal cortex (mPFC), Part II of this thesis explores how early life experiences impact development.

In Part I, I demonstrate that rats form partner preferences when playing in groups, and that the criteria rats use to select partners is context dependent, changing depending on the social milieu in which they find themselves. For example, when partnered with individuals that range in familiarity, factors such as weight and dominance influence the selection of partners (Chapter 2). However, if all the partners are unfamiliar, these factors no longer play a role, instead, rats prefer to play with individuals with whom they play more symmetrically (Chapter 3). Similarly, in groups composed of familiar individuals, rats prefer partners who engage in reciprocal and symmetrical play, with weight and dominance again having no influence on partner selection (Chapter 4). However, it should be noted that there is variation in preference strength irrespective of the group composition, with certain rats being very particular in their partner choice and others less selective.

To explore the influence of play style on partner selection further, Chapter 5 examined play in mixed strain groups using three rat strains: Long Evans (LE), Sprague Dawley (SD), and Fischer 344 (F344). These strains were selected because they engage in different frequencies of RTP and they tend to use different defensive tactics to protect their

napes when attacked. SD rats are relatively high playing compared to LE while F344 are low playing (S. M. Himmler, Modlinska, et al., 2014; Siviy et al., 1997, 2003; Stark et al., 2021). SD and F344 rats are also more likely to evade a playful attack than roll to supine compared to LE rats (S. M. Himmler, Modlinska, et al., 2014; Orsucci et al., 2024; Siviy et al., 2023). Each group contained two LE rats, one SD, and one F344, allowing LE rats to choose between similarly playing (LE), higher-playing (SD), and lower-playing (F344) partners. When given the choice, LE rats preferred same-strain partners over SD and F344 rats. However, when tested in dyads, LE rats played equally with all strains, suggesting that preferences only emerge when choice is available. Thus, some, but not all, social situations elicit partner preferences. While Chapter 5 confirmed the existence of preferences in mixed-strain groups, the long-term consequences of these early social experiences remained unclear.

To address this, Part II of the thesis shifts focus to investigate how variations in juvenile RTP experiences shape behavioral outcomes and brain development later in life. To do so, three different experiments were conducted. While it is well established that limiting RTP reduces socio-cognitive development and mPFC synaptic pruning (e.g., Bell et al., 2009; Einon & Morgan, 1977; B. T. Himmler, Pellis, & Kolb, 2013; Marquardt et al., 2023; Stark et al., 2023), deprivation methodologies differ, with some impacting experiences beyond play more than others (Pellis, Pellis, Ham, et al., 2023). To limit the loss of experiences as much as possible to play itself, the Pellis laboratory has more recently adopted the Schneider group's play reduction model, in which a high-playing strain is reared with a low-playing one (Schneider, Bindila, et al., 2016; Schneider et al., 2014; Schneider, Pätz, et al., 2016). In doing so, Stark et al. showed that both male and female

LE rats reared with low-playing F344 rats exhibit social skill deficiencies and excessively large dendritic arbours of the neurons in the mPFC (Stark et al., 2023; Stark & Pellis, 2020, 2021). Given that a low-playing strain can negatively impact a higher playing strain, it raises the question: can a low-playing strain benefit from being reared with a high playing strain? If so, this would have implications for how rats choose partners when living in groups.

To test this, I reared F344 rats with LE partners in pairs during the juvenile period (Chapter 6). Despite exposure to high-playing partners, F344 rats still developed social deficits and atypical mPFC anatomy. Analysis of F344-LE juvenile play revealed limited RTP experiences, fewer role reversals, and reduced symmetry compared to F344-F344 RTP. Interestingly, same-strain F344 dyads played 2.5 times more than expected (Siviy et al., 1997, 2003), suggesting that prior studies may have underestimated F344 playfulness due to cross-strain testing (Siviy et al., 1997, 2003, 2011, 2017, 2023). These findings suggest that F344 rats are incompatible with other strains, leading to decreased RTP quality and frequency.

Given the consequences of impoverished juvenile RTP experiences (Chapter 6; Bell et al., 2009; Bijlsma et al., 2022, 2023; Himmler et al., 2013; Schneider, Bindila, et al., 2016; Stark et al., 2023; Stark & Pellis, 2020, 2021; Whitten et al., 2025) and that not all rats are equally playful (Chapter 2, 3; Achterberg et al., 2023; Lampe et al., 2017; Lesscher et al., 2021; Melotti et al., 2014; Poole & Fish, 1976) and not all rats living in a group are preferred and popular (Chapter 4), Chapter 7 tested whether natural variation in juvenile RTP predicts later social competence and mPFC neuron anatomy. While RTP frequency did not differ between juveniles that as adults were socially competent and incompetent,

individuals that were socially incompetent engaged in less turn-taking behavior during juvenile RTP. As adults, these rats were more aggressive and had larger pyramidal neurons in the mPFC, suggesting that cooperative play, not mere frequency, is critical for typical development.

To explore potential early-life origins of this less cooperative play style, Chapter 8 examined the effects of early life adversity (ELA), specifically chronic short-term isolation, on RTP and adult behavior. While ELA rats showed reduced RTP with unfamiliar partners, this deficit disappeared when playing with cage mates. Also, when playing with familiar partners, the ELA rats engaged in as much turn-taking as the controls. Moreover, when given a choice between unfamiliar ELA and control rats, ELA rats preferred other ELA rats. As adults, ELA rats were more aggressive, similar to those reared with incompatible strain partners (Chapter 6) and the group living rats that exhibited low levels of turn-taking during RTP (Chapter 7). Inconsistent with rats from these other conditions, the ELA rats had dendritic arbors of pyramidal neurons in the mPFC similar to control animals, but even so, the ELA rats had fewer spines on those neurons than controls, suggesting that this rearing manipulation did alter neuronal organization.

### 9.1 How does play change the brain?

The effects of juvenile RTP on brain development are well documented in rats, and evidence from hamsters suggests that these neural changes may not be limited to rats (Burleson et al., 2016). In rats, juvenile RTP changes the architecture of the pyramidal neurons found in the mPFC (e.g., Chapter 6, Bell et al., 2009; Himmler, Pellis, & Kolb, 2013; Stark et al., 2023), as well as the electrophysiology (Bijlsma et al., 2022, 2023, 2024),

and biochemistry of these cells (Baarendse et al., 2013; Hermes et al., 2011; van Kerkhof et al., 2013). While the mechanism by which these changes are achieved remains unclear, emerging evidence suggests that specific aspects of RTP may be critical for promoting the development of mPFC and socio-cognitive skills (Pellis et al., 2019; Pellis, Pellis, Ham, et al., 2023).

