WHAT ARE THEY GOOD FOR? ULTRASONIC VOCALIZATIONS AS SOCIAL COMMUNICATION SIGNALS IN RATS

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DEDICATION

To my daughter Madeline Rose, if science has taught me anything, it is to stay curious and question everything. I love you always.

ABSTRACT

Rats emit ultrasonic vocalizations in a variety of appetitive and aversive contexts. Although the reason why these calls are emitted is yet unknown, two prominent theories exist, the affective state theory and the social communication theory. This thesis will provide evidence to support the theory that these calls are emitted as a form of social communication. Four experiments will be presented, each testing a facet of the social communication theory. These experiments provide evidence that the calls are linked to social stimuli over non-social reward, and are emitted in divergent patterns depending on strain, regardless of depressive state. Further, they demonstrate that not all appetitive rewards elicit the same calling response, and that the calls emitted have a direct impact on conspecific behaviour. Overall, it is concluded that, although some vocalizations may be an indicator of the rat's affective state, they are also used as a form of social communication.

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TABLE OF CONTENTS

Dedication…… iii

Abstract…… iv

Acknowledgments…… v

List of Tables…… xii

List of Figures……..… xiii

List of Abbreviations…… xv

Chapter 1: Introduction of the Usage of Ultrasonic Vocalizations.…… 1

1.1. Background Information…… 1

1.2. History of Ultrasonic Vocalization Research…… 1

1.3. Call Categorization…… 2

1.3.1. Vocal Production in Rats…… 2

1.3.2. Ultrasonic Vocalization Frequency Distinction…… 3

1.3.3. Categorizing 50-kHz USVs…… 4

1.4. Theories of Ultrasonic Vocalization Function.…… 4

1.4.1. Mechanical by-product Theory…… 5

1.4.2. Affective State Theory.…… 6

1.4.2.1. 22-kHz USVs…… 6

1.4.2.2. 50-kHz Ultrasonic Vocalizations.…… 6

1.4.3. Social Communication Theory…… 7

1.4.3.1. Anticipation of Play…… 8

1.4.3.2. Devocalization Studies…… 8

1.4.3.3. Adult Aggression …… 8

1.4.3.4. Juvenile Conspecific Play 9

1.5. Summary: Thesis Objectives 10

Chapter 2: Are 50-kHz USVs emitted in the same context for social and non-social rewards?..... 12

2.1. Abstract..... 12

2.2. Introduction..... 12

2.3. Methods..... 15

2.3.1. Subjects..... 15

2.3.2. Behavioral Procedures..... 15

2.3.3. Anticipation of Play Test..... 17

2.3.4. Food Restriction..... 18

2.3.5. Anticipation of Food Test 18

2.3.6. Ethics 18

2.3.7. Behavioral Analyses 19

2.3.8. Vocalizations Analyses..... 20

2.3.9. Statistical Analyses 21

2.4. Results..... 22

2.4.1. Vocalizations..... 22

2.4.1.1. Vocalization Counts 22

2.4.1.2. Vocalization Analyzed by Category 23

2.4.2. Behavior 28

2.4.3. Vocal-Behavioral Associations 30

2.5. Discussion 33

2.6. Conclusions…… 40

2.7. Acknowledgments …… 40

Chapter 3: Are 50-kHz social anticipatory USV different in a depressed phenotype? 41

3.1. Abstract …… 41

3.2. Introduction …… 41

3.3. Methods …… 44

3.3.1. Subjects and experimental procedures …… 44

3.3.2. Behavioral analyses …… 46

3.3.2.1. Play behavior …… 46

3.3.2.2. Anticipation of play …… 46

3.3.3. Vocalizations analyses …… 48

3.3.4. Correlation of particular calls with particular behaviors …… 48

3.3.5. Statistical analyses …… 49

3.4. Results …… 50

3.4.1. Play behavior …… 50

3.4.2. Anticipation of play …… 51

3.4.2.1. Activity levels …… 51

3.4.2.2. Frequency of vocalizations …… 53

3.4.2.3. Frequency of types of calls emitted …… 55

3.4.2.4. Behavior-vocalization associations …… 56

3.5. Discussion …… 56

3.6. Conclusion …… 61

3.7. Acknowledgments …… 62

Chapter 4: Are 50-kHz USVs the same in both tickling and social play? 63
4.1. Abstract 63
4.2. Introduction 63
4.3. Materials and Methods 67
4.3.1. Subjects 67
4.3.2. Experimental procedures 67
4.3.2.1. Conspecific play procedure 67
4.3.2.2. Tickling procedure 68
4.3.3. Behavioral analyses 69
4.3.3.1. Conspecific play 69
4.3.3.2. Heterospecific play 70
4.3.4. Vocalization analyses 72
4.3.5. Statistical analysis 72
4.3.5.1. Vocalization counts 72
4.3.5.2. Vocal-behavioral correlates 73
4.3.6. Data Accessibility 74
4.4. Results 74
4.4.1. Vocalization counts 74
4.4.2. Vocalization types 75
4.4.3. Vocal-behavioral correlates 76
4.5. Discussion 77
4.6. Acknowledgments 82
Chapter 5: Do 50-kHz USVs impact the behaviour of a conspecific? 83

5.1 Abstract 83
5.2. Introduction 83
5.3. USVs as play signals 85
5.4. What calls are associated with what behavior? 87
5.5. Communicating play via USVs 92
5.6. The functions of signaling during play 95
5.7. Conclusion 98
5.8. Acknowledgments 99
6. Discussion 100
6.1. General Discussion 100
6.1.1. Summary of Thesis Objectives 100
6.1.2. Comparisons Across Experiments 101
6.1.2.1. Calling is not random 101
6.1.2.2. Context of calls 102
6.1.2.3. Trill calls and social context 103
6.1.3. Evaluating the Affective State Theory 104
6.1.3.1. Evidence from social and non-social rewards 104
6.1.3.2. Evidence from depressive phenotypes 105
6.1.3.3. Evidence from heterospecific and conspecific play 105
6.1.3.4. Evidence from the behavioural effects of USVs 106
6.1.4. Neural Implications 106
6.1.5. Caveats 108
6.2. Conclusions 109

6.3. References …… 111

LIST OF TABLES

Table 2.1. Description of the anticipatory behaviors that were scored. 21
Table 3.1. Description of the behavior scored during the anticipatory period, adapted from Burke et al., 2017 50
Table 3.2. Attack behavior during play trials is compared across the two strains on day 7 of testing (presented as the mean and standard error of the mean for each variable). 53
Table 4.1. Description of conspecific play behaviours scored adapted from [1]. 72
Table 4.2. Description of the heterospecific play ("tickling") behaviours scored 74
Table 5.1. Description of the social behaviors scored (reprinted from Burke et al 2018). 92

LIST OF FIGURES

Figure 2.1. The average rate of 50-kHz vocalizations produced on day 1 and 7 of anticipatory testing. All error bars are standard error of the mean. 25

Figure 2.2. The average proportion of all commonly used call categories for each experimental group (Play Reward, Play Control, Food Reward, Food Control) on day 1 and 7 of testing are shown. Numbers show percentage of all calls for that group and testing day. 27

Figure 2.3.Comparison of the increase/decrease in call rates from day 1 to day 7 for each category of commonly produced calls. (**A**) Comparison of the change in call rates for Play Reward and Play Control groups. Asterisks denote comparisons that were statistically significant ($p < 0.05$). (**B**) Comparison of the change in call rates for Food Reward and Food Control groups. 28

Figure 2.4. Comparison of the rates of calls on day 1 for all food and play groups. Play includes both Play Reward and Play Control while food includes both Food Reward and Food Control. Asterisks denote comparisons that were statistically significant ($p < 0.05$). 30

Figure 2.5. The average proportion of time spent in each of several key behaviors on day 1 and day 7 for each experimental group. Upper left shows the proportion of the 2 min test period spend in slow locomotion (single step, turn or walk). Upper right shows proportion of time spent in fast locomotion (running or jumping). Lower left shows time spent in exploratory behaviors. Lower right shows time spent rearing on hind legs. All error bars are standard error of the mean. 31

Figure 2.6. Association between types of calls and types of behavior are shown for the four groups on day 1 and 7 of the anticipation trials. Each matrix shows the strength of association, as a z-score, for each combination of behavior (x-axis) and vocalization category (y-axis). Deep red indicates the strongest positive association and deep blue the strongest negative association. The white sections indicate that either the behavior or the vocalization in that category did not have sufficient instances to run the analysis. 34

Figure 3.1. The duration (in seconds) spent active during the two minute anticipation trial is compared between strains and between days 1 and 7. The average duration of performing all active behaviors is combined and is shown for juveniles (A) and adults (B). The WI are shown in red and the WKY in blue. The average duration of walking (solid lines) and the duration of vigorous activities (dashed lines) is shown for juveniles (C) and adults (D). The data are expressed as means and the standard error of the mean. 55

Figure 3.2. The number of calls emitted during the two-minute anticipation trial is compared between the strains on days 1 and 7 in the juvenile (A) and adult (B) period. The WI are shown in red and the WKY in blue. The data are expressed as means and the standard error of the mean. 56

Figure 3.3. The percentage of use of the different vocalization categories is shown for the two strains as juveniles and adults on day 7 of the anticipation trials. 57

Figure 3.4. Association between types of calls and types of behavior are shown for the two strains at both ages on day 7 of the anticipation trials. Each matrix shows the strength of association, as a z-score, for each combination of behavior (x-axis) 58

and vocalization category (y-axis). Deep red indicates the strongest association and deep blue the weakest. The white sections indicate that either the behavior or the vocalization in that category did not have enough instances to run the analysis.
Figure 4.1. The different types of USV emitted are compared between heterospecific and conspecific play as percentages (mean ± SD). ***p < .001. 78
Figure 4.2. The matrix shows call types on one axis and the types of behaviour on the other, with z scores showing the strength of the associations between particular calls and particular behaviours, spanning from deep red for the strongest positive association, green for no association and deep blue for the strongest negative association. A z-value of 1.96 gives a p-value of < 0.05 and a z-value of 2.58 gives a p-value of < 0.01. Because some of the behaviours scored in the two play contexts were different, some of the behaviours in panel A and panel B differ. The description of the relevant behaviours scored can be found in Tables 4.1 and 4.2. 79
Figure 5.1. Examples are shown of the different types of ultrasonic calls (derived from Wright et al., 2010) that are used in the present study (reprinted with permission from Burke et al. (2017b)). 90
Figure 5.2. Visual representation of the Monte Carlo shuffling technique used to identify significant associations between behaviors and vocalizations. 93
Figure 5.3. The matrix shows call types on one axis and the types of behavior on the other, with z-scores showing the strength of the associations between particular calls and particular behaviors, spanning from deep red for the strongest and deep blue for the weakest. This matrix shows the associations between calls and behaviors by actors (A) and recipients (B) (see text). The data are derived from pairs in which one partner was devocalized (reprinted with permission from Burke et al., 2018). 94
Figure 5.4. The matrix shows the vocalizations emitted by one partner and the actions performed by the recipient across four time bins: the time when the call is being emitted (call emission), from the end of call emission to 300 ms after the call, from 301-600 ms after the call and finally, from 601-900 ms after the call. The data are derived from pairs in which one partner was devocalized. M.U denotes mutual uprights and sniff m denotes mutual sniffing (i.e, the pair sniffs each other) 96
Figure 5.5. A simplified representation illustrates the temporal changes in the associations between calls by one animal and the behavior of the recipient, as represented in the matrices shown in Figure 4. Note that for this figure, a more stringent cut-off value of $z \geq 2.58$ was used. 98

LIST OF ABBREVIATIONS

AMPH	Amphetamine
CCBN	Canadian Centre for Behavioral Neuroscience
FM	Frequency modulated
Hz	Hertz
kHz	Kilohertz
M	Mean
NSERC	Natural Science and Research Council
S.D	Standard Deviation
USVs	Ultrasonic vocalizations
WI	Wistar Rat
WKY	Wistar Kyoto Rat
VTA	Ventral Tegmental Area

Chapter 1: Introduction of the Usage of Ultrasonic Vocalizations

1.1. Background Information

Ultrasonic vocalizations (USVs) in rats have been studied for over 60 years; however, it is still unknown why rats make these calls. Historically, it was thought that USVs were used for echolocation or thermoregulation. However, more recently, three theories have emerged to explain the occurrence of USVs: movement by-product theory, affective state theory and social communication theory. This thesis will focus on contrasting the affective state and social communication theories. Ultimately, this thesis will provide evidence in support of the social communication theory through the evidence from four experiments. The experiments contrast USV production in anticipation of food and play, anticipation of play in a depressed strain of rats, contrasting being tickled by a human with social play between peers, and how USVs alter the behaviour of conspecifics. Each of the experiments provide a piece of evidence indicating that USVs are used for more than the simple expression of affective state, but rather, for social communicative purposes.

1.2. History of Ultrasonic Vocalization Research

In 1954, the first paper was published indicating that laboratory rats could produce ultrasonic calls along with the known audible sounds they could make [2]. This early study noted that the USVs included both frequency modulated and flat calls, which ranged across frequencies from 20 – 80 kHz. At the time, bats were the primary animal known to make ultrasounds, thus it was theorized that, like bats, the calls were used for

echolocation [2]. An experiment in 1955 showing that blinded rats were able to traverse an elevated plus maze to obtain a food reward was considered to support this echolocation function. Nevertheless, ultrasonic calls were incredibly rare during the task, and the researchers concluded that it was the audible calls that were more likely to be used for echolocation [3]. Because neither claim could be substantiated, the field moved on to an alternate theory of USV production, that of homoeothermic regulation.

In 1956, Zippelius and Schleidt discovered that mice and vole infants emitted ultrasounds when they were removed from the nest and hypothesized that they made these calls because they were either cold or hungry [4]. It was found that these calls, which are typically emitted at a frequency of 40-kHz, were produced when the pups experienced a drop in body temperature. This led to the proposition that these calls are a by-product of the rat's homoeothermic regulation [5]. Furthermore, evidence emerged that these calls may have a communicative function because they are emitted when pups are separated from their mothers [6]. When these 40-kHz calls were played back to dams, they retrieve their pups [7]. Although a tight link exists between breathing, warmth, and the production of USVs, the homoeothermic regulation theory cannot account for all ultrasounds produced by the rat pups [8]. Also, although 40-kHz calls may be unique to pups, they emit calls across a wide range of frequencies, 5–120 kHz, but with most calls concentrated around two frequency ranges, 35–40 kHz and 50–70 kHz [9-11]. This thesis will focus on the calls produced by juvenile and adult rats that occur in the 5 – 120 kHz range.

1.3. Call Categorization

1.3.1. Vocal Production in Rats

Rats produce both audible and ultrasonic calls. The audible calls are produced by vibrations of the vocal folds, resulting in squeals of 2-4 kHz [12]. These calls are generally emitted when the animal is experiencing, or anticipating physical pain or discomfort [13]. Wild rats have been shown to produce audible calls when confronted with a threatening stimulus, such as an approaching human. The calls are associated with baring of the teeth and jump attacks toward the threat [14]. Rats may also direct audible calls at humans when anticipating or while experiencing rough handling [15]. In contrast to audible calls, to emit USVs, rats stabilize the larynx and whistle through a small opening in the vocal cords [16], which are tightly constricted and so cannot vibrate [17]. The air column passing through the small opening is like that of a whistle, except on a reduced scale, creating the ultrasonic calls.

1.3.2. Ultrasonic Vocalization Frequency Distinction

USVs are emitted in a number of contexts, both social and non-social. The calls are categorized as belonging to two distinct classes: 22-kHz calls are emitted in negative contexts and are thus often referred to as alarm calls, 50-kHz that are emitted in positive contexts [15]. The 22-kHz calls are long duration calls lasting 100 - 3000ms with a peak frequency of 20-23 kHz. Frequency modulation is almost absent in the 22-kHz calls, so they appear on a sonogram as flat lines that occur in bursts of 2-7 consecutive calls [18, 19]. In contrast, 50-kHz calls are brief, with an average duration of 30-40ms, with a peak frequency of 45-55 kHz, although the peak frequency can reach 70 kHz [15]. Another distinguishing feature of 50-kHz calls is that many involve the frequency modulation. Because 50-kHz calls have such a variable profile, a variety of distinct calls can be recognized, and this has led to several different ways to categorize these calls.