One facet of play that appears especially important for the development of the mPFC and socio-cognitive skills is the ability to inject cooperative elements into the inherently competitive framework of RTP (Pellis et al., 2019). Taking turns during play often requires rats to voluntarily place themselves at a disadvantage (Foroud & Pellis, 2002, 2003), temporarily relinquishing control. In doing so, rats may be inadvertently training themselves to navigate uncertain and unpredictable social situations (Špinka et al., 2001). This skill may be reflected in their ability to interact effectively with unfamiliar conspecifics, as assessed by the stranger test.

In both Chapters 6 and 7, rats that engaged in fewer role reversals during juvenile RTP exhibited poor social skills as adults, often escalating playful encounters into overt physical aggression. The atypical morphology of their mPFC neurons suggests possible deficits in emotional regulation and inter-animal coordination (Pellis & Bell, 2020). Supporting this, rats with bilateral mPFC lesions have difficulty coordinating social actions with partners (Bell et al., 2009; B. T. Himmler et al., 2014). These findings suggest a strong link between the social competence and the function of the mPFC.

Although ELA reduced role reversals and play frequency among juveniles, these changes were only observed when the rats were paired with unfamiliar individuals. When tested with their cage mates, ELA rats displayed typical RTP frequencies and similar rates

of role reversals compared to controls. From a developmental perspective, the RTP that occurs with cage mates is likely more consequential then the play measured during acute tests with unfamiliar individuals, as rats engage in RTP within their home cage throughout the day (Thiels et al., 1990). Therefore, the RTP observed with cage mates is likely representative of their broader juvenile play experience. This is supported by the absence of neuron size differences between ELA and control rats, suggesting that their overall play exposure was similar.

Interestingly, ELA rats had fewer dendritic spines than controls, opposite to the pattern typically observed in rats experiencing RTP deprivation (Table 9.1). This further supports the notion that role reversals, rather than RTP frequency alone, are key drivers of pyramidal cell pruning in the mPFC. Moreover, it suggests that ELA affects brain development through mechanisms distinct from those of impoverished play experiences.

**Table 9.1** Summary of the results on role reversals, neuron size, and number of spines from Chapters 6, 7, and 8. F344s reared with LEs and incompetent rats engaged less turn taking than control rats and had larger neurons, while no difference was seen between ELA and control rats in either role reversals or neuron size. The number of spines was greater in incompetent rats compared to competent rats, while ELA rats had less spines compared to control rats.

| Chapter | Role reversals | Neuron size | Number of spines |
|---|---|---|---|
| Ch. 6: F344-LE | ↓ | ↑ | ? |
| Ch. 7: Incompetent | ↓ | ↑ | ↑ |
| Ch. 8: ELA | = | = | ↓ |

Though not directly examined here, the group-rearing and testing paradigm offers a way to examine whether having more connections and play partners during development benefits the brain. Not all rats are equally popular in group settings (Chapter 4), so diversity in social interactions may differentially shape brain outcomes. For example, rats reared with multiple partners develop larger dendritic arbors in the orbitofrontal cortex (OFC) than those reared in pairs (Bell et al., 2010). Damage to the OFC impairs the ability to tailor social responses to the identity of a partner; rats with OFC lesions treat dominant and subordinate partners similarly (Pellis et al., 2006). It is plausible that interacting with a variety of partners during development trains the OFC to adaptively regulate social behavior based on partner identity.

Although the structure of the neurons found within the OFC remains plastic into early adulthood, changing with transitions between group and pair housing (B. T. Himmler et al., 2018) and with exposure to novel partners (Hamilton et al., 2020), it remains unknown whether more social experiences with diverse partners in the juvenile period produce lasting enhancements in social cognition. Perhaps diverse social interactions during the juvenile period primes increased flexibility in the OFC. This may help explain why rats often play with all available partners in a group, even those that are suboptimal playmates (Chapter 4). This is something I plan to investigate in the future.

### 9.2 Why study group RTP?

Given the limited research on rats playing in groups (e.g., Holman et al., 2019; Kahana et al., 1997; Meaney & Stewart, 1981), I wish to briefly discuss a few of the advantages and drawbacks of studying group play, as well as highlight a few unanswered questions that emerged from my work.

### 9.2.1 Advantages

Studying RTP in groups offers several advantages. First, it more closely mirrors the natural social ecology of rats (Calhoun, 1963; Schweinfurth, 2020). Group housing allows for the emergence of complex social hierarchies, networks, and interactions to form between individuals. It also introduces naturalistic competition for resources, such as food, allogrooming partners, and preferred huddle locations, making the setting more ethologically valid. As such, results obtained from group play paradigms are likely more generalizable to real-world social dynamics of rats.

Moreover, for translational purposes, group housing and testing better simulates the complex and often unpredictable social interactions that humans must navigate across development. As demonstrated in Chapter 8, group play settings also provide a powerful tool for identifying social deficits. Because social cues in rats can be subtle, observing who an individual chooses to interact with, or maybe more importantly, who they avoid, can provide insights into social preferences and/or impairments. This paradigm may prove useful for evaluating rodent models of autism, mood disorders, and other developmental conditions.

### 9.2.2 Draw backs

Despite these strengths, studying RTP in group contexts presents several challenges. First among them is the time-intensive nature of behavioral scoring. In groups of six (as in Chapter 4), each individual rat must be scored independently (Pellis, Pellis, Burke, et al., 2022), meaning a single play session must be analyzed six times. In contrast, a dyadic session requires only two rounds of scoring. Additionally, group studies require a

significantly larger number of animals. For example, if six rats are needed per group and at least eight unique groups are required, the experiment demands a minimum of 48 animals, compared to only 16 in a similar dyadic study.

Tracking individuals in larger groups also becomes increasingly difficult, especially when rats lack distinct markings. Determining which individual initiated or received a play attack often requires frame-by-frame review and frequent rewinding to verify identities. This makes microstructural analysis of play laborious and technically demanding.

It is nearly impossible to record ultrasonic vocalizations during group play as the individuals emitting the calls cannot be disentangled without complex triangulation. Indeed, this is a major downside as the rats could potentially be selecting partners that are the best communicators or those that use vocalizations to signal a mutual preference for one another (C. J. Burke et al., 2018, 2020; Kisko, Euston, et al., 2015). This loss of valuable social information is a major limitation.