1.3.3. Categorizing 50-kHz USVs

The first distinction among 50-kHz calls is between those that have a flat profile and those that are frequency modulated (FM) [20, 21]. The flat 50-kHz calls have been hypothesized to play a social coordinating function, as they are used in socially ambivalent or aggressive situations [22, 23]. In contrast, the FM calls are linked to positive contexts, and are even thought to be a type of laughter [24].

The next level of distinction is between the different types of FM calls. Based on distinctive profiles on spectrographs of the calls, several categories have been recognized. First, the most distinctive, non-overlapping calls are treated as unique and so scored separately, these are calls with steps, trills, step trill combination calls. The remaining calls have a more complicated structure and may contain elements unique to the call. They are grouped into a miscellaneous category, giving a total of four call categories [25]. Second, the 'miscellaneous' category is resolved by identifying key structural features that distinguish unique calls within the category. This approach has yielded 14 types of 50-kHz calls (see figure 5.1) [26]. There is little agreement in the field about categorization [27]. Further research will need to be done to understand which calls have biological relevance to the rats.

1.4. Theories of Ultrasonic Vocalization Function

There are three theories relating to the function of ultrasonic vocalizations: the movement-by-product theory, affective state theory and social communication theory. The movement-by-product theory posits that USVs are created simply as a by-product of thoracic pressure as the animals move, and thus have no communicatory value [28]. The affective state theory hypothesizes that USVs are used by the animals to express either a

positive or negative affective state, depending on the frequency of the call emitted [29]. The social communication theory posits that USVs are used beyond the expression of affective state, with at least some of the calls being used as signals to communicate with conspecifics [1, 22, 30-32]. The following section will focus on evaluating evidence related to the various theories, with a focus on contrasting the affective state theory with the social communication theory.

1.4.1. Mechanical by-product Theory

There are two different mechanical by-product theories. The first posits that adult 22-kHz USVs are a developmental remnant of infant 40-kHz USVs developed as a part of an "abdominal compression reaction" mechanism that increases an infant's blood pressure during periods of maternal isolation [33]. The second posits that 50-kHz USVs are produced by thoracic compression during periods of vigorous activity [28].

The first hypothesis fails to explain why the 22-kHz calls are only emitted in conjunction with specific behavioural contexts (generally aversive), nor why that they can be produced whether stationary or moving [34]. Several lines of evidence also argue against the second hypothesis. First, although sniffing and walking are associated with increased respiration, rats can move and sniff without vocalizing [35]. In fact, it is suggested that vocalizations require the active contraction of the larynx muscles during the exhalation [36]. Second, rats in which the laryngeal nerves have been transected do not vocalize even though they actively move about [37]. Third, it has also been demonstrated that vocalizations often occur at the onset of locomotion, not when the animal is stopping, making thoracic compression an unlikely mechanism [38]. Finally, the presence of another rat can influence the rate of vocalizing independently of the rate

of movement [38]. Altogether, it seems unlikely that the movement-by-product theory accounts for the majority of the USVs produced, although it cannot be completely excluded from contributing to some USVs.

1.4.2. Affective State Theory

The second theory to explain the function of USVs is the affective state theory. This theory is based on the finding that the main two categories of USVs, 50-kHz and 22-kHz, represent two affective states, positive and negative, respectively. There is a wide breadth of literature in support of this theory.

1.4.2.1. 22-kHz Ultrasonic Vocalizations

The 22-kHz calls are elicited in both social and non-social contexts, and in anticipation of such contexts. Socially, 22-kHz calls are emitted during aversive interactions such as fighting or confrontations with dominant conspecifics and, in the latter, are primarily emitted by the subordinate rat [39-41]. Male rats will also emit 22-kHz calls at the end of copulation, or if the female is resisting the sexual interaction [42, 43]. Non-socially, 22-kHz calls are emitted in contexts that are unpleasant, such as when exposed to the odour of a predator, or when confronted with an anxiety inducing stimulus, such as a startling sounds or puffs of air [14, 44, 45]. They are also emitted in anticipation of foot shock, and during drug withdrawal [46, 47]. Clearly, these calls are emitted in negative contexts, and 22-kHz calls do appear to fit well with the affective state theory.

1.4.2.2. 50-kHz Ultrasonic Vocalizations

The 50-kHz calls, which will be the focus of this thesis, are also elicited in both social and non-social contexts. Anticipation of non-social stimuli, such as food and drugs like amphetamine (AMPH) and morphine, induce the emission of 50-kHz calls [48, 49 1997, 50]. Anticipation of social rewards, such as males gaining access to a sexually receptive female, also causes rats to elicit 50-kHz USVs [51-53]. Socially rewarding interactions, such as rough and tumble play, sexual encounters or being tickled by a human, also cause rats to elicit 50-kHz USVs [15, 51, 54, 55].

1.4.3. Social Communication Theory

Much of the evidence that supports the social communication theory, also fits with the affective state theory, thus, these theories need not be mutually exclusive, perhaps, some calls are used to communicate affective state while others are used for social communication. This section will provide an overview of the current evidence that supports a social communicative function for USVs.

The first pieces of evidence that indicated communication derived from studies demonstrating a link between 50-kHz USVs and social stimuli. During mating, male 50-kHz USVs cause females to respond with darting and ear wiggling, toward the male caller [56]. Playback studies show that juvenile rats in a radial arm will approach 50-kHz USVs over pure tones of the same frequency. In adulthood, rats will show the same preference for the 50-kHz USVs [57]. Indeed, it was first hypothesized that 50-kHz, specifically, flat 50-kHz calls, played a social coordinating function, when it was shown that 50-kHz flats were emitted when rats were separated from their cage mates [22]. This phenomenon was then explored by measuring vocalizations while rats anticipated a social partner.

1.4.3.1. Anticipation of Play

When rats are separated by a screen, they emit more 50-kHz USVs after play reward, compared to before. Further, the rats also vocalized more when in a chamber associated with play, compared to a neutral chamber [53]. My Masters project explored anticipation of play over seven days and found that USVs increase over the seven days of training, indicating the increase in USV is likely due to the upcoming arrival of a partner. This project also showed that specific sub-types of rat 50-kHz vocalizations are strongly associated with specific behaviors during play anticipation. More specifically, USVs occurred with active behaviours, such as running, walking and jumping, while less active behaviours were negatively associated with USVs [30]. Thus, these calls are not random and there appears to be a link between the calls emitted and an upcoming social partner, indicating a potential communicative function. Nevertheless, we cannot exclude that the upcoming positive reward does not simply increase the overall positive state of the rat.

1.4.3.2. Devocalization Studies

Vocalizations are not required for play as deafening [58] or devocalizing [37, 52] juvenile rats does not prevent play. Although not required for play, devocalization diminishes the frequency of play and cooperative actions that sustain play [37]. Furthermore, intact rats paired with devocalized rats produce calls at twice the baseline rate of pairs when both rats can vocalize while the devocalized initiates double the attacks [52]. This evidence suggests that USVs facilitate playfulness [59 & Pellis, 2017].

1.4.3.3. Adult Aggression

The risk of aggression among unfamiliar adult male rats is elevated when one of the pair has been devocalized [37]. This indicates that USVs could play a role in

coordinating and de-escalating these aggressive encounters. A follow up study indicated that lower frequency (20–30 kHz) calls with a flat component are critical for de-escalating encounters [31]. Further, it appears that during these aggressive encounters, coordinating USVs emitted in a complementary fashion (e.g., nape attacks, being attacked on the nape) pose an additional way to coordinate and de-escalate these interactions.

1.4.3.4. Juvenile Conspecific Play

As stated above, 50-kHz USVs are frequently emitted during rough and tumble play, however, all types of 50-kHz calls are produced during play [54]. There is a temporal association between calling and play behaviour, with most calls being uttered immediately before contact [52, 54]. Further, specific calls have been linked to specific defensive tactics during play, all leading to social communication as a potential explanation for this phenomenon. Analysis from my Master's project revealed that there were strong associations between types of calls and types of social contact. Further, different types of USVs were associated with different play behaviours, with most calls uttered by the initiator of the action, not the recipient. Finally, the vocal-behavioural associations appeared to have a complementary function, with attacker and defender having complementary calls [1]. In summary, the calls are used specifically during play, have a temporal relationship with behaviour, and are emitted in a complementary fashion, all which support a form of social communication.

1.5 . Summary: Thesis Objectives

Theory. The social communication theory of rat vocalization proposes that rat vocalizations are used for communication in social situations.

Species that display extensive vocal behaviour, including primate and many bird species, mainly do so in social situations [60-64]. Vocalizations are used in courtship, for communicating information about food resources, to alert conspecifics about danger, and to sustain social organization. The evidence summarized above in the main supports the social communication theory of rat vocalization. It follows that, for the rat, vocalization serves similar purpose. It is this theory that will be tested in this thesis with the following four experiments.

Experiment 1. Are 50-kHz USVs emitted in the same context for social and non-social rewards?

To examine whether 50-kHz USVs emitted in the same context for social and non-social rewards, I will look at ultrasonic calls in juvenile male rats during the expectation of either play or food. Behaviour and vocalizations will be recorded while rats are in a test chamber awaiting the arrival of a play partner or food over seven days of testing. Control groups will be included for the non-specific effects of food deprivation and social isolation. If the different rewards cause different vocal profiles this would indicate that 50-kHz calls are used for more than expression of affect.

Experiment 2. Are 50-kHz social anticipatory USV different in a depressed phenotype?

To test whether 50-kHz social anticipatory USV different in a depressed phenotype, I will use anticipation of social reward (i.e., a play partner) to assess behavioural and vocal differences between the Wistar-Kyoto (WKY) and normal Wistar (WI) rats in the juvenile period. The WKY rat was developed as a control for the

spontaneous hypertensive rat but has subsequently also been used as a genetic animal model of depression due to its hyper-responsiveness to stress. I would expect that a depressed phenotype of rat would simply have a reduction of calls as their general affect should be reduced. However, if a different call pattern emerges, this could imply USV usage beyond affective state.

Experiment 3. Are 50-kHz USVs the same in both tickling and social play?

The 50-kHz USVs emitted during social play are thought to be an expression of a positive affective state ('laughter'), which in some situations may also function as communication signals. Heterospecific play - 'tickling' by an experimenter - is thought to simulate conspecific play. Given that tickling evokes substantial amounts of USV, I will investigate whether heterospecific play is simulating conspecific play by comparing USV behaviour associations in both contexts. If the 50-kHz calls are merely an expression of 'laughter', then the pattern and type of emission in both contexts should be similar. In contrast, as playing with a conspecific involves a two-way exchange of signalling, the additional demands on communication should lead to a different pattern of calling.

Experiment 4. Do 50-kHz USVs impact the behaviour of a conspecific?

Communication during play fighting can be crucial in facilitating and sustaining contact. As juvenile rats play mostly in the dark, visual signals are unlikely to fulfill this function. However, during play, rats have a variety of ultrasonic calls that are emitted, and there is growing evidence to support the idea that these USVs are used to communicate. It is established that particular calls are associated with particular actions, and in this experiment, we will explore how the impact of USVs emitted by one rat changes the probability of the actions taken by another.

Chapter 2: Are 50-kHz USVs emitted in the same context for social and non-social rewards? *

* Burke, C. J., Markovina, M., Pellis, S. M., & Euston, D. R. (2021). Rat 50-kHz trill calls are tied to the expectation of social interaction. *Brain Sciences, 11*(9), 1142. https://doi.org/10.3390/brainsci11091142

2.1. Abstract

Rats emit a variety of calls in the 40–80 kHz range (50-kHz calls). While these calls are generally associated with positive affect, it is unclear whether certain calls might be used selectively in certain contexts. To examine this, we looked at ultrasonic calls in 30–40 day old male rats during the expectation of either play or food, both of which are reinforcing. Behaviour and vocalizations were recorded while rats were in a test chamber awaiting the arrival of a play partner or food over seven days of testing. Control groups were included for the non-specific effects of food deprivation and social isolation. Play reward led to an increase in 50-kHz vocalizations, generally, with specific increases in trill and "trill with jump" calls not seen in other groups. Expectation of food reward did not lead to a significant increase in vocalizations of any type, perhaps due to the young age of our study group. Further, rats that were food deprived for the food expectation study showed markedly lower calls overall and had a different profile of call types compared to rats that were socially isolated. Taken together, the results suggest that trill-associated calls may be used selectively when rats are socially isolated and/or expecting a social encounter.

2.2. Introduction

A predominate theory about the purpose of USVs in rats is that these calls signal the affective state of the animal [25, 29]. Two main categories of calls have been

described: 50-kHz calls associated with appetitive situations and positive affect and 22-kHz calls associated with threatening situations and negative affect [25]. While 22-kHz calls are mainly long and flat, 50-kHz calls come in a variety of shapes, including trills, ramps and jumps [26]. Whether the different types of 50-kHz calls have different functional roles is a topic of active research [1, 26]

Vocalizations of the 50-kHz type are strongly associated with non-social rewarding stimuli. There is a significant increase in 50-kHz vocalizations emitted when rats are placed in a chamber in which they have received amphetamine (AMPH) [48, 49]. Interestingly, the amount of AMPH administered has a direct relationship with the amount of 50-kHz USVs produced [65]. Anticipation of self-administration of electrical stimulation to brain reward centers, such as the ventral tegmental area (VTA) and lateral hypothalamic area, also elicits high rates of 50-kHz calls [50]. The animal in that study showed a marked increase in 50-kHz USVs to cues associated with the electrical stimulation as well as to the stimulation itself. Finally, 50-kHz calls have also been associated with cues indicating food reward [50, 66, 67] or during anticipation of daily feeding [68]. These findings suggest that 50-kHz USVs signal positive affective states associated with rewarding contexts, independent of social context.

Fifty kHz USVs are also emitted during, and in anticipation of, a variety of rewarding social interactions. Significant increases in 50-kHz calls have been found in males during the anticipatory period before introduction of a female [51]. During copulation, both male and female rats produce 50-kHz vocalizations [23, 69]. Interestingly, the number of 50-kHz vocalizations appears to relate to the level of sexual motivation in the respective vocalizing party [51, 70]. Juvenile male rats will also emit 50-kHz vocalizations when anticipating the presence of a conspecific, and these

vocalizations will increase over days of testing in rats that are socially isolated before testing [30, 53]. At least one study has failed to find anticipatory calling in juveniles, but that study used only limited social isolation [71]. Rats will also emit these vocalizations when entering an area frequently visited by other rats [72].

One social context that is known to be particularly rewarding and associated with high numbers of 50-kHz vocalizations is rough and tumble play in juvenile rats. The calls are most common before contact is made [54]. Further, these calls have also been elicited by rats tickled by human handlers and are more common in isolated than socially housed animals, possibly reflecting the greater value of this hetero-species contact when other social interactions are lacking [23, 55]. Rats will also produce 50-kHz vocalizations when introduced to an immobilized and, therefore, easily approachable conspecific and when being introduced to a conspecific after separation [73].

In summary, the 50-kHz USVs are emitted during acquisition and anticipation of non- social and social rewards and elicit a response from conspecifics. To complicate matters, these calls are also elicited during negative social contexts such as during aggression and when a resident initially meets an intruder [15, 37]. Rats also emit 50-kHz calls when a companion is taken away [21]. One explanation for the variety of usage is the 14 potential categories of calls existing in the 50-kHz range [74]. Indeed, the specific calls have been linked to anticipation of play behaviours [30], to mitigate aggression [31], signal play [1], feeding [75] and social contact signaling [22]. Thus, rather than signaling a general positive state, different 50-kHz calls may serve different functional roles.

In this study, we sought to contrast anticipatory calling in juvenile rats to both social and non-social stimuli using play and food, respectively. Two recent studies have attempted similar comparisons. Willey et al. [76] compared vocalizations in male rats to

the presence of either food or a female rat on the other side of a wire mesh barrier. The social stimulus elicited far more vocalizations than the food reward. Similarly, Mulvihill and Brudzynski [77] compared vocalizations in males to food reward and to exploration of space recently vacated by an estrous female. The estrous female elicited an increase in 50-kHz calls, especially trill calls, whereas the food reward did not cause an overall change in vocalization rate, but rats did produce more flat calls in the 50-kHz range. This latter finding is consistent with previous reports that feeding is associated with flat calls in the 40 kHz range [75]. These studies show clearly that social stimuli elicit more calls than food reward, but a detailed comparison of calls during *anticipation* of both food and social reward has not yet been reported.

To investigate if anticipation of different types of reward elicited different patterns of calling, we compared the vocalizations of food restricted animals anticipating food to socially isolated animals anticipating play. To ensure that the vocalizations were not due to the restrictions or to the chamber, we had control animals, who were either socially isolated or food restricted, run in the same paradigm as the test subjects but without food or play reward. If a particular 50-kHz call communicates positive affect, we would expect to see elevated rates of this particular call type during anticipation of both food and play. Trill calls are a likely candidate, given their frequency and strong association with drug reward [74]. Any differences in call types or usage, on the other hand, would indicate that 50-kHz vocalizations are more nuanced, signaling specific features of the anticipated reward.

2.3. Methods

2.3.1. Subjects

Thirty juvenile male Long Evans aged 30–40 days obtained from Charles River (Kingston, NY, USA) at 22 days old, were used. These animals were pair housed and given five days to acclimatize to the facility. Eighteen animals were used in the anticipation of play paradigm, 6 in the Play Reward group, who received a play partner after a two minute waiting period, 6 in the Play Control group, who similarly waited for a partner that never came, and 6 as play partners for the Play Reward group. The remaining 12 animals were used for the anticipation of food paradigm, 6 in the Food Reward group, which received food in the test chamber after a two minute waiting period, and 6 in the Food Control group who did not receive food. All animals were maintained on the Lab Diet Enriched Rat Chow (Lab Diet, St. Louis, MO, USA). Housing rooms were lit during the day and dark at night and all testing occurred during the day.

2.3.2. Behavioural Procedure

The testing enclosure was a Plexiglas box (50 × 50 × 50 cm), which was situated inside a soundproof chamber (61 × 61 × 83 cm) lined with acoustic foam. The floor of the chamber was covered with 2 cm of paper-based bedding (Care Fresh, Ferndale, WA, USA) which we found to facilitate play while causing very low levels of ultrasonic interference. Ultrasonic vocalizations were collected using a specialized microphone (Model 4939, Brüel & Kjaer, Denmark) with a frequency response of 4 Hz to 100 kHz. The microphone was located in the ceiling of the chamber and was approximately 15 cm above the center of the play enclosure. The microphone was connected to a Soundconnect™ amplifier (Listen, Inc., Boston, MA, USA) and sound waves were recorded at 195,313 Hz using 16-bit resolution via a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, FL, USA). Video was recorded using a USB

webcam (Microsoft Lifecam Studio, Redmond, WA, USA) with its infrared filter removed, positioned directly above the animal

2.3.3. Anticipation of Play Test

Data presented were taken from a 2 min anticipation period during which a target animal either waited in the testing enclosure for the arrival of a familiar play partner (i.e., his former cage mate) or received no partner. For the Play Reward group, once the play partner was introduced, animals were allowed to play for 10 min, following previously established methods [78]. After testing, rats were returned to their original home cages for an additional hour of play and then separated. The Play Control animals, who received no partner, waited in the chamber for 10 min, and then were placed back in their home cage. One hour later, these animals were introduced to their former cage mate for 1 h and 12 min of play before separation. Prior to testing, animals were individually habituated to the enclosure for 10 min each day for 3 consecutive days. On the 3rd day all subjects were socially isolated from their cage mates for 24 h prior to play testing and isolation continued until after all 7 days of testing were complete, in order to increase overall playfulness [79-81]. Both habituation and testing sessions were conducted in complete darkness, as this has been shown to facilitate USV production [53]. Audio and video recordings began after the target rat was placed in the test enclosure. Because both audio and video data were recorded on separate devices, a custom-made beeper with an LED light was used to emit a simultaneous light/sound cue at the beginning and end of each recording session and these times were used to synchronize audio and video recordings during subsequent analysis. Following each session, the apparatus was thoroughly cleaned with Virkon, a broad-spectrum disinfectant (Virkon Disinfectant Technologies, Sudbury, United Kingdom), and bedding was replaced to avoid any odors

from other subjects. The data analyzed comes from day 1 and day 7 in all animals apart from one animal in the Play group who was not separated from his cage mate after testing on day 6. For this one animal, we use data from day 6 instead.

2.3.4. Food Restriction

In order to food restrict animals at such a young age, we used the play animals as weight controls. Each food-restricted animal was matched based on weight to a play animal when handling started. The target weight was calculated based on that of the play animal. The food restriction animals were restricted to maintain 85% of the weight of play controls. The animals were separated for three hours to eat the appropriate amount of food and then were placed back in with their cage mate, with any food remaining in their isolated chambers being placed in with both the animals.

2.3.5. Anticipation of Food Test

The Food Reward group consisted of 6 animals who anticipated food reward in the chamber for 2 min and subsequently received half a semi-sweet chocolate chip each 30 s for 10 min. The chocolate chips were dropped by the experimenter from the top of the sound chamber. The animals were then brought back to their individual feeding cages, which had their allocated food, and then were given 3 h to eat before being return to their shared cages. The remaining 6 animals, the Food Control group, were placed in the chamber for 12 min while the experimenter remained in the room; however, no food was given. These animals were similarly isolated and given 3 h to eat before being return to their shared cages. One hour into this period, the Food Control rats were given 10 chocolate chips so as to equate both the quantity and type of food eaten each day between the Food Reward and Food Control groups.

2.3.6. Ethics

All procedures were in accordance with the University of Lethbridge institutional animal care and use committee and Canadian Council on Animal Care recommendations and guidelines.

2.3.7. Behavioural Analyses

The 2 min anticipatory period was analyzed in each group. The behaviours were coded using recorded video sequences and were evaluated at normal speed, slow motion and frame-by-frame to manually code these behaviours [37]. To capture the range of possible actions, behaviour patterns associated with anticipation were scored (Table 2.1).

Both the type of behavior and duration of that particular behavior were scored manually. Importantly, we assigned a behavioral category at every video frame, so that no time was left unaccounted for. This meant that the video frame of the termination of each behavior was the beginning of the next behavior. Frame-by-frame analysis of video was performed using Avidmux software, and the behaviors scored are shown in Table 2.1.

Table 2.1. Description of the anticipatory behaviors that were scored.

Behaviour	**Description**
Step	Removal of at least two paws from the ground in an alternating manner
Walk	Removal of all four limbs off the ground in an alternating manner (left paw and right hind limb move simultaneously followed by right paw and left hind limb) OR significant shift from one location to another (if all limbs are not visible)
Run	Only two limbs touch the ground at any given time; the rat may alternate two limbs at a time (as is seen during walking behavior) OR the rat may move two paws followed by two hind limbs at any given time; such movement is accompanied by the extension of the torso as the front limbs reach forward followed by flexion of the torso as the hind limbs are removed from the ground and placed under the body
Jump	Up jump: the two front limbs leave the ground followed by the hind limbs while body is lifted into the air, then all limbs touch the ground simultaneously or closely one after the other. Forward jump: the two front limbs are extended forward and removed from the ground followed by the removal of the hind limbs from the ground; this behavior is accompanied

	by the extension of the torso as the front limbs reach forward followed by flexion of the torso as the hind limbs are removed from the ground
Turn	Turn with one or both front limbs at a 45-, 90- or 180-degree angle OR turn with three or more limbs at a 360-degree angle. Turning may also be preceded by a stepping or walking pattern or followed by a rear (see below for the operational definition of rearing behavior)
Explore	Immobile; may extend one front limb; turning of head so as to examine the surrounding area
Dig	Vigorous forward and backward motion of front limbs while significantly displacing bedding
Rear	Standing on rear limbs with both front paws off ground (either free standing or against wall)
Shake	Vigorous side-to-side shudder of head, neck and trunk
Groom	Licking of paws; wipes/rubs face and nose; wipes behind ears, neck and/or downward to either side of the body; may grab fur and nibble with teeth. Grooming may consist of a variation of these behaviors many consecutive times. However, grooming is typically initiated by wiping of the nose or face and followed by grooming of the neck and body
Scratch	Rapid movement of hind limb with the claws rubbing against head, neck or side
Rest	Immobile; may turn head, but significantly less than is seen during exploration

2.3.8. Vocalization Analyses

Acoustic data were analyzed using Raven Pro 1.4 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY, USA). The Raven Pro software generated spectrograms with a 256-sample Hann window from which the experimenter manually selected 50-kHz vocalizations. The 14 different 50-kHz vocalizations characterized by Wright et al. [26] were scored as distinct calls as we have done previously [31]. The occurrence of these calls was used to compare rates of calling, types of calling and whether different types of calls were associated with particular types of actions.

To compute the proportion of each call category emitted by each group, we summed the total number of each call for that group (e.g., total number of trills across all six Food Reward rats) and divided by the total number of all calls emitted by that group

and expressed the result as a percentage. Analysis was based on the entire 2 min anticipation period. We also analyzed the change in vocalization rate from day 1 to day 7 for each call category. For this analysis, we first computed the rate of calling on day 1 and day 7 for each rat for each call, and then expressed this as a difference score (i.e., day 7 rate—day 1 rate). Difference scores were then averaged to compare the change in vocalization rate for each call category for each group. A similar method was used to analyze the vocalization rates for each call when the two play and two food groups were combined, except that all data was from day 1 and results are shown as raw vocalization rates.

2.3.9. Statistical Analyses

To evaluate the associations between all the behaviors and call types a Monte Carlo Shuffling method was used [30]. We first counted the number of co-occurrences of each vocalization type with each of the coded behavioral categories. A vocalization was counted as occurring during a particular behavior if the mid-point of the call occurred between the start and stop time of the behavior. To allow for small errors in coding of the start and stop times of behaviors, the window for counting a call as associated with a behavior was extended to 200 milliseconds before the start of the behavior and 200 milliseconds after the end of the behavior. Shuffling was achieved by assigning each vocalization a random time within the duration of the 2 min observation period. Hence, the relative frequency of vocalizations was kept the same for each shuffle. This shuffling was done 10,000 times and the total number of co-occurrences of each vocalization type with each type of behavior was tabulated. Based on the distribution of these counts, a z-score was calculated for each of the actual co-occurrences of each call–behavior pairing. The higher the z-score, the more likely that specific combination of call and behavior

could have occurred by chance (i.e., for $p \leq 0.05$ the z score is +1.96 and for $p \leq 0.01$ the z score is +2.58). Large negative z-scores, on the other hand, indicate that the call and behavior are associated much less than expected by chance. Shuffling was performed separately for each animal and the z-scores averaged across animals in the same group to generate the final, average z- score values.

2.4. Results

2.4.1. Vocalizations

2.4.1.1. Vocalization Counts

To gage anticipation, we calculated the average vocalizations produced during the two-minute period of anticipation for each group. As is evident in Figure 2.1 by day 7 of testing the Play Reward group had a significantly greater average vocal production than the other conditions. A repeated measures ANOVA was conducted on the influence of group (Play Reward, Play Control, Food Reward and Food Control) on the vocalizations produced on day 1 and 7 of testing. The effect for testing day was not significant $F(1, 4) = 0.233$, $p = 0.654$, partial $\eta^2 = 0.055$, but the group $F(3, 12) = 9.10$, $p = 0.002$, partial $\eta^2 = 0.695$ and testing day X group interaction were significant $F(3, 12) = 5.49$, $p = 0.013$, partial $\eta^2 = 0.578$. The Play Reward group increased vocalizations production in the anticipatory period, explaining the significant effect of group, however, overall the other groups did not show an increase; in fact, the two control groups actually decreased over days.

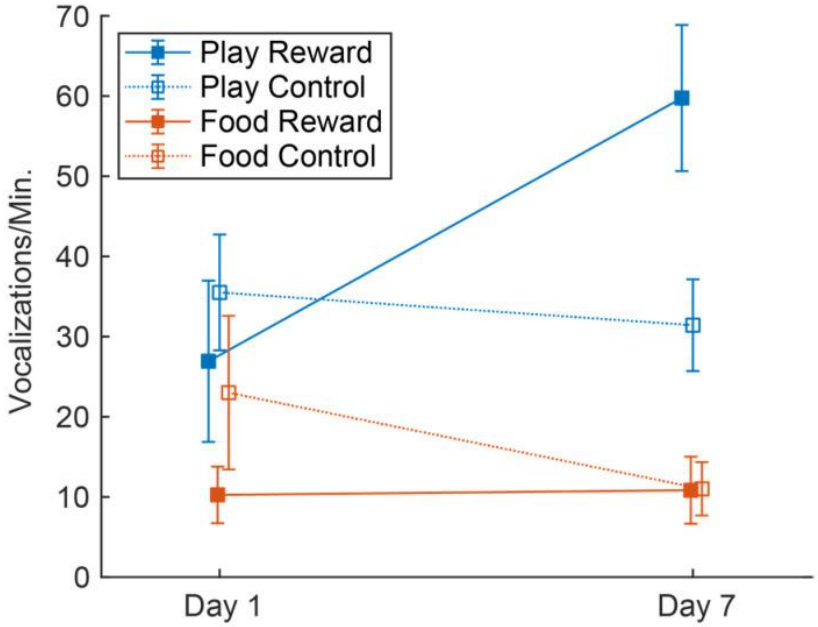

Figure 2.1. The average rate of 50-kHz vocalizations produced on day 1 and 7 of anticipatory testing. All error bars are standard error of the mean.

2.4.1.2. Vocalization Analyzed by Category

To assess if anticipation of different rewards impacted the type of vocalizations produced, we calculated the average number of each call subtype emitted over the entire 2 min anticipation period in each condition and expressed this as a proportion of all calls (Play Reward, Play Control, Food Reward, Food Control). To assess if the calls emitted changed over days of testing we performed this analysis on both day 1 of testing, when the animals had been habituated to the chamber but had not experienced reward, and day 7 when the reward groups had received 7 days of experience with rewards and the chamber and the control groups had 7 days experience with the test chamber. The analysis, shown in Figure 2.2 reveals several interesting patterns. First, both food deprivation and social deprivation appear to influence the types of calls produced on both days. Secondly, the pattern of calling on day 1 is similar for the Play Reward and Play Control groups, as is the pattern of calling in the Food Reward and Food Control groups,

but the play and food conditions differ markedly. In particular, the rats in the food conditions exhibited a much higher proportion of flat and upramp calls and proportionally fewer trills than the rats in the play conditions. Thirdly, while the rats in the play groups mostly had minor changes in call distribution from day 1 to day 7, the rats in both food groups had a large increase in trill and trill with jumps and a reduction in flat calls. In fact, by day 7, the majority of calls from these rats were trills, trills with jumps and upramps. The rats in the play conditions, in contrast, had a wider variety of call types on day 7. This reduction in the variety of calls in the two food conditions on day 7 was also validated by a comparison of Gini coefficients [82]. On day 1 in the Play Reward and Play Control conditions, the Gini coefficients were 0.60 and 0.58, respectively, while on day 7, they were barely different at 0.58 and 0.54. In contrast, in the Food Reward and Food Control conditions on day 1, the Gini coefficients were 0.54 and 0.58, respectively, but then increased to 0.70 and 0.79.