### 9.2.3 What we still don't know

A key question that emerged from this work concerns how motivation to play interacts with partner preference strength. Are some individuals inherently more social and therefore less selective in their interactions? This issue arose repeatedly during peer review of Chapters 2–5, particularly in relation to the use of brief social isolation periods to increase play motivation—a standard procedure when testing play (B. T. Himmler, Pellis, & Pellis, 2013; Pellis, Pellis, Burke, et al., 2022). We were repeatedly asked: does the time spent in social isolation reduce partner preferences because the rats are more motivated to play?

To test this, I conducted an experiment involving eight trios of juvenile male rats, each unfamiliar with one another. Each group included two LE rats and one F344 rat. As we found that LE rats prefer same-strain partners when playing in groups of four (Chapter 5), I hypothesized that increasing RTP motivation through social isolation (0 h, 2.5 h, 12 h, or 24 h) would weaken this preference for same strain partners. Instead, however, I found that while increasing the time spent in social isolation led to more nape attacks (a proxy for play motivation), partner preference remained consistent across conditions (Figure 9.1). Interestingly, preference strength was generally low, regardless of isolation time, and was not always for a same-strain partner.

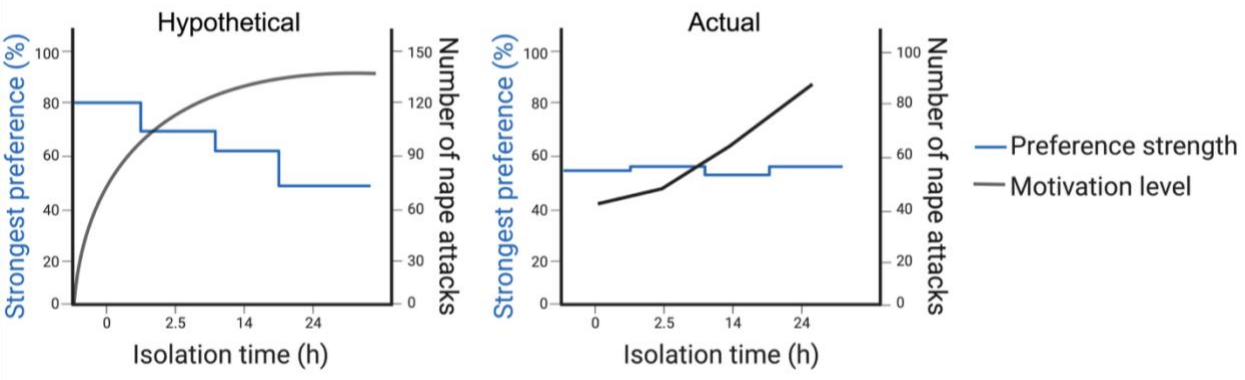


**Figure 9.1** Hypothetical data (left) and actual data (right) are presented comparing the effect of isolation time on the preference strength for the preferred partner and the number of nape attacks. It was predicted that as motivation to play increased, preference for partners would decrease. I found instead that while motivation to play does increase, preference strength remained stable.

This raises another methodological consideration: how many rats should be included in a group to effectively detect partner preferences? Based on my findings, the optimal group size depends on the research question. To detect individual developmental differences, six rats per cage may be necessary as (typically) only one individual per group will show deficits (Chapter 7). For strain comparison studies (e.g., Chapter 5), groups of four appear ideal. In trios, one rat is often excluded when the other two are playing. The 'rat left out' then tries to fight its way back in to the playing dyad and in doing so potentially disrupts the natural play dynamics and confounding results. This is because once attacked, rats are inclined to engage in counter attacks or role reversals. That is, a LE might prefer an LE partner but plays with the F344 because it was attacked by that partner. Groups of four reduce this issue by allowing two dyads to be playing simultaneously, minimizing social exclusion and enhancing the expression of partner preferences.

However, this is not to say that testing trios does not have its advantages. Chapter 8 demonstrated that ELA rats showed a preference for playing with other isolated rats when tested in trios. By selecting one individual as the focal rat, trios allow you to ask the very simple question: if you have a choice between Rat A and Rat B, do you play with one more than the other? This greatly limits the complex social relations and hierarchies that emerge in groups of six. Further exemplifying the advantages of trio testing, preliminary data from mixed-sex groups has revealed sex differences in play frequency and partner preferences. When male Wistar rats were tested in male + male + female trios, males consistently preferred to play with the female over the other male in the group (Figure 9.2a). However, when the roles were reversed (female + female + male), some females preferred to play with females while others preferred males (Figure 9.2b). Interestingly, females played more

when a male was present compared to all-female groups (Figure 9.2c). Together, these results suggest that partner preferences are expressed by both sexes and are sensitive to social context and stress that depending on the question, trios can be suitable.

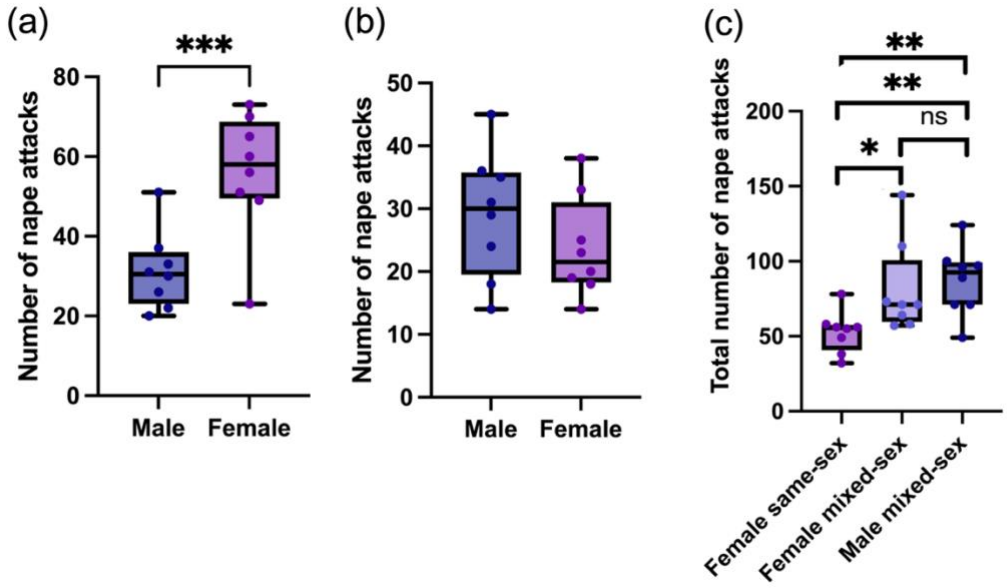


**Figure 9.2** Wistar male rats playing in trios preferred to play with females over males (a). In contrast, when in mixed-sex groups, female rats did not prefer either sex (b). Females did, however, play more when in mixed sex-groups compared to when they were in same-sex groups, playing just as much as males suggesting that sex differences in RTP may be contingent on social groupings (c).