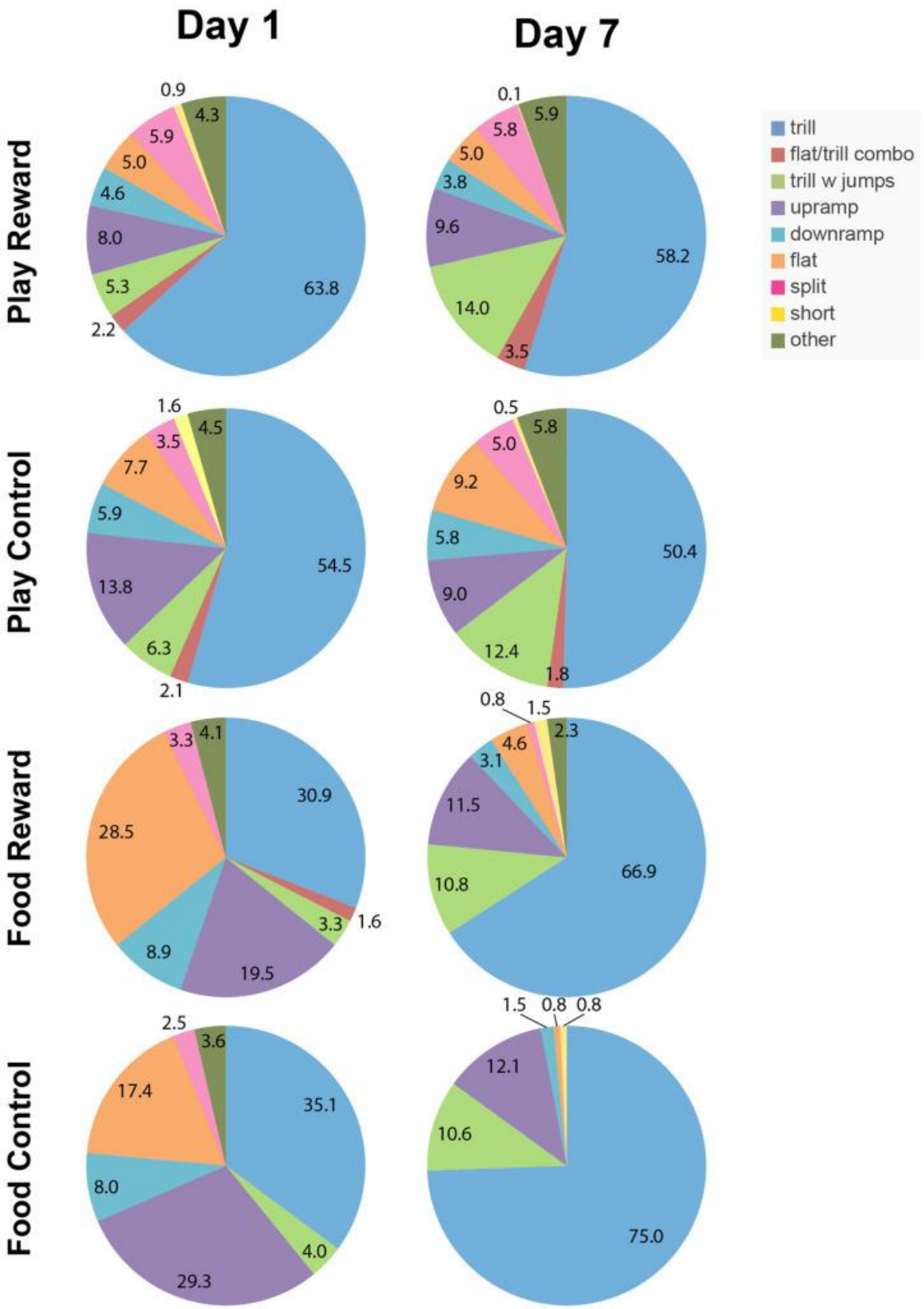

Figure 2.2. The average proportion of all commonly used call categories for each experimental group (Play Reward, Play Control, Food Reward, Food Control) on day 1 and 7 of testing are shown. Numbers show percentage of all calls for that group and testing day.

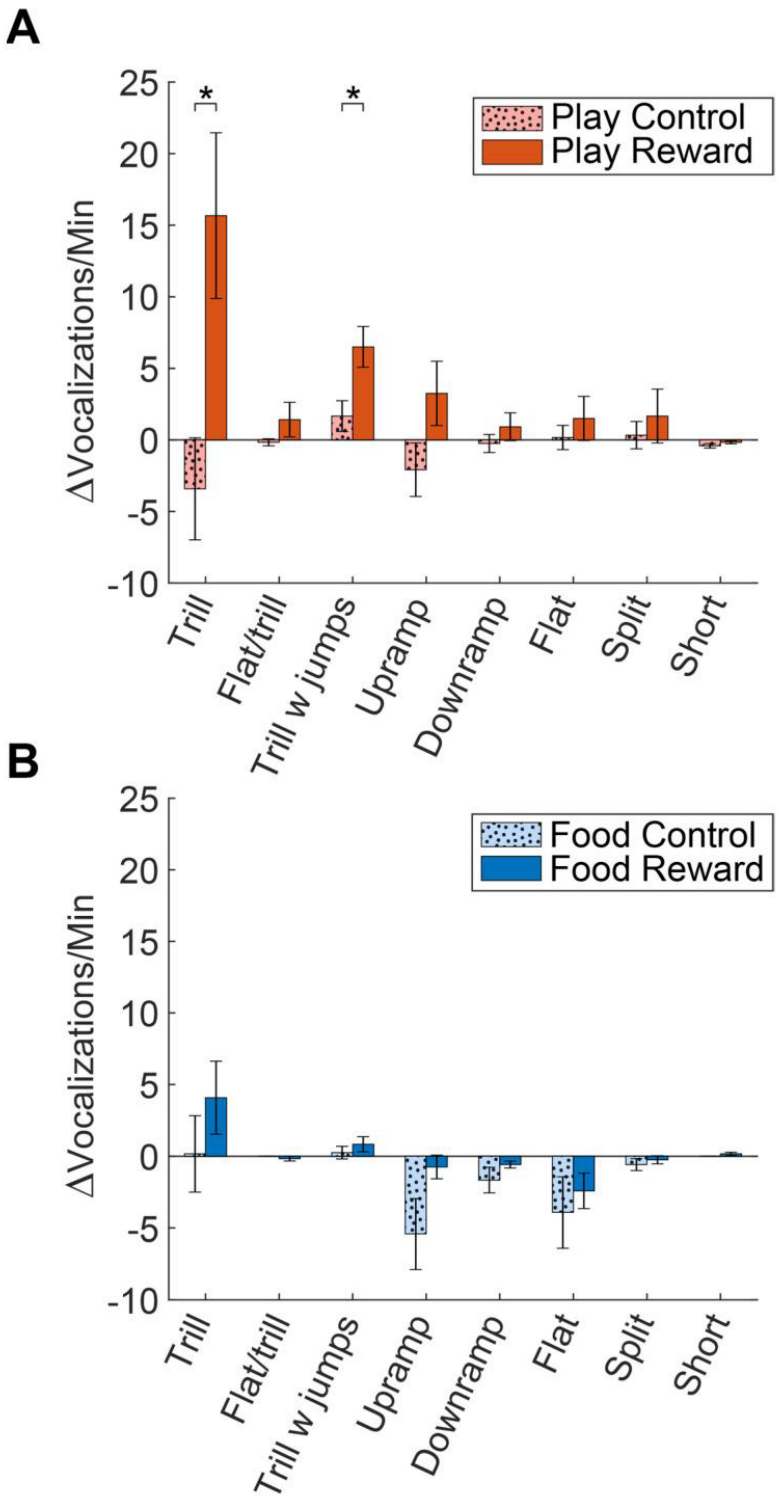

Figure 2.3. Comparison of the increase/decrease in call rates from day 1 to day 7 for each category of commonly produced calls. (**A**) Comparison of the change in call rates for Play Reward and Play Control groups. Asterisks denote comparisons that were statistically significant ($p < 0.05$). (**B**) Comparison of the change in call rates for Food Reward and Food Control groups

To quantify these effects, we also compared the change in the average number of vocalizations of each type from day 1 to day 7, computing a change score for each vocalization. In Figure 2.3 A, it is apparent that the Play Reward group showed increases in trills, trills with jumps and upramps. A two-tailed *t*-test was used to compare these change scores for control and reward groups for each vocal category. Compared to the Play Control group, the increase in calls was significant for both the trill ($t(10) = 2.81$, $p = 0.019$) and trill with jump ($t(10) = 2.70$, $p = 0.022$) calls. In contrast, Figure 2.3 B shows that the Food Reward group showed an increase in trills and a decrease in upramps and flats, but none of these were statistically different compared to the changes in the Food Control group. Hence, the anticipation of social reward seems to lead to an increase in calls with trills (trills and trills with jumps), whereas the anticipation of food does not cause unique changes in the number of any types of calls.

As previously mentioned, our qualitative analysis (Figure 2.2) revealed dramatic differences between play and food groups in the types of calls used on day 1. To examine this effect in more detail, we combined the day 1 data from the Food Reward and Food Control groups and separately combined the data from the Play Reward and Play Control groups. As none of these groups had yet to experience the associated reward, there is no reason to suspect differences within the Play Reward/Play Control or Food Reward/Food Control supergroups. Hence, the only difference between the play and food supergroups is that one was socially isolated (play groups) and the other food deprived (food groups). Figure 2.4 shows a comparison of the average number of calls emitted by each group during the anticipation period, broken down by category. A two-tailed *t*-test was used to compute the probability of a difference between the play groups and food groups

for each call category. Both groups emitted more trills than any other calls, but the play groups emitted far more trills than the food groups ($t(22) = 3.10$, $p = 0.005$). Significant differences were also seen between play and food groups in trills with jumps ($t(22) = 2.39$, $p = 0.026$) and short calls ($t(22) = 3.08$, $p = 0.005$) although the strength of this latter effect is due to the fact that there were zero short calls emitted by the food deprived animals on day 1. In sum, when placed in a new environment, rats that have been socially isolated emitted more trill, trill with jumps and short calls compared to rats that had been food deprived but not socially isolated.

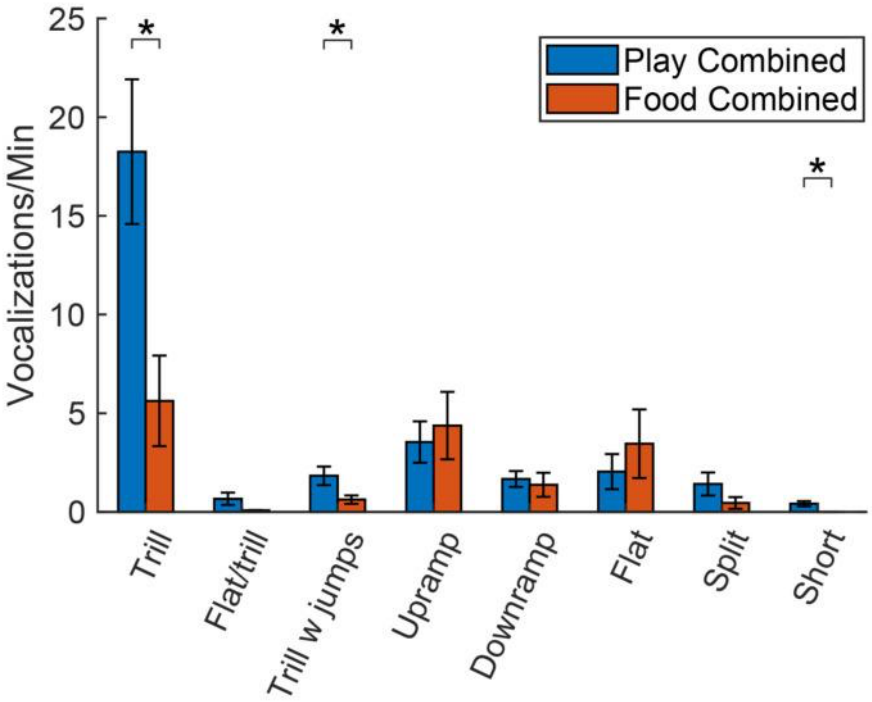

Figure 2.4. Comparison of the rates of calls on day 1 for all food and play groups. Play includes both Play Reward and Play Control while food includes both Food Reward and Food Control. Asterisks denote comparisons that were statistically significant ($p < 0.05$).

2.4.2. Behavior

We compared the mean time spent in each of the coded behaviors on day 1 and day 7 for each of the four treatment groups. We then grouped these measurements into

slow locomotion (step, turn or walk) and fast locomotion (run or jump). The latter is of relevance because it could indicate the level of arousal. As shown in Figure 2.5, the two food groups showed no change in the average time spent in slow locomotion from day 1 to day 7, while both play groups showed a slight decrease. A two-way ANOVA with between-subjects factor group (Play Reward, Play Control, Food Reward, Food Control) and repeated-subjects factor day (1 or 7) showed only an effect of group ($F(3, 20) = 3.64$, $p = 0.03$, partial $\eta^2 = 0.35$). Tukey's HSD tests for multiple comparisons showed

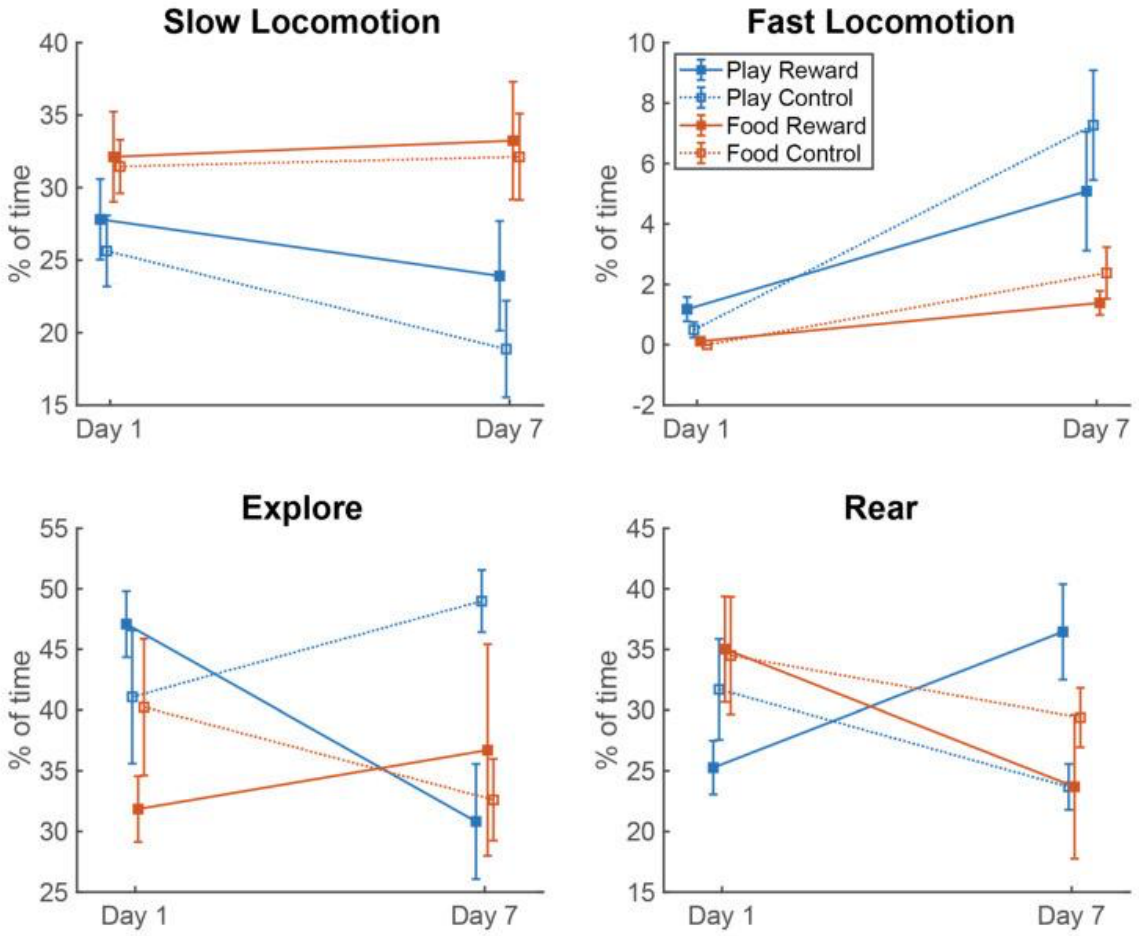

Figure 2.5. The average proportion of time spent in each of several key behaviors on day 1 and day 7 for each experimental group. Upper left shows the proportion of the 2 min test period spend in slow locomotion (single step, turn or walk). Upper right shows proportion of time spent in fast locomotion (running or jumping). Lower left shows time spent in exploratory behaviors. Lower right shows time spent rearing on hind legs. All error bars are standard error of the mean.

that the primary reason for this group effect was a significant difference between the Food Control and Play Control groups ($p = 0.046$). With fast locomotion, all groups showed an upward trend from day 1 to day 7 and this was borne out in a two-way ANOVA (group x day), which showed a significant effect of day ($F(1,20) = 24.20$, $p < 0.001$, partial $\eta^2 = 0.548$). There were also differences between groups ($F(3,20) = 4.458$, $p = 0.015$, partial $\eta^2 = 0.401$), which Tukey's HSD tests revealed were primarily due to a significant difference between the Food Control and Play Control groups ($p = 0.026$). Hence, we see effects of group on both slow and fast locomotion with play groups showing less slow locomotion and more fast locomotion but no significant differences in the rate of change of either variable from day 1 to day 7.