## 9.3 Translational value

Although it is a leap to go from rats to children, the findings presented in Chapter 7 suggest that some rats, and potentially some animals, including humans, despite ample play opportunities, do not engage in the type of play necessary to derive developmental benefits. As discussed above, the key deficit appears to be in turn-taking, a social skill also implicated in the development of behavioral flexibility in children (Pellegrini, 1988). This raises the question: can play experiences be tailored to improve outcomes for these individuals?

If some rats fail to benefit simply because of incompatible group composition, then social structuring could be optimized to enhance outcomes. However, if they fail to benefit regardless of group structure, then earlier developmental interventions may be needed. This has important implications for human children, particularly those who are neurodivergent. While inclusive education policies promote integration of neurodivergent children with neurotypical peers, the data remain unclear on whether these environments benefit neurodivergent children, and whether all neurodivergent children benefit equally (Cologon, 2022). Observational work has shown that some children fail to engage in peer play during recess (Blurton Jones, 1976), much like the socially incompetent rats described in Chapter 7. The mere availability of the opportunity for social interaction might not guarantee that such interactions take place and even if they did, that they would lead to the desired developmental gains. Indeed, social groups might need to be engineered so as to maximize the quality of social interactions. The use of "integrated play groups" to tailor social experiences for children on the autism spectrum provides such an example (Wolfberg et al., 2012).

Children with autism, while motivated to play, often do so atypically with social overtures that are unique to themselves (Jordan, 2003; Wolfberg et al., 2012). Children with autism often appear aloof to peers as they avoid or withdraw from playful interactions, and their attempts to solicit interaction is often too ambiguous for potential play partners to recognize and respond appropriately (Wolfberg et al., 2012). This leads to difficulty in soliciting, coordinating, and sustaining play with peers (Schuler, 2003; Sigman & Ruskin, 1999). However, with aid, these children can learn social cues and tactics that allow them to meld into the group.

This requires coaching and guidance within social groups specifically tailored to provide mutually engaging play experiences. In such groups, known as "integrated play groups," children with autism are partnered with more capable peers—typically neurotypical children or siblings who demonstrate competent social abilities and have an interest in participating—under the supervision of an experienced adult coach (Wolfberg et al., 2012). In these settings children with developmental delays show improvements in the quality of their social interactions (Wolfberg et al., 2012). Moreover, children with autism who participate in integrated play groups being begin to refine their social skills and contextualize their idiosyncratic behavior in ways that are more understandable to peers (Wolfberg & Schuler, 2006). This suggests that when social groups are intentionally structured to maximize positive social interactions, children with developmental delays benefit.

## 9.4 Conclusion

This thesis aimed to deepen our understanding of juvenile RTP in rats by examining how individual variation and group dynamics shape developmental outcomes. By

developing and applying more naturalistic methods to study play in group settings, several key insights emerged.

First, rats exhibit partner preferences, however, these preferences vary depending on the social context in which the rats find themselves. This suggests that individual recognition and social selection significant roles in shaping peer interactions. Second, not all rats benefit equally from engaging in play. Some appear incapable of participating in the nuanced, reciprocal interactions required for developmental gains, raising questions about the underlying causes and potential for remediation.

Together, these findings emphasize the importance of social context. Who an animal plays with, and under what conditions, can fundamentally alter the developmental trajectory. Group play paradigms, while challenging, provide a valuable lens through which to investigate individual differences in social behavior and their neural and behavioral consequences. As such, group play represents not only a richer, more ecologically valid approach to studying social development but also a powerful translational model for understanding variability in social functioning across species.