Two other behaviors, rearing and exploring, stood out because they apparently showed different patterns for the Play Reward group compared to the other groups. For exploration, the Play Reward group showed a reduction in duration from day 1 to day 7, but a two-way ANOVA (group × day) showed no significant effects of group, day or their interaction. Similarly, rearing duration increased from day 1 to day 7 only in the Play Reward group, but a two-way ANOVA (group × day) failed to show any significant differences, although the day X group interaction was close, with a p-value of 0.055.

2.4.3. Vocal-Behavioral Associations

The vocal-behavior correlations shown in Figure 2.6 demonstrate several differences both between groups and days tested. First, on day 1, the Play Reward and Play Control groups had very similar profiles, with the strongest associations being between trill calls and walking, and between downramp and running. When comparing between groups on day 7, the Play Reward and Play Control groups were less similar. For

the Play Reward group, the majority of the strong behavior-call associations involving running and jumping, whereas for the Play Control group, the majority of strong associations were with walking and running. More specifically, in the Play Reward group there were strong associations of downramp, flat and split with jumps. Trills, flat/trill combinations and trills with jumps were associated with running. In the Play Control group, on the other hand, the strongest associations were between trills and walking, and between downramps and running. On day 1, the Food Reward and Food Control also had similar profiles, with the trill-walk, upramp-walk and flat-walk being the predominant associations. Arguably the most interesting finding is that by day 7, both the Food Reward and Food Control groups did not have any significant vocal-behavior associations.

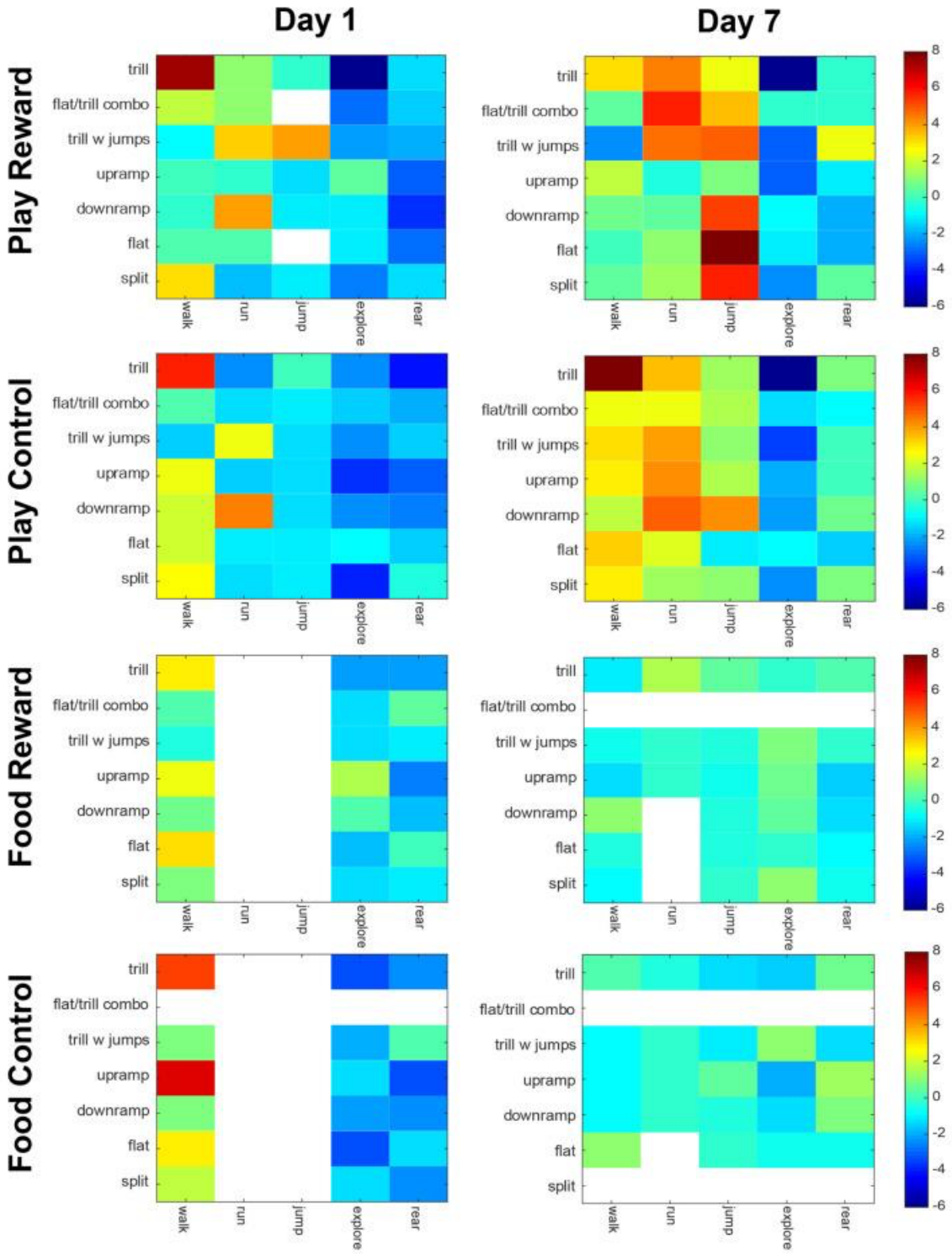

Figure 2.6. Association between types of calls and types of behavior are shown for the four groups on day 1 and 7 of the anticipation trials. Each matrix shows the strength of association, as a z-score, for each combination of behavior (x-axis) and vocalization category (y-axis). Deep red indicates the strongest positive association and deep blue the strongest negative association. The white sections indicate that either the behavior or the vocalization in that category did not have sufficient instances to run the analysis.

2.5. Discussion

The primary goal of this study was to compare the vocalizations emitted by male rats in anticipation of two types of reward: food and social play. Care was taken to equate the age of the animals being tested, as social behavior and vocalizations change dramatically with age [83]. We also included controls for the effects of social isolation and food deprivation, as both might be expected to affect the production of vocalizations irrespective of the presence of rewards. Over seven days, repeated pairing of the recording chamber with the reward of a play partner led to an increase in 50-kHz vocalizations, a change not present in the social isolation control group. There were also trends towards greater high-intensity movement, less exploration and increased rearing in the group rewarded with play, although none of these behavioral changes were statistically significant. Examining each call category individually showed that both the trill and trill with jump calls increased with increased training, suggesting that these two calls in particular may have a social role. In contrast, repeated pairing of the reward chamber with food did not lead to any discernable changes in either vocalizations or behavior.

A secondary finding was the robust, but different effect of social isolation and food deprivation on vocalizations. Hungry rats produced fewer and different calls than socially isolated ones. The lower number of calls in the food-deprived animals is apparent in Figure 2.1 and this finding is consistent with previous reports that food-deprived animals call less [66, 84]. Differences in the distribution of calls is apparent in Figure 2.2 on both days 1 and 7, where distributions look similar within the two play conditions and within the two food conditions, but very different between the food and play super-

groups. A quantitative comparison of call rates on the first day of testing showed that the primary difference between play and food groups was in trills and trills with jumps, the same two calls whose prevalence correlates with the expectation of social reward. This adds to the evidence that these calls have a social role. The increased drive for play induced by social isolation increases their prevalence and the expectation of the arrival of a play partner increases their prevalence further.

Our finding that trills and trills with jumps are tied to the expectation of social reward is broadly consistent with the findings of others. Earlier studies have shown that the expectation of social play in juveniles increases the emission of 50-kHz calls, generally [53]. While this early study did not categorize calls, a later study showed that frequency-modulated 50-kHz calls predominate during play itself, at least among juvenile males [23]. Our results are also similar to the pattern reported by Wright et al. [26] who found that two male adult rats placed in a chamber together after saline injection emitted, as the most frequent call categories, 20% trills, 17% flat/trills and 20% flats. The increased frequency of flat-containing calls in that study may be either because of recent injection or because adult males tend to have more aggressive encounters, which have been linked to 50-kHz flat calls [23]. More recently, Mulvihill and Brudzynski [77] showed that trill calls, in particular, are more common as male rats explore a space recently vacated by an estrous female. Mating is also commonly associated with 50-kHz calls in the period before ejaculation and 22-kHz calls afterwards [85]. It would be interesting to compare the types of 50-kHz calls emitted during sex with those during play and other affiliative behaviors, but most studies of sexual vocalizations were conducted well before the common use spectroscopic analysis. One relatively recent

report shows that the calls during sex are frequency modulated, but does not categorize calls any further [23]. In studies very similar to the present one, we have previously found high rates of both trills and trills with jumps in male rats anticipating social reward (but there we did not demonstrate that rates were modulated by social expectation) [30, 32]. Further, when male juvenile rats play, the most commonly emitted calls are trills and trills with jumps [1]. Taken together, the data suggest that trills and possibly trills with jumps play a role in calling to other rats, possibly to broadcast a general state of positive affect and/or to attract them [25, 29, 86, 87]. Trills, in particular, are the most common call detected in many studies, suggesting that, although trill rate is modulated by social expectation, rats may be set, by default, for constant social signaling [26, 30, 88].

The lack of anticipatory vocalizations or behavior in our food condition is puzzling, especially in light of several other studies showing an increase in 50-kHz calls during the expectation of food. Willey and Spear [76] showed elevated 50-kHz calling when male rats were placed in a chamber with food on the other side of a barrier. Rats also show an increase in 50-kHz calls in the 15 min before their daily feeding [68]. Several studies have shown that 50-kHz vocalizations increase after presentation of a tone or light cue that predicts food delivery [50, 66, 67, 84]. The one exception to this pattern was a study by Tripi et al. [89], which showed that Pavlovian conditioning with lever, light and food did not lead to elevated cue-related calling , though the anticipatory period was relatively short (8 s). The weight of the evidence, however, suggests that rats will elevate their calling when context or cues predict the arrival of food.

Why did others find increases in 50-kHz vocalizations associated with food expectation while we did not? The contrast is most striking with Burgdorf et al. [50],

upon which our study was modelled. Both studies used the same strain of rat, the same 2 min expectation window, and similar methods of food deprivation. Burgdorf et al. did use a mix of male and female rats while we used solely males, but previous studies have shown that sex differences in vocalizations are either subtle or non-existent [90-92] (see below). Hence, the most notable difference was that we used juvenile rats while they, and all the other studies cited above, used adults. The idea that anticipatory vocalizations for food are age-specific is consistent with a previous report that male adolescent rats show lower levels of food-associated vocalizations than adults [76].

Another explanation for the lack of food expectancy calls, may be is that our rats were not as motivated by food. The use of juveniles was necessary because we wanted to compare the effects of food and play in similar groups of animals and juveniles exhibit a pronounced peak in play activity between 30–40 days of age [81, 93, 94]. Unfortunately, this imposed constraints on our ability to food-deprive our animals, because prolonged caloric restriction at this age leads to stunted growth. In our study, a control group given free access to food (in this case, the two play groups) were used as a control to set weight targets for rats as they grew. However, as the food-deprived rats probably did slow their growth, the freely fed rats may have served as an overly generous target for our 15% weight reduction. As a consequence, the motivation to seek out food reward may have been reduced in our food groups, resulting in the lack of anticipatory behavior for food reward. On the other hand, we used a highly palatable food reward (chocolate chips) that at least one other study showed was sufficient to induce 50-kHz vocalizations even in rats that were not food deprived [76].

A third possible explanation for our lack of food expectancy calls is that we simply lacked the statistical power to detect increased vocalizations for food reward. There are intriguing non-significant differences in the change in vocalizations from day 1 to day 7 between the Food Reward and Food Control groups (Figure 2.3 B) that suggest that a larger number of subjects might have allowed detection of some differences. On the other hand, even with the low number of animals, there were very clear differences in the Play Reward and Play Control conditions (Figure 2.3 A), suggesting that low power was not a critical limitation. To us, the most likely explanation is simply that rats at this age, due to some biological programming, simply care far more about social activities than they do about food.

While our study did not allow us to determine which calls are tied to food reward, there is considerable evidence from other studies. Many studies use only broad categories of calls. For example, Opiol et al. [68], showed an increase in frequency modulated calls tied to a tone that predicted the delivery of food. Other studies are more specific. In one, male rats were trained for 24 days to expect food after a light cue [67]. In the 2 min anticipatory window, the calls related to the expectation of food were “other frequency modulated” (which excludes calls with trills), “step frequency modulated” (which look like Wright et al.’s category, split) and 50-kHz flat calls. Similarly, Brenes and Schwarting [66] found an increase in step calls over flat and trill calls during cued anticipation of food reward. In a recent study that directly examined the different calls elicited by food and social stimuli, male rats were allowed to explore a space with either a highly palatable food reward or an empty space recently occupied by an estrous female [77]. Flat calls were more common in the food group while calls with trills were more

common in response to the female. The elevated flat calls are consistent with previous reports of 40 kHz flat calls related to food consumption [68, 75]. Taken together, the evidence suggests that the expectation of food elicits flat, step, split and other frequency-modulated calls but notably, not calls with trills. This is a striking contrast with the social-related calls we observed, both of which include trills.

Further insights into the function of different 50-kHz calls can be gleaned from studies with amphetamine, a highly reinforcing drug. Amphetamine induces a robust increase in 50-kHz vocalizations both from acute administration or to contextual cues associated with the drug [48, 95-97]. Some studies have found that amphetamine increases all types of 50-kHz calls [98]. Another study by the same first author found increases in flat, trill, complex, inverted U, short, step up, multistep, upward ramp calls [88]. Other studies have found more selective effects on specific vocalizations. Two separate studies showed that injection of amphetamine causes a selective increase in trills and a decrease in 50-kHz flat calls [26, 99]. In sum, amphetamine induces trills and possibly other frequency-modulated calls. From this perspective, amphetamine elicits vocalizations very similar to those associated with social reward while food seems to elicit a non-overlapping set consisting of flat, step and other calls without trills. This suggests that vocalizations may be specific to certain types of reward, but more study is clearly needed.

We have previously provided evidence that certain categories of rat vocalizations are selectively emitted when rats are performing specific actions [1, 30]. In the current study, we provide further evidence of the selective emission of calls with respect to behavior (Figure 2.6). The data are roughly consistent with findings from our previous

study with the same strain of rats on the anticipatory period before play [30]. Admittedly, the plots are not identical because certain calls were omitted from each analysis due to low numbers, and the omitted calls were different in the two studies. Looking at the data from Day 7 in the Play Reward condition, we can see that walking is associated with trills, running with any trill call (trill, flat/trill combo and trill with jumps) and jumping is associated with a wide range of calls, most notably split, flat and downramp. In contrast, the data from Burke et al., [31], Figure 2.3 C, largely agree for walk and partially agree for runs. In that study, running was associated with composite and trill with jumps, both of which have trills. The current associations between split calls and jumps agrees with Burke et al. [31] but that study included composite and multi-step, neither of which was common enough in the present dataset to analyze. Both studies agree that exploration and rearing are negatively correlated with vocalizations. Finally, it is interesting to note that the food-deprived groups show a marked lack of correlation between vocalizations and specific behaviors, especially on Day 7 (Figure 2.6, bottom two rows). Our data suggest that food deprivation not only reduces the number of vocalizations, but also may desynchronize their association with behaviors. We cannot rule out the possibility that this de-synchronization is due to low numbers of calls, but our shuffling method is robust and, if anything, tends to overestimate associations when vocal counts are low (which is why many rows and columns in that data are left blank). Taken together, this data strengthens the case that specific calls are tied to specific behaviors, but studies with a much higher number of vocalizations may be needed to iron out the specifics.