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## APPENDIX A: CHAPTER 4 SUPPLEMENTARY MATERIAL

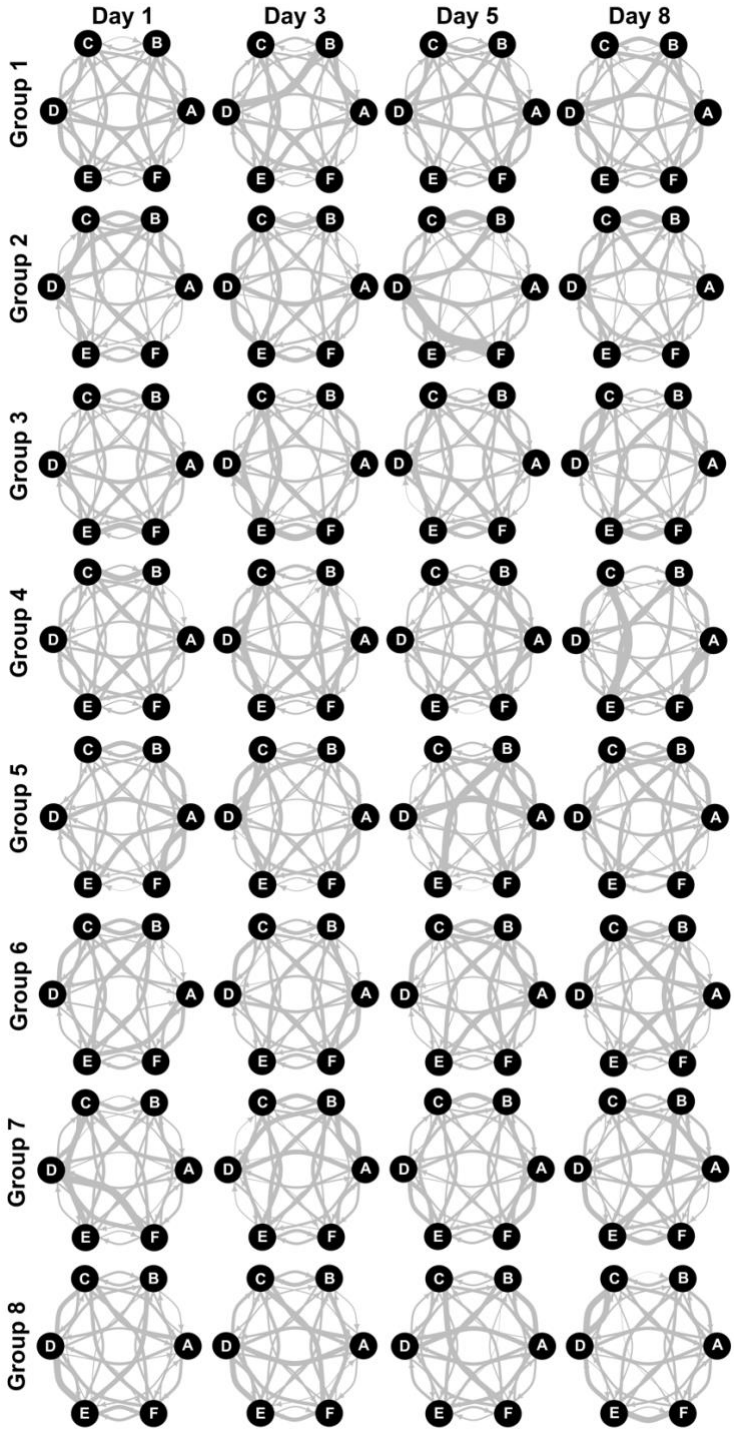


**Figure A.1** Directed social networks for each group on all four days. The circles or nodes represents the individual rats in the groups. The lines or edges connecting the nodes illustrates the proportion of play that individual directed towards the rats in the group.

**Table A.1** Results from the tube test, for each group. For every possible pair in the group, five trials were conducted. The maximum number of trials a rat could have been 25 and would indicate dominance over all individuals while a 0 would indicate complete subordinance. Rows represent wins while columns represent losses.

**Group one**

| Rat | A | B | C | D | E | F | Wins |
|---|---|---|---|---|---|---|---|
| A | X | 0 | 4 | 3 | 1 | 1 | 9 |
| B | 3 | X | 2 | 3 | 4 | 1 | 13 |
| C | 1 | 2 | X | 3 | 2 | 0 | 8 |
| D | 2 | 1 | 2 | X | 5 | 0 | 10 |
| E | 3 | 0 | 2 | 0 | X | 1 | 6 |
| F | 3 | 4 | 4 | 5 | 4 | X | 20 |

**Group two**

| Rat | A | B | C | D | E | F | Wins |
|---|---|---|---|---|---|---|---|
| A | X | 3 | 2 | 0 | 0 | 0 | 5 |
| B | 2 | X | 0 | 0 | 0 | 0 | 2 |
| C | 3 | 5 | X | 1 | 1 | 0 | 10 |
| D | 5 | 5 | 4 | X | 5 | 0 | 19 |
| E | 5 | 5 | 4 | 0 | X | 5 | 19 |
| F | 4 | 5 | 5 | 5 | 0 | X | 19 |

**Group three**

| Rat | A | B | C | D | E | F | Wins |
|---|---|---|---|---|---|---|---|
| A | X | 3 | 4 | 5 | 3 | 2 | 17 |
| B | 2 | X | 2 | 5 | 3 | 5 | 17 |
| C | 1 | 3 | X | 2 | 1 | 1 | 8 |
| D | 0 | 0 | 2 | X | 4 | 2 | 8 |
| E | 2 | 2 | 4 | 0 | X | 2 | 10 |
| F | 3 | 0 | 3 | 3 | 2 |  | 11 |

**Group four**

| Rat | A | B | C | D | E | F | Wins |
|---|---|---|---|---|---|---|---|
| A | X | 1 | 2 | 0 | 0 | 0 | 3 |
| B | 4 | X | 5 | 1 | 2 | 5 | 17 |
| C | 2 | 0 | X | 0 | 1 | 3 | 6 |
| D | 5 | 4 | 5 | X | 1 | 3 | 18 |
| E | 5 | 3 | 4 | 4 | X | 5 | 21 |
| F | 5 | 0 | 2 | 2 | 0 | X | 9 |

**Group five**

| Rat | A | B | C | D | E | F | Wins |
|---|---|---|---|---|---|---|---|
| A | X | 5 | 5 | 2 | 2 | 4 | 18 |
| B | 0 | X | 4 | 2 | 2 | 3 | 11 |
| C | 0 | 1 | X | 0 | 1 | 3 | 5 |
| D | 3 | 3 | 5 | X | 4 | 5 | 20 |
| E | 3 | 3 | 4 | 1 | X | 3 | 14 |
| F | 1 | 2 | 1 | 0 | 2 | X | 6 |

**Group six**

| Rat | A | B | C | D | E | F | Wins |
|---|---|---|---|---|---|---|---|
| A | X | 0 | 2 | 5 | 4 | 5 | 16 |
| B | 5 | X | 4 | 5 | 5 | 5 | 24 |
| C | 3 | 1 | X | 5 | 3 | 4 | 16 |
| D | 0 | 0 | 0 | X | 0 | 1 | 1 |
| E | 1 | 0 | 2 | 5 | X | 2 | 10 |
| F | 0 | 0 | 1 | 3 | 3 | X | 7 |

**Group seven**

| Rat | A | B | C | D | E | F | Wins |
|---|---|---|---|---|---|---|---|
| A | X | 1 | 1 | 2 | 4 | 0 | 8 |
| B | 4 | X | 5 | 5 | 5 | 3 | 22 |
| C | 4 | 0 | X | 2 | 5 | 2 | 13 |
| D | 3 | 0 | 3 | X | 5 | 2 | 13 |
| E | 1 | 0 | 0 | 0 | X | 1 | 2 |
| F | 5 | 2 | 3 | 3 | 4 | X | 17 |

**Group eight**

| Rat | A | B | C | D | E | F | Wins |
|---|---|---|---|---|---|---|---|
| A | X | 4 | 0 | 5 | 4 | 4 | 17 |
| B | 1 | X | 0 | 1 | 2 | 3 | 7 |
| C | 5 | 5 | X | 4 | 3 | 4 | 21 |
| D | 0 | 4 | 1 | X | 3 | 4 | 12 |
| E | 1 | 3 | 2 | 2 | X | 2 | 10 |
| F | 1 | 2 | 1 | 1 | 3 | X | 8 |