One limitation of our study is that it was restricted to male rats, as were the majority of studies cited above. Studies of the effects of sex differences suggest that, at

least in juvenile play, sex differences in vocalizations are, if anything, subtle. Certainly, juvenile male rats play more than female rats [94, 100]. It is hence not surprising that one recent study in Sprague-Dawley rats found lower overall vocalizations in juvenile females [92]. The authors also found decreases in specific calls (flat and step) but not others (trill). On the other hand, Gzielo et al. [91], also working with adolescent Sprague-Dawley rats, found no sex differences in either the number of vocalizations or their structure (duration, frequency and bandwidth). Similarly, we have recently compared vocalization sub-types in juvenile Long Evans rats during play and found no sex differences [101]. Adding a female group would certainly be valuable; however, as our study also used Long Evans rats, we have reasonable grounds to assume that their results would be substantively the same as those reported here.

2.6. Conclusions

In conclusion, our data add to the evidence that calls with trills are associated with social reward. It may not be possible to do a strict apples-to-apples comparison between food and social reward because the motivation for social interaction peaks in adolescence while food deprivation studies work better with adults. However, future studies that compare vocalizations to both forms of reward in rats of different ages but keeping all other parameters equivalent would be helpful to elicit exactly which calls are tied to social reward and which to food reward.

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Chapter 3: Are 50-kHz social anticipatory USV different in a depressed phenotype?*

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3.1. Abstract

The Wistar-Kyoto (WKY) rat was developed as a control for the spontaneous hypertensive rat but has subsequently also been used as a genetic animal model of depression due to its hyper-responsiveness to stress. We used anticipation of social reward (i.e., a play partner) to assess behavioural and vocal differences between the WKY and normal Wistar (WI) rats in the juvenile period. We found marked differences between groups; the WKY rats, were less active, vocalized less, and used significantly fewer types of 50-kHz calls in comparison to their WI counterparts. The animals were re-tested in adulthood and the same differences existed in overall activity, types of vocalizations and the behavioural vocal profiles used by the two groups of animals. These findings provide a robust baseline for an animal model of depression using a social paradigm. This paradigm may be useful to evaluate the efficacy of pharmaceutical interventions as potential treatments of depression in WKY rats.

3.2. Introduction

Depression is a debilitating mental disorder affecting up to 350 million people globally [102]. Animal models are useful to test novel pharmaceuticals to remediate depression and the Wistar-Kyoto (WKY) rat provides one of only a small number of established genetic models of depression (for review, see Alexandrova et al., [103]). Initially bred from the Wistar (WI) rat as the control strain for the spontaneously

hypertensive rat [104], WKY rats demonstrate an exaggerated response to stress compared to other strains [105]. For example, they exhibit passive coping and behavioral inhibition in several tests, such as immobility in the open field [106, 107] and in the forced swim test (FST) [108-111], plus freezing in the learned helplessness paradigm [106, 108, 112]. Although WKY rats exhibit deficits in a variety of behavioral measures, the majority of tests currently used involve training the rats to perform a specific task. Therefore, the differences identified between the WKY and WI rats could reflect difficulties in learning or in executing the tasks, rather than being a direct result of the depressive-like phenotype of WKY rats.

Social play is a positive affective behavior that occurs spontaneously in rats without the need for formal training [113]. WKY rats exhibit abnormalities in social interaction tests; compared to WI rats, they are socially withdrawn [106] and exhibit lower levels of play [114]. Play is usually studied when the animals are juveniles (post natal days 30 – 40), the time period when play is most frequent [115, 116], although play does continue into adulthood [81]. Given that the majority of pre-clinical studies of depression treatments are performed with adult rodents [117-119], in order for play to be useful to assess depression in rats, it needs to be comparably diminished in adults as well as juveniles. A complication in evaluating play is the mutual influence that partners exert on each other's actions [120], and the contagious nature of play, with one partner inducing play in the other [121]. Consequently, even a depressed rat may be induced to engage in more play than it otherwise would due to the influence of a conspecific.

Three main methods are used to assess the motivation of rats to engage in play. The first involves directly measuring how many nape contacts are initiated during play trials. This first method is commonly used to test the effects of strain and sex differences

and experimental manipulations on play motivation [81, 93, 122-124]. The second method is to indirectly measure play motivation by using conditioned place preference [125-127]. The motivation to access a chamber with a potential play partner can be further assessed by adding an operant conditioning component, such as having to press a lever [128]. The final approach is to train rats to anticipate the imminent arrival of a play partner and evaluate the rat's behaviour prior to the introduction of the partner [30, 53]. It is this last approach that will be the focus of the present paper.

The subject's motivation to engage in play with the forthcoming partner is manifested as an increase in activity in the enclosure and by the increase in the emissions of positively affective 50-kHz calls [53]. Ultrasonic vocalizations (USVs) are spontaneous calls which, in addition to signaling social context and status, are now known to be emitted in response to anticipation of stimuli that are either rewarding (e.g. food, social play, drugs with rewarding properties) or aversive (e.g. predator, social defeat, drugs with aversive properties), as in the case of frequency-modulated 50-kHz and 20-kHz USVs, respectively. Thus, USVs are gaining traction as a putative window into an animal's affective state [29, 86]. In this experimental paradigm, particular calls become progressively more closely associated with particular behaviors [30]. The anticipation of play paradigm (AOP) thus provides a promising method for assessing the motivation to play without confounds arising from partner-induced changes by actually engaging in play.

Given that WKY rats initiate less social play than WI rats [114], the implication is that the WKY rats are less motivated to engage in play with a peer. Reduced motivation to play relative to WI rat controls may reflect the depressive-like state of WKY rats. Indeed, social withdrawal and reduced motivation to engage socially are common symptoms of

depression (e.g., [129-131]). Accordingly, in the present study, we used previously established protocols for testing AOP [30, 53] in WKY and WI rats. Based on the well documented evidence that WKY rats have a depressive-like phenotype compared to WI rats, we hypothesized that WKY rats would show reduced motivation to play with the prospective partner. Specifically, we predicted that WKY rats would 1) be less active, 2) utter fewer ultrasonic vocalizations and 3) exhibit a weaker association between particular calls and particular actions. Moreover, this should be the case for both juveniles and adults. Confirmation of these predictions would establish a baseline for the WKY strain which could then be used to assess improved social motivation, and so reduced depressive-like behavior when treated with antidepressant interventions.

3.3. Methods

3.3.1. Subjects and experimental procedures

A total of 20 male, juvenile Wistar (WI) rats and 20 male, juvenile Wistar Kyoto (WKY) rats were used in this study. All animals were obtained from Charles River Laboratories (Kingston, New York) shortly after weaning (around 24 days of age) and were housed in pairs of the same strain until testing. Testing occurred from post-natal days 32 – 38, within the age in which play is most frequent [81, 115, 116, 132], and again in adulthood from days 90 – 97 when play is still present, but less frequent [81].

The testing enclosure was a Plexiglas box (50 x 50 x 50 cm), which was situated inside a soundproof chamber (61 x 61 x 83 cm), lined with acoustic foam. The floor of the chamber was covered with 2 cm of paper bedding (Carefresh, Healthy Pet, Ferndale, WA). Ultrasonic vocalizations were collected using a specialized microphone (Model 4939, Brüel & Kjaer, Denmark) with a frequency response of 4-Hz to 100-kHz. The microphone

was located in the ceiling of the chamber and was approximately 15 cm above the middle of the play enclosure. The microphone was connected to a Soundconnect™ amplifier (Listen, Inc, Boston, MA) and sound waves were recorded at 195,313 Hz using 32-bit resolution via a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, FL). Video was recorded using a USB webcam (Microsoft Lifecam Studio, Redmond, WA) with its infrared filter removed, positioned directly above the animal. Illumination was provided by an array of 16 100 mW infrared LEDs (Osram SFH 4557, Regensburg, Germany) mounted on the ceiling of the soundproof chamber. Each animal was habituated to the testing enclosure for ten minutes each day for three consecutive days.

In the 'anticipation of play (AOP) paradigm', the target animal was socially isolated for 24h prior to testing, as this has been shown to increase the motivation to play [81, 116], and was then placed in the testing enclosure two min prior to the introduction of a familiar play partner (i.e., its cage mate). Once the play partner was introduced, the animals were allowed to play for 10 min. Audio and video recordings began when the target rat was placed in the test enclosure. Because both audio and video data were recorded on separate devices, a custom-made beeper with an LED light was used to emit a simultaneous light/sound cue at the beginning and end of each recording session and these times were used to synchronize audio and video recordings during subsequent analysis [54]. Following each session, rats were returned to their home cages, and the apparatus was thoroughly cleaned with Virkon, a broad-spectrum disinfectant (Virkon Disinfectant Technologies, Sudbury, United Kingdom), and bedding was replaced to avoid any odors from other subjects. Testing was conducted for seven consecutive days.

3.3.2. Behavioral Analysis

3.3.2.1. Play behavior

To confirm that WKY rats initiated less play than WI rats, as previously demonstrated [114], we analyzed the frequency of playful interactions. The interaction sessions were 10 minutes in duration and the last day of testing, day 7, was analyzed. Play fighting involves rats attacking and defending the nape of the neck, which is nuzzled with the snout if contacted [58, 120]. In most strains of rats, including WI, over 90% of attacks are clearly oriented toward the nape [133]. When the snout of one rat was either in contact with or moved towards the nape of the other rat, a playful attack was scored [78]. Occasionally, rats may initiate a playful interaction by gently biting their partner's rump [134, 135], which is more similar to serious fighting [120, 136]. In case the WKY animals were more likely to initiate aggression, the frequency of such attacks was also scored. From these values, the proportion of nape to rump attacks could also be compared. The WKY rats were compared with the WI controls as both juveniles and adult. The attacks of the subject rat (i.e., the one that spent the preceding two min period alone in the test enclosure) were scored and compared across ages and strains. The subject rat that was scored had its tail marked with ink (1 line) so that its actions could be distinguished from those of its partner, whose tail was marked with 2 lines.

3.3.2.2. Anticipation of play

For each animal, the type of behavior and duration of that particular behavior were scored during the 2 min session prior to the introduction of the partner. Behaviors were scored by using a frame-by-frame analysis of video records. Behaviors included in the analyses are described in Table 1, which also describes the criteria used in categorization.

Overlapping behaviors were allowed (e.g., if the animal turned while walking, that time interval would be coded as both a turn and a walk). To compare overall changes in activity when in the 2 min anticipation period, those behaviors that involved active movement were summed and the level of activity on the seventh day was compared to that of the first day. The behaviours in Table 1 included in the scoring of active movement were walk, jump and run.

Table 3.1. Description of the behavior scored during the anticipatory period, adapted from Burke et al., 2017 [30]

Behavior	Description
Step	The rat alternates lifting at least two of its paws from the ground
Walk	The rat steps with all four of its limbs in an alternating manner (e.g., left forelimb and right hind limb, right forelimb and left hind limb). If the stepping pattern cannot be seen by the observer, walking can be detected when the rat significantly shifts its body from one location to another.
Run	This can be achieved by the rat in two ways: (1) by increasing the speed of its walking gait or (2) by galloping, in which the rat alternates the contact of its forepaws and hind paws with the ground. In galloping, the rat elongates its torso as it reaches forward with its forelimbs, and then contracts its torso once it contacts the ground with its forepaws and thrusts its hind paws forward, placing them beneath its body [137]
Jump	Two types of jumps are recognizable [138]: (1) Up jump: the rat propels itself directly upward by pushing downward, simultaneously, with all four of its limbs; (2) Forward jump: the rat lifts its forepaws from the ground and pushes forward with its hind paws. The rat elongates its torso as it leaps forward and then contracts its torso once it lands on the ground with all four of its paws.
Turn	A turn can be achieved by the rat in one of three ways: (1) turns limited to the forequarters (45-180^{o}), in which the rat steps with one or both of its forepaws; (2) whole body turns (360^{o}), in which the rat steps with its hind paws as well; (3) more complex turns, which can be preceded by the rat stepping, walking or rearing (see below)
Explore	The rat remains in the same location, but may turn its head so as to examine the surrounding area
Dig	The rat vigorously performs repeated forward and backward movements with its forelimbs with the substrate noticeably disturbed
Rear	The rat stands on its hindlimbs with both of its forepaws off the ground (either free standing or against a wall)

Shake	The rat makes vigorous side-to-side shudders of its head, neck, and trunk [139]
Groom	In a typical grooming sequence, a rat licks its forepaws, wipes its face & nose, then wipes its ears, neck, followed by licking and nibbling downwards, along its flanks. The sequence may be repeated or truncated before completion [140]
Scratch	The rat makes rapid upward and downward movements of a hindlimb resulting in its claws rubbing against its head, neck or flank
Rest	The rat remains immobile; it may turn its head, but significantly less so than is seen during exploration

3.3.3. Vocalization analysis

Acoustic data were analyzed using Raven Pro 1.4 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). The Raven Pro software generated spectrograms with a 256-sample Hann window from which the experimenter manually selected 50-kHz vocalizations. Some putative vocalizations were not perceptually distinct from the background noise and were removed from further analyses. After manual scoring, the Raven Pro software provided the beginning and end times of each vocalization. Based on its frequency over time, each call was categorized according to the schema provided by Wright et al. [26] in which 14 distinct categories of 50-kHz calls are recognized (for example of some of these call types, see Figure 1 in Burke et al., [30]). To compare overall changes in vocalizing when in the two minute anticipation period, the total number of calls emitted on the seventh day was compared to that of the first day. Similarly, the proportion of different types of calls was compared between the first and seventh day of testing.

3.3.4. Correlation of particular calls with particular behaviors

The vocalizations were manually selected and categorized using the Wright et al. [26] criteria, with the start and stop of each vocalization recorded along with the categorization of each call. The various behaviors shown in Table 1 were scored manually,

so that every second of the anticipation period was assigned with a behavior. The start and stop of each behavioral event were recorded along with the categorization of each behavior. The start and stop time of each call was then superimposed on the behavioral events to identify the calls and behaviors that co-occurred.

3.3.5. Statistical analyses

To evaluate the associations between all the behaviors and call types, a Monte Carlo Shuffling method was used [30]. First, we counted the number of co-occurrences of each vocalization type with each of the coded behavioral categories. Next, for each animal pair, vocalizations were repeatedly shuffled, and the number of behavior-vocalization co-occurrences computed. A vocalization was counted as occurring during a particular behavior if the mid-point of the call occurred between the start and stop time of the behavior. To allow for errors in the coding of the behavior times, we expanded the time window for each behavior by 200ms in each direction. Shuffling was achieved by assigning each vocalization a random time within the duration of the two-minute observation period. Hence, the relative frequency of vocalizations was kept the same for each shuffle. Shuffling was done 10,000 times and the total number of co-occurrences of each vocalization type with each type of behavior was tabulated (for a graphical illustration of the method, see Figure 2 in Burke et al. [141]. Based on the distribution of these random-shuffle counts, we assigned a z-score to the actual number of occurrences. The higher the z-score, the higher likelihood that a specific combination of vocalization and behavior did not occur by chance (i.e., a z-value of 1.96 gives a p-value of < 0.05 and a z-value of 2.58 gives a p-value of < 0.01). Shuffling was performed separately for each animal pairing and the z-scores averaged across play pairings to generate the final, average z-score values.

To compare differences in call numbers, overall activity levels and specific activity levels across the two strains (i.e., WI pairs *versus* WKY pairs), a repeated measures ANOVA was used to assess the baseline level of calls and behaviors on day 1 and then the anticipatory behaviors and calls on day 7. Partial η^2 was used to measure-effect size, shown only in cases in which the comparisons were significant. The effect size for non-significant comparisons was low (range: 0.002 – 0.239). To compare playful attacks and types of such attacks between the two strains, independent t-tests were used. Significance was set at $p \leq 0.05$.

3.4. Results

3.4.1. Play behavior

Consistent with previously published findings, WKY rats launched significantly fewer nape attacks than did WI rats, and this was the case for both juveniles (Table 2A) and adults (Table 2B). Moreover, rump attacks tended to be more frequent in the WI rats and were significantly so in adulthood. Therefore, with regard to initiating playful interactions, WKY were less playful, and they were not likely to initiate more aggressive forms of attack.

Table 3.2. Attack behavior during play trials is compared across the two strains on day 7 of testing (presented as the mean and standard error of the mean for each variable).

A. Juveniles

Measures during play trials	**Wistar**	**Kyoto-Wistar**	**t-test**
Number of nape attacks per 10 min	52.80 $\pm$ 5.91	35.10 $\pm$ 2.20	t = 1.19 p = 0.006
Number of rump attacks per 10 min	1.90 $\pm$ 0.69	1.00 $\pm$ 0.94	t = 1.19 p = 0.124
% of attacks directed at the nape	97.07 $\pm$ 0.91	96.91 $\pm$ 0.94	t = 0.13 p = 0.451

B. Adults

Measures during play trials	**Wistar**	**Kyoto-Wistar**	**t-test**

Number of nape attacks per 10 min	17.50 ± 2.46	11.60 ± 1.60	t = 2.01 p = 0.029
Number of rump attacks per 10 min	3.90 ± 1.02	1.60 ± 0.40	t = 2.11 p = 0.025
% of attacks directed at the nape	81.81 ± 5.10	88.82 ± 2.70	t = 1.21 p = 0.120

3.4.2. Anticipation of play

3.4.2.1. Activity levels

The average time each animal spent being active in the anticipation period was compared between the strains (WI, WKY) on days 1 and 7 of testing. For juveniles, the activity level was similar over days and between strains. A repeated measures ANOVA with the factors testing day and strain were non-significant $F(1, 9) = 0.014, p = .908$, , strain $F(1, 9) = 2.33, p = .162$, nor was day*strain interaction $F(1, 9) = 0.14, p = .907$ (Figure 1A). Similarly, for adults, a repeated measures ANOVA between testing days and strain indicated a significant main effect for day $F(1, 9) = 21.70, p = .001, \eta^2 = .707$, but not strain $F(1, 9) = 0.77, p = .403$, or the day*strain interaction $F(1, 9) = 0.29, p = .607$ (Figure 1B).

The activities were sub-divided into the vigorous forms of locomotion (jump and run) and walking and were compared between the juvenile WKY and WI rats over days. A repeated measures ANOVA was run between testing day, strain and activity type. For juveniles, there was no significant main effect for day $F(1, 9) = 0.04, p = .838$, strain $F(1, 9) = 2.55, p = .145$, or day*strain interaction $F(1, 9) = 0.05, p = .829$. However, there was a significant main effect for activity type $F(1, 9) = 254.2, p < .001$, partial $\eta^2 = .966$, as well as a significant day*activity type interaction $F(1, 9) = 26.83, p = .001$, partial $\eta^2 = .749$, strain*activity type interaction $F(1, 9) = 8.38, p = .018$, partial $\eta^2 = .482$, and

day*strain*activity type interaction $F(1, 9) = 20.87$, $p = .001$, partial $\eta^2 = .699$. As shown in Figure 1C, walking was much more common than vigorous locomotion, however, there was an increase in vigorous activity on day 7 compared to that of day 1 for both strains. Further, the WI group walked less and began performing more vigorous activities over days, while the activity of the WKY group remained the same.

Similarly, for adults, there was a significant main effect for day $F(1, 9) = 21.4$, $p = .001$, partial $\eta^2 = .704$, but not strain $F(1, 9) = 1.70$, $p = .225$, or day*strain interaction $F(1, 9) = 24.1$, $p = .371$. However, there was a significant main effect for activity type $F(1,$

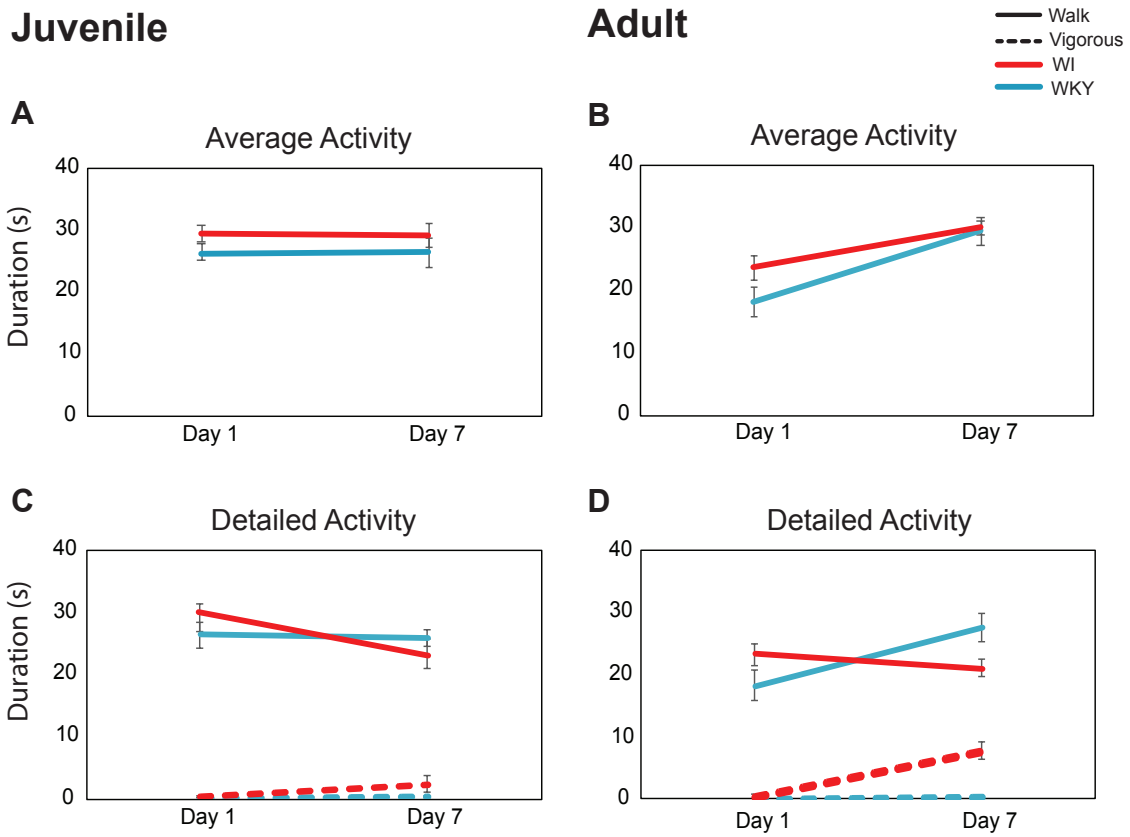

Figure 3.1. The duration (in seconds) spent active during the two minute anticipation trial is compared between strains and between days 1 and 7. The average duration of performing all active behaviors is combined and is shown for juveniles (A) and adults (B). The WI are shown in red and the WKY in blue. The average duration of walking (solid lines) and the duration of vigorous activities (dashed lines) is shown for juveniles (C) and adults (D). The data are expressed as means and the standard error of the mean.

9) = 540.2, $p > .001$, partial $\eta^2 = .984$, as well as a significant day*activity type interaction $F(1, 9) = 0.237$, $p = .018$, partial $\eta^2 = .896$, and day*strain* activity type interaction $F(1, 9) = 12.6$, $p = .006$, partial $\eta^2 = .584$, but no strain* activity type interaction $F(1, 9) = 2.83$, $p = .127$. As shown in Figure 1D, the WI group began at a higher level of walking, but then reduced the time they spent performing this activity in favor of more vigorous activities over time, while the WKY group increased their time spent walking, but were not engaged in the more vigorous activities.

3.4.2.2. Frequency of vocalizations

The vocalizations emitted were compared between the juvenile WKY and WI groups over days (Figure 2A). A repeated measures ANOVA with testing days and strain as factors indicated a significant main effect for day $F(1, 9) = 78.03$, $p < .001$, partial $\eta^2 = .897$, strain $F(1, 9) = 21.92$, $p = .001$, partial $\eta^2 = .709$, and day*strain interaction $F(1, 9) = 14.82$, $p = .004$, partial $\eta^2 = .622$. Similarly, for adults, a repeated measures ANOVA with testing days and strain as factors indicated a significant main effect for day $F(1, 8) = 13.77$, $p = .006$, partial $\eta^2 = .632$, strain $F(1, 8) = 60.42$, $p < .001$, partial $\eta^2 = .883$, but no day*strain interaction $F(1, 8) = 0.224$, $p = .649$. Clearly, both strains increased the frequency of vocalizations over days, but the number of calls emitted by WI remained greater than that of WKY rats on both days (Figure 2B). It should be noted that, for one pair of animals, their vocalization files were missing and were excluded from the vocal count analysis.

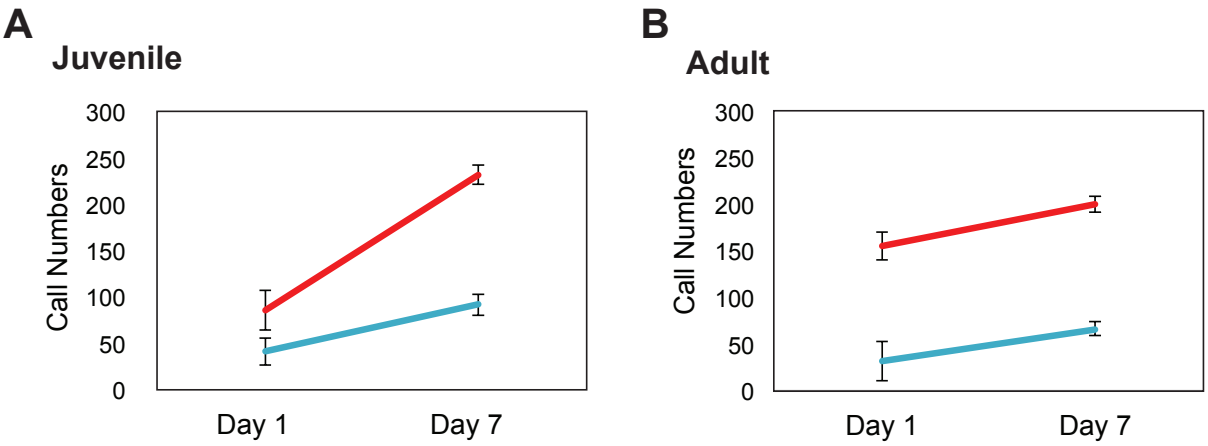

Figure 3.2. The number of calls emitted during the two-minute anticipation trial is compared between the strains on days 1 and 7 in the juvenile (A) and adult (B) period. The WI are shown in red and the WKY in blue. The data are expressed as means and the standard error of the mean.

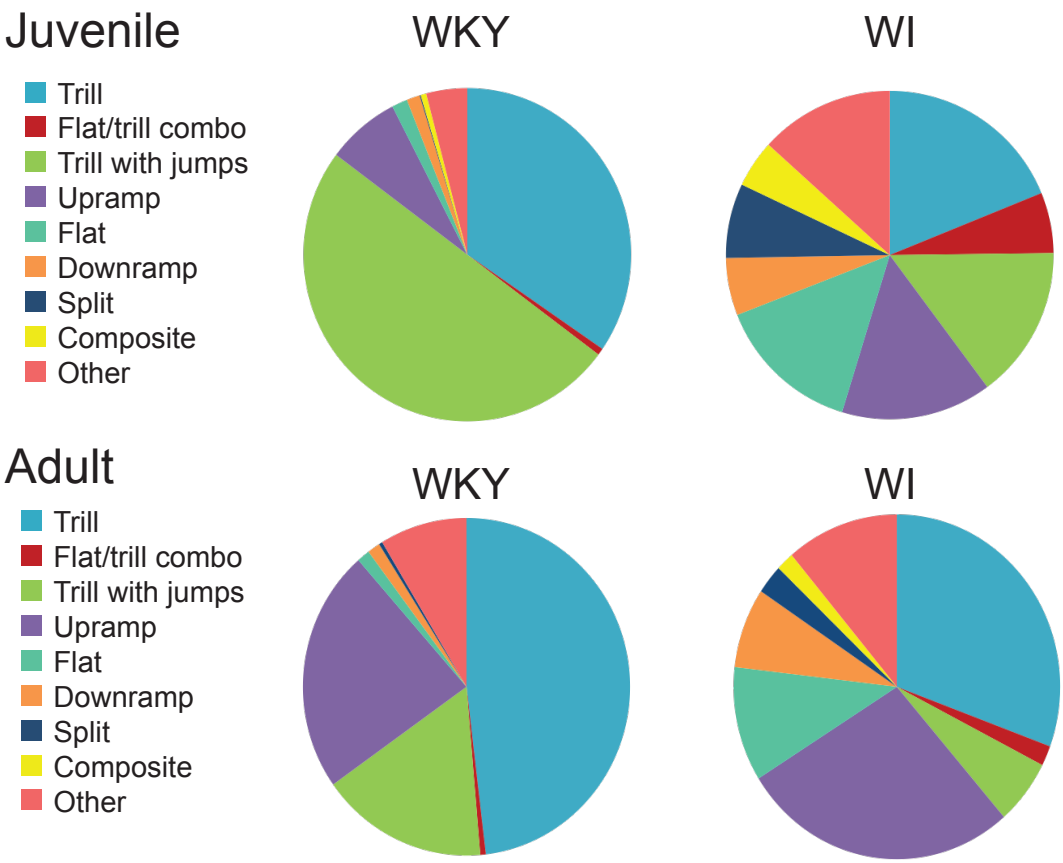

Figure 3.3. The percentage of use of the different vocalization categories is shown for the two strains as juveniles and adults on day 7 of the anticipation trials.