## APPENDIX B: CHAPTER 7 SUPPLEMENTARY VIDEO

Link to video: https://youtu.be/Eh4gf1Z5g50

## APPENDIX C: CHAPTER 8 SUPPLEMENTARY MATERIAL

**Table C.1** Play measures for the pair, when tested with a same sex and condition, unfamiliar partner. How the rats defended themselves, when they defend themselves, is compared. Behavioral measures are provided as a percentage (% ± SD) and in bold when significant. Unlike the behavioral measures, the play symmetry scores range from 0-1, with 1 being perfectly symmetrical (mean ± SD).

| | Females | | Males | | Two-way ANOVA | | | |
|---|---|---|---|---|---|---|---|---|
| Behavior | Control | Isolated | Control | Isolated | Condition | Sex | Interaction | Effect |
| % Defense | 53.68 ± 10.62 | 38.74 ± 13.80 | 63.86 ± 5.23 | 65.28 ± 9.20 | $F(1,25) = 0.144$, $p = .708$ | $F(1,25) = 2.197$, $p = .151$ | $F(1,25) = 0.265$, $p = .611$ | |
| % Evasion | 44.08 ± 8.20 | 32.34 ± 13.99 | 43.89 ± 4.66 | 41.18 ± 11.86 | $F(1,25) = 0.215$, $p = .647$ | $F(1,25) = 0.036$, $p = .851$ | $F(1,25) = 0.043$, $p = .838$ | |
| % Pin | 26.32 ± 8.36 | 27.52 ± 12.52 | 39.92 ± 5.72 | 40.22 ± 11.30 | $F(1,25) = 0.072$, $p = .791$ | $F(1,25) = 1.219$, $p = .280$ | $F(1,25) = 0.057$, $p = .814$ | |
| % Mutual upright | 17.10 ± 6.99 | 2.64 ± 1.73 | 16.18 ± 4.22 | 18.60 ± 13.71 | $F(1,25) = 0.533$, $p = .472$ | $F(1,25) = 0.842$, $p = .368$ | $F(1,25) = 1.066$, $p = .312$ | |
| Symmetry in role reversals | 0.83 ± 0.06 | 0.25 ± 0.16 | 0.90 ± 0.03 | 0.55 ± 0.15 | **$F(1,25) = 13.51$, $p = .0011$** | $F(1,25) = 1.859$, $p = .185$ | $F(1,25) = 1.066$, $p = .441$ | **Isolated♀< control♀+♂** |

**Table C.2** Play measures for the pair, when tested with a same sex but opposite condition, unfamiliar partner. How the rats defended themselves, when they defend themselves, is compared. Behavioral measures are provided as a percentage (% ± SD) and in bold when significant. Unlike the behavioral measures, the play symmetry scores range from 0-1, with 1 being perfectly symmetrical (mean ± SD).

| | Females | | Males | | Two-way ANOVA | | | |
|---|---|---|---|---|---|---|---|---|
| Behavior | Control | Isolated | Control | Isolated | Condition | Sex | Interaction | Effect |
| % Defense | 41.89 ± 4.92 | 34.13 ± 6.14 | 38.74 ± 13.80 | 39.73 ± 6.68 | $F(1,52) = 0.449$, $p = .506$ | $F(1,52) = 0.097$, $p = .756$ | $F(1,52 = 0.384$, $p = .538$ | |
| % Evasion | 34.70 ± 7.28 | 42.25 ± 5.99 | 45.92 ± 6.72 | 24.12 ± 7.16 | $F(1,52) = 4.652$, $p = .036$ | $F(1,52) = 0.258$, $p = .614$ | $F(1,52) = 1.097$, $p = .300$ | ns after correction |
| % Pin | 51.04 ± 8.33 | 51.20 ± 5.48 | 46.30 ± 5.31 | 73.45 ± 7.15 | **$F(1,52) = 4.162$, $p = .046$** | $F(1,52) = 1.708$, $p = .197$ | **$F(1,52) = 4.083$, $p = .049$** | **control♂ <isolated♂** |
| % Mutual upright | 6.54 ± 1.88 | 7.08 ± 4.75 | 7.77 ± 2.98 | 2.44 ± 1.34 | $F(1,52) = 0.627$, $p = .432$ | $F(1,52) = 0.318$, $p = .575$ | $F(1,52) = 0.940$, $p = .337$ | |

**Table C.3** Play measures for the pair, when tested with a same sex, same condition, familiar partner (cage mate). How the rats defended themselves, when they defend themselves, is compared. Behavioral measures are provided as a percentage (% ± SD) and bolded when significant. Unlike the behavioral measures, the play symmetry scores range from 0-1, with 1 being perfectly symmetrical (mean ± SD).

| | Females | | Males | | Two-way ANOVA | | | |
|---|---|---|---|---|---|---|---|---|
| Behavior | Control | Isolated | Control | Isolated | Condition | Sex | Interaction | Effect |
| % Defense | 63.73 ± 10.51 | 75.54 ± 5.18 | 58.20 ± 13.31 | 68.30 ± 7.31 | $F(1,27) = 0.118$, $p = .734$ | $F(1,27) = 0.579$, $p = .454$ | $F(1,27) = 0.016$, $p = .901$ | |
| % Evasion | 22.39 ± 6.65 | 15.88 ± 6.29 | 35.58 ± 12.58 | 38.82 ± 8.51 | $F(1,27) = 0.131$, $p = .720$ | $F(1,27) = 2.639$, $p = .116$ | $F(1,27) = 0.020$, $p = .889$ | |
| % Pin | 44.71 ± 9.58 | 40.27 ± 12.53 | 42.04 ± 11.20 | 48.57 ± 9.30 | $F(1,27) = 0.343$, $p = .563$ | $F(1,27) = 2.615$, $p = .118$ | $F(1,27) = 0.269$, $p = .608$ | |
| % Mutual upright | 10.67 ± 3.83 | 6.35 ± 3.35 | 9.88 ± 3.35 | 12.61 ± 2.60 | $F(1,27) = 0.209$, $p = .651$ | $F(1,27) = 0.090$, $p = .766$ | $F(1,27) = 0.724$, $p = .402$ | |
| Symmetry in role reversals | 0.46 ± 0.15 | 0.39 ± 0.15 | 0.41 ± 0.16 | 0.66 ± 0.15 | $F(1,27) = 2.028$, $p = .166$ | $F(1,27) = 2.344$, $p = .137$ | $F(1,27) = 0.616$, $p = .439$ | |

**Table C.4** Average measurements (± standard error of the mean) of pyramidal neurons found in Cg3, layer III, of rats.

| Measurement | Female control | Female isolated | Male control | Male isolated |
|---|---|---|---|---|
| Convex hull volume ($cm^3$) | 918.52 ± 60.99 | 994.64 ± 57.38 | 905.74 ± 58.89 | 972.09 ± 73.31 |
| Apical convex hull volume ($cm^3$) | 321.89 ± 50.01 | 301.64 ± 32.91 | 276.91 ± 27.16 | 346.46 ± 45.94 |
| Basilar convex hull volume ($cm^3$) | 127.28 ± 12.87 | 137.17 ± 13.22 | 160.58 ± 28.55 | 140.52 ± 20.44 |
| Convex hull surface area ($cm^2$) | 30.24 ± 1.36 | 33.15 ± 1.40 | 30.23 ± 1.43 | 32.78 ± 1.74 |
| Apical convex hull surface area ($cm^2$) | 14.70 ± 1.27 | 15.05 ± 1.10 | 13.83 ± 0.90 | 16.32 ± 1.44 |
| Basilar convex hull surface area ($cm^2$) | 13.52 ± 0.98 | 13.86 ± 0.91 | 18.57 ± 4.00 | 13.55 ± 1.22 |
| Total length (mm) | 5.13 ± 0.20 | 5.21 ± 0.17 | 5.33 ± 0.21 | 5.37 ± 0.23 |
| Apical length (mm) | 1.69 ± 0.11 | 1.55 ± 0.10 | 1.64 ± 0.10 | 1.76 ± 0.12 |
| Basilar length (mm) | 2.50 ± 0.12 | 2.64 ± 0.12 | 2.66 ± 0.16 | 2.57 ± 0.16 |
| Cell body length (mm) | 0.94 ± 0.05 | 1.03 ± 0.04 | 1.03 ± 0.04 | 1.04 ± 0.04 |
| Total volume ($mm^3$) | 11.18 ± 0.70 | 11.74 ± 0.52 | 12.87 ± 0.68 | 12.04 ± 0.67 |
| Apical volume ($mm^3$) | 2.84 ± 0.22 | 2.83 ± 0.21 | 3.00 ± 0.23 | 3.12 ± 0.25 |
| Basilar volume ($mm^3$) | 2.33 ± 0.15 | 2.17 ± 0.14 | 2.70 ± 0.23 | 2.29 ± 0.20 |
| Cell body volume ($mm^3$) | 6.00 ± 0.51 | 6.74 ± 0.34 | 7.18 ± 0.43 | 6.66 ± 0.41 |
| Total branch number | 27.88 ± 1.14 | 27.42 ± 1.03 | 29.73 ± 1.42 | 28.46 ± 1.43 |
| Apical branch number | 11.43 ± 0.81 | 10.54 ± 0.65 | 11.27 ± 0.77 | 11.79 ± 0.84 |
| Basilar branch number | 16.44 ± 0.90 | 16.88 ± 0.89 | 18.46 ± 1.13 | 16.67 ± 1.11 |
| Apical distal spine density (#/40µm) | 21.47 ± 1.40 | 10.84 ± 1.01 | 20.38 ± 1.34 | 13.81 ± 0.80 |
| Apical proximal spine density (#/20µm) | 0.48 ± 0.12 | 0.08 ± 0.06 | 0.44 ± 0.18 | 0.14 ± 0.06 |
| Basilar distal spine density (#/40µm) | 27.49 ± 1.79 | 10.12 ± 0.79 | 21.51 ± 13.84 | 13.84 ± 1.23 |
| Basilar proximal spine density (#/20µm) | 1.28 ± 0.28 | 0.36 ± 0.09 | 2.14 ± 0.38 | 0.86 ± 0.21 |

**Table C.5** Average measurements (± standard error of the mean) of stellate-like neurons found in dLS.

| Measurement | Female control | Female isolated | Male control | Male isolated |
|---|---|---|---|---|
| Convex hull volume ($cm^3$) | 81.75 ± 11.48 | 113.14 ± 17.63 | 75.55 ± 12.28 | 89.79 ± 16.70 |
| Convex hull surface area ($cm^2$) | 5.46 ± 0.55 | 6.22 ± 0.68 | 4.87 ± 0.56 | 5.55 ± 0.62 |
| Total length (mm) | 1.94 ± 0.13 | 2.45 ± 0.15 | 1.81 ± 0.14 | 2.18 ± 0.14 |
| Dendrite length (mm) | 0.79 ± 0.07 | 0.99 ± 0.10 | 0.75 ± 0.08 | 0.85 ± 0.09 |
| Cell body length (mm) | 1.15 ± 0.10 | 1.47 ± 0.09 | 1.06 ± 0.09 | 1.33 ± 0.09 |
| Total volume ($mm^3$) | 10.84 ± 1.28 | 13.82 ± 1.09 | 8.85 ± 0.98 | 12.23 ± 1.09 |
| Dendrite volume ($mm^3$) | 2.51 ± 0.31 | 3.07 ± 0.34 | 1.55 ± 0.20 | 2.59 ± 0.37 |
| Cell body volume ($mm^3$) | 8.32 ± 1.13 | 10.76 ± 0.91 | 7.30 ± 0.84 | 9.63 ± 0.86 |
| Branch number | 5.22 ± 0.70 | 7.79 ± 0.84 | 4.83 ± 0.65 | 5.65 ± 0.74 |

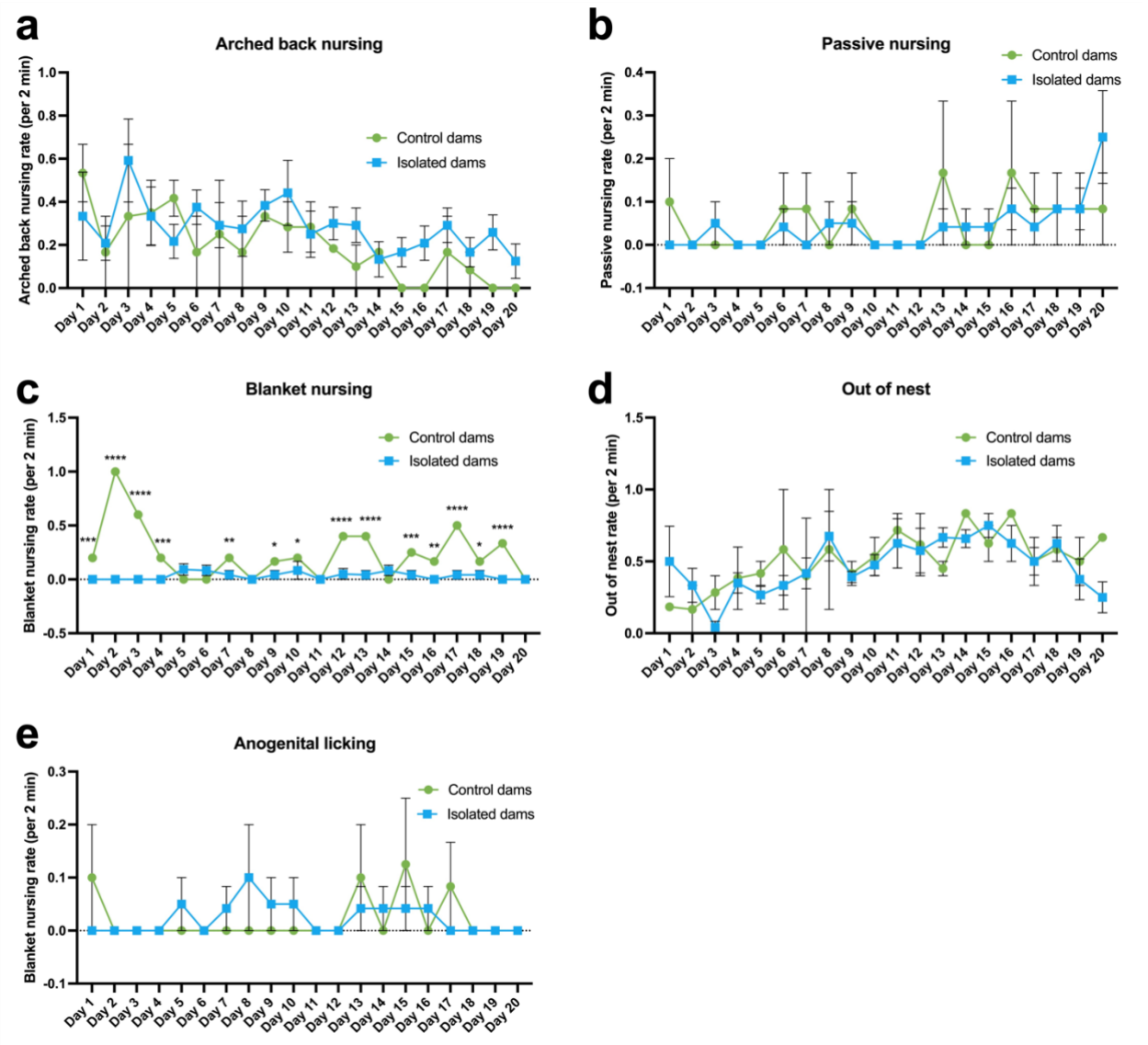


**Figure C.1 Maternal care measures.** Arched back nursing did not vary significantly between conditions (control $n$ =2; isolated $n$ = 4) ($F(1,4) = 2.487$, $p = 0.190$), day ($F(19,76) = 1.603$, $p = 0.077$), or condition × day interaction ($F(19,76) = 0.524$, $p = 0.943$) (a). Passive nursing did not vary significantly between conditions ($F(1,4) = 0.797$, $p = 0.652$), day ($F(19,76) = 1.359$, $p = 0.174$), or condition × day interaction ($F(19,76) = 0.628$, $p = 0.874$) (b). Blanket nursing did vary significantly between conditions ($F(1,4) = 1073.00$, $p < 0.0001$), day ($F(19,76) = 18.63$, $p < 0.0001$), or condition × day interaction ($F(19,76) = 21.71$, $p < 0.0001$) (c). Time spent out of the nest did not vary significantly between conditions ($F(1,4) = 1.175$, $p = 0.339$), or condition × day interaction ($F(19,76) = 0.665$, $p = 0.842$), but did by day ($F(19,76) = 2.197$, $p = 0.0085$) with dams of isolated pups spending more time out of the nest on day 15 compared to day 3 ($p = 0.0257$) (d). Anogenital licking of pups did not vary significantly between conditions ($F(1,4) = 0.007$, $p = 0.936$), day ($F(19,76) = 0.861$, $p = 0.630$), or condition × day interaction ($F(19,76) = 0.860$, $p = 0.631$) (e).

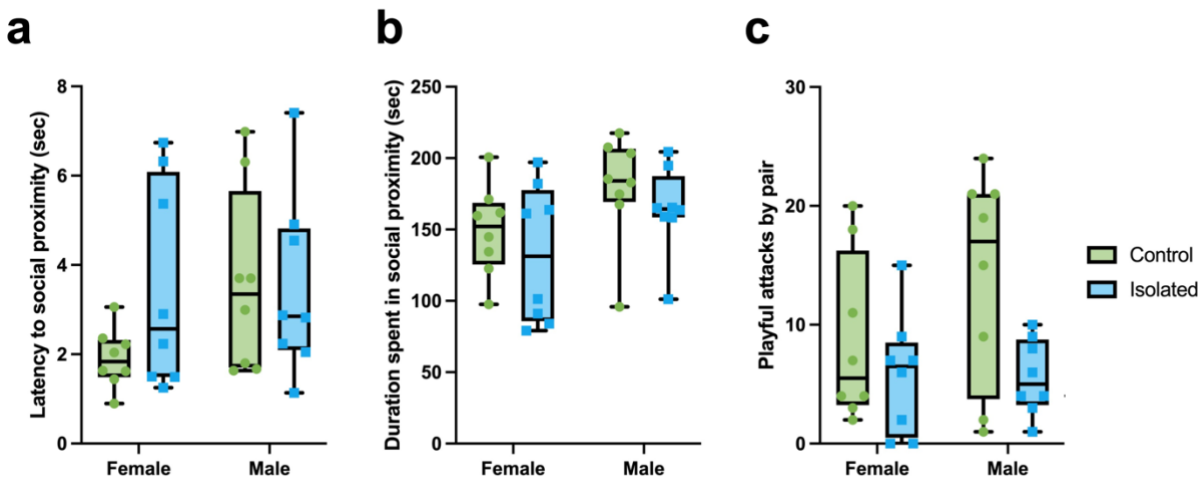


**Figure C.2 Social interaction test.** Results from the adolescent social interaction test did not differ with latency to be within social proximity (a), the duration spent in social proximity (b), and the number of playful attacks not differing among pairs of same-condition and -sex, unfamiliar partners (c). Eight pairs were tested for each condition × sex.