3.4.2.3. Frequency of types of calls emitted

We also categorized the vocalizations to see if the types of calls emitted in the anticipatory period were different between groups on day 7. The call categories used were from the Wright et al. (2010) [26] schema, with the more infrequent calls being summed

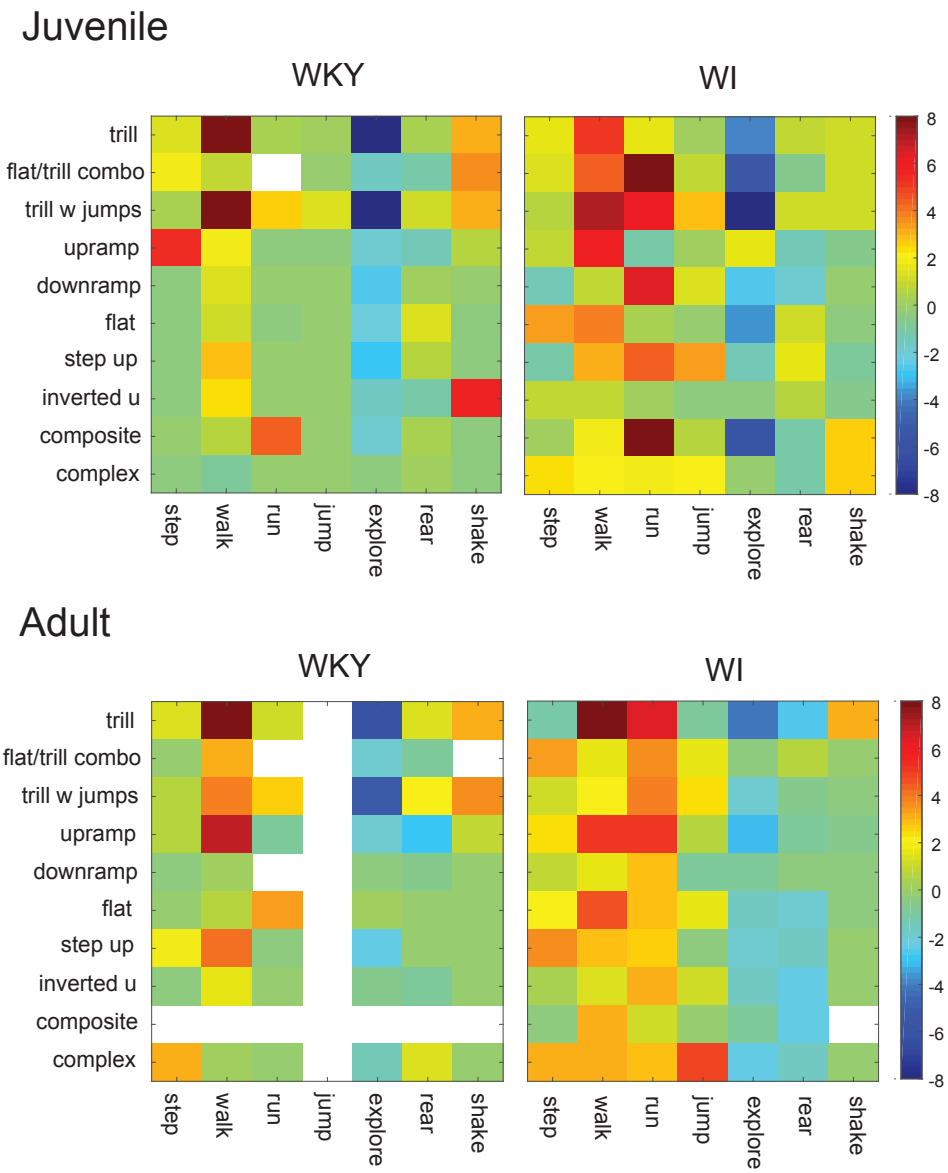

Figure 3.4. Association between types of calls and types of behavior are shown for the two strains at both ages on day 7 of the anticipation trials. Each matrix shows the strength of association, as a z-score, for each combination of behavior (x-axis) and vocalization category (y-axis). Deep red indicates the strongest association and deep blue the most negative. The white sections indicate that either the behavior or the vocalization in that category did not have enough instances to run the analysis.

in an “other” category containing multi step, short, complex, down ramp, step up and step down calls. At both ages, the WKY and WI rats exhibited different call profiles, with the WKY rats using trill type calls (trill, and trill with jumps) (Figure 3) most frequently, whereas the WI rats used a more diverse range of calls.

3.4.2.4. Behavior-vocalization associations

At both ages, both strains exhibited higher vocal associations with active behaviors (e.g., trill calls with walking, composite calls with running) compared to passive behaviors (e.g., no call associations with exploring or rearing). However, the diversity of associations differed between groups (Figure 4). Using a z-score of 2.3 as a cut-off for significant positive behavior-vocalization associations, the WKY rats had fewer associations than the WI rats (juveniles: 8 *versus* 14; adults: 10 *versus* 21).

3.5. Discussion

Consistent with a previous report [114], the juvenile WKY rats in the present study initiated less play than the WI juveniles. This strain difference persisted into adulthood. Moreover, at both ages, the nape of the neck was the main target for initiating playful interactions as is typical of rats from various strains (e.g., [58, 120, 133, 142, 143]). Initiating play with gentle bites to the rump, likely reflecting a more aggressive state [120, 135], was infrequent in both strains, with such initiations being more frequent in the WI rats (see Table 2). This latter finding suggests that the lower frequency of initiating play in the WKY strain ([114]; present study) was not due to these rats being more irritable or agonistic in social situations. Rather, the reduced frequency of initiating play in this strain was likely due to a lower motivation to engage in such behavior, as has been shown for some other genetically selected strains of rats (e.g., [144, 145]). In the case of the WKY rats, this lower motivation for social engagement was consistent with the depressive-like

phenotype of this strain.

That this reduced initiation of play in the WKY rats reflects a reduced motivation to engage in play rather than a reduced capacity to execute such behavior, is shown by the differences in behavior of these rats in the anticipation of play paradigm (AOP). Compared to the WI rats, the WKY rats perform less vigorous activities and emit fewer vocalizations in anticipation of the imminent arrival of a play partner (see Figures 1 and 2). An increase in both physical activity and ultrasonic vocalizing is typical as rats learn to expect the arrival of a partner in the AOP paradigm [30, 53]. In addition, the WKY have an altered pattern of emission of vocalization types (see Figure 3), and do not develop as many associations between calls and behaviors (see Figure 4). As the AOP measures assess the animal's motivation to play independently of the confounding effects of the partner's playful actions, this is a novel testing method to assess depressive-like deficits related to social behavior and their therapeutic amelioration. Most critical for its use as a test paradigm for depression research, the differences between the WKY and WI rats are reliably present in adults, the age at which pharmacological interventions are typically tested (e.g., [103, 146]).

Anticipatory behavior can be characterized by an increased level in activity resulting from quick transitions between behaviors, with locomotion and exploration being the most frequent [147]. In social paradigms, WKY rats have a reduced activity level, such as reduced social exploration and contact behavior [114]. Further, when faced with a novel conspecific, WKY rats spend more time avoiding the conspecific than do control animals [106]. However, WKY rats do not show a deficit when tested in the running wheel, and actually outperform Sprague Dawley rats [148]. This indicates that the reduction in activity

level was likely due to reduced motivation and not an inability to initiate motor behavior. Therefore, we predicted that, in anticipation of play, the WKY rats would have a reduced activity level compared to the WI rats in both the juvenile and adult periods and this is what was found (see above). Most critically, the WKY rats were less likely to engage in more rigorous movements, such as running and jumping, which became clearly differentiated by day 7 of testing (see Figures 2C and 2D). The lack of differences in the types of activity on day 1 between the groups indicates that the WKY rats did not have a motor problem in their baseline activity, but instead, was consistent with a between-group difference in the reward salience.

Vocalizations can be used to gauge a rat's motivation, as they are elicited in anticipation of social stimuli such as a sexual partner or a play partner [37, 51, 53]. We predicted that the WKY group would emit a reduced number of calls in the anticipatory period, as they are likely less motivated to engage in play. Indeed, this was the case, with both the juvenile and adult WKY rats producing significantly less vocalizations on both day 1 and 7 of testing. Our findings were consistent with a previous study demonstrating that WKY rats emit lower numbers of USVs in a variety of testing paradigms [149]. Further, to assess if both groups learned to anticipate the impending arrival of a partner, vocalizations were compared between days 1 to 7. In both the juvenile and adult period, the WKY animals showed a significant increase in the number of vocalizations emitted, from day 1 to 7. However, in the WI animals, the significant increase was only present in the juvenile period, with a trend towards a significant increase in the adults.

We suspect that the lack of significant increase in vocalizations from day 1 to 7 in the WI adult group may be due to them retaining a memory of the experience when tested

as juveniles. This interpretation is supported by the WI adults displaying a very high level of USV emission on day 1 of testing. The finding that WKY rats emitted significantly fewer vocalizations compared to the WI group, while still showing an increase from day 1 to 7 in USV emission, indicates that this difference cannot be due to a learning deficit and is most likely due to a baseline difference in vocalization emissions. Measuring the increase in the number of calls from day 1 to day 7 of testing could provide a novel way to measure the effects of antidepressant treatments. If the treatment works, the rate of increase should be greater than that in the untreated control rats.

Different types of vocalizations generally have different utility, as we know from previous work using the Long Evans strain. In this strain, during the anticipation of play, trill and trill with jumps are the most commonly emitted calls. However, other common calls, like composite, flat/trill combo, upramp and short calls, each make up roughly 7% of the overall call emissions [30]. Thus, even though trill calls are the most frequent, a wide variety of calls are used in the anticipatory period. As demonstrated in Figure 3, WKY rats primarily use trill type calls similar to Long Evans rats [54], whereas WI rats use a wide variety of calls. While this is the first study to identify differences in the frequency of emission of different call types between WI and WKY rats, one previous study reports that WKY rats have significantly longer, mostly flat, calls when exposed to a novel environment away from their home cage [149]. We did not see an increase in flat type calls in the WKY group compared to the WI group, but this difference is likely due to the familiar context in which the animals were tested. The flat calls are emitted after removal from the home cage, whereas in anticipation of play, the animal waits for a partner, often eliciting 50-kHz frequency-modulated calls [30]. The types of calls emitted provide

important insight into the difference between WI and WKY rats, where the lack of variety in the types of calls implies that the WKY rats are experiencing the anticipatory period differently than WI controls. If this is true, then we can predict that WKY rats will be using the vocalizations in different contexts from the WI rats. This seems to be the case.

There were fewer and different vocalization-behavior associations in the WKY rats compared to the WI rats. Many of the differences in the vocalization-behavior associations between the two strains may be explained by the behavior of the WKY rats. WKY rats were less likely to engage in vigorous activities, such as running and jumping, and this could have affected the types of calls emitted. An alternative explanation is also possible. The vocalization-behavior associations present in WI rats (this study) and Long Evans rats [30] are similar. For these strains, the test subjects were rewarded with a same-strain partner; that is, the social reward was a partner highly motivated to play. In contrast, the WKY test subjects were presented with a same strain partner that was less motivated to play, so the impoverished vocalization-behavior associations in the WKY rats compared to the WI rats could reflect the WKY rats being rewarded less. We think this is unlikely to fully account for the behaviour of the WKY rats as the frequency of emission of call types is more similar between the WKY and Long Evans rats than between WKY rats and WI rats. This suggests that the difference in the associations present in WKY rats reflects a motivational deficiency, and not a deviant pattern of call emission, in this strain. Furthermore, the lower frequency of initiating playful attacks during play fighting by WKY rats is consistent with a lower motivation to engage in play. Nonetheless, future studies with this paradigm should provide WKY test subjects with a more playful partner, such as a WI rat, to increase the social reward. This would test

whether the deficiency in the WKY rats found in the present study may be due to an intrinsic lower motivation to play or instead to the reduced social reward provided by a WKY partner. Further, since the play partner is familiar to the test subject, we cannot rule out the possibility of their home cage experiences with the same animal causing some of the changes in the behaviour of the test subjects anticipating a partner. Given that, test subject familiarity was the same for both WI and WKY subjects, this is unlikely to fully account for the observed strain differences.

3.6. Conclusion

In summary, we compared the behavioral and vocal similarities and differences between depressive-like WKY and normal WI rats when tested in the AOP paradigm. While both strains showed that they learned to anticipate the arrival of a social partner, there were also some marked differences between the strains. The WKY rats were less active, vocalized less, and used significantly fewer types of 50-kHz calls in comparison to their WI counterparts. Even though adult rats were less motivated to play than juveniles [81], the same pattern of differences between strains was present in both the juvenile period and in adulthood. These findings of significant reductions in objective measures of ecologically relevant conspecific play behaviour in WKY rats provide a robust baseline for a valid genetic animal model of depression. Furthermore, this social paradigm amenable for use with WKY rats and WI controls expands the range of motivational tests with which to evaluate the efficacy of novel pharmaceutical candidates as potential treatments of depression.

3.7. Acknowledgments

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Chapter 4: Are 50-kHz USVs the same in both tickling and social play? *

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4.1. Abstract

Social play in rats is a highly rewarding, energetic form of social interaction and important for development of the brain and social skills. The 50-kHz ultrasonic vocalizations (USV) emitted during social play are thought to be an expression of a positive affective state ('laughter'), which in some situations may also function as communication signals. Heterospecific play, 'tickling' by an experimenter, is thought to simulate conspecific play, and has been used to improve welfare and to study the neurobiology of positive affect. Given that tickling evokes substantial amounts of USV, we investigated whether heterospecific play is simulating conspecific play by comparing USV-behaviour associations in both contexts. If the 50kHz calls are merely an expression of 'laughter' then the pattern and type of emission in both contexts should be similar. In contrast as playing with a conspecific involves a two-way exchange of signalling, the additional demands on communication should lead to a different pattern of calling. While calling was prevalent in both types of play, how the different types of 50kHz calls are used in the two contexts differed markedly. The findings suggest that while conspecific and heterospecific play are positive experiences, tickling is not the equivalent of conspecific play.

4.2. Introduction

In rats and several other species of mammals social play behaviour during the juvenile period is critical for the development of skills that are important for navigating the social world [150, 151]. Engaging in social play is highly rewarding, as has been

demonstrated with place preference and operant conditioning paradigms [113]. In rats, social play mainly involves play fighting, a vigorous form of behaviour containing components of other social behaviour, especially that from sex, that are displayed in an altered and/or out-of-context form [152-154]. Social play mostly involves attack and defence of the nape of the neck, which is nuzzled with the snout if contacted [58, 155]. After a successful pounce that leads to nape contact, the recipient may protect its nape by rotating to supine, with the partner standing on top (i.e. pinning). From the pinned position, the rat can launch a counterattack for access to the nape. The back-and-forth interaction leads to reciprocity, thus alternating which partner has the advantage. This requires not only cooperation between the playing rats, but also seems to be one of the factors that makes play enjoyable [156].

Because social play is unpredictable, dynamic and complex, many species rely on play signals to prevent misinterpretation of each other's actions and potential escalation to serious fighting [157]. Given that rats mostly play in low light conditions, visual cues are of limited use, as are olfactory cues, which quickly saturate the play arena [158]. However, rats have a rich repertoire of vocalizations, especially ultrasonic vocalizations (USV) in the 50-kHz range [25, 26, 159], many of which are emitted both when anticipating the arrival of a play partner [30, 53] and during social play [1, 23]. While 50-kHz calls are emitted in both social and non-social positive affective contexts [15, 29], during play many calls are emitted in contexts that suggest that they are being used to communicate with one another [57, 160]. Indeed, if both partners are devocalized, the amount of play initiated declines, as do the role reversals indicative of reciprocity [37]. Moreover, when adult male rats, who are unfamiliar with one another, play together in a neutral arena, if one of the pair is devocalized there is an increased risk of escalation to

serious fighting [37], which seems to arise from the failure to use specific ultrasonic calls to de-escalate the encounter [31].

Therefore, USV seem important as play signals that are used to negotiate interactions and avoid misinterpretation, much as is the case for some of the more intensively studied visual signals used by canids and primates, such as play bows and open mouth facial expressions [31]. Some signals used during play may simply have a tonic effect, that of sustaining the playful mood of emitters and receivers [161, 162]. A good example of such a mood effect emanating from a vocal play signal has recently been shown in keas (Nestor notabilis). When keas were exposed to the playback of play-typical warbles of keas, play behaviour was increased [163]. In this regard, some play signals can be like human laughter, a signal that induces a positive affective state [164]. Indeed, by focusing on the types of 50-kHz calls most commonly emitted during play, Jaak Panksepp and Jeff Burgdorf emphasized the role of calling as an expression of a positive affective state, and suggested that this could be a homologue of human laughter [165, 166]. That juvenile rats, when 'tickled' by an experimenter, emit the same types of 50-kHz calls as they do when playing with other rats [24], supports this view. Since these initial insights and findings, rat tickling or 'heterospecific play' has been widely viewed as mimicking social play and has been used extensively to assess and improve animal welfare [167, 168] and to study the neurobiology of positive affect and communication [20, 169-171].

However, we think there are three problems with regarding heterospecific play as mimicking conspecific play, two of which have also been raised in a recent opinion paper by Bombail and colleagues [168]. First, there are individual differences among rats in both their motivation to engage in conspecific and heterospecific play, with rats that

engage in more conspecific play not being the same as those that prefer to engage in heterospecific play [172]. This suggests that the rats do not consider these two forms of play as the same.

Second, part of heterospecific tickling involves turning the rat onto its back to simulate the pinning typical of conspecific play [24], the implication being that the rat emits positively affective 50-kHz calls in this context. Using pairs of conspecifics, in which one member is devocalized the calling by the rats in the pin configuration could be assessed [59]. It is the pinning rat, not the partner being pinned that emits most of 50-kHz calls. Again, this would suggest that heterospecific play does not directly mimic conspecific play. Bombail and colleagues [168] argue that tickling methods focusing on the pin configuration and following a protocol that loosely resembles social play loses some essential elements of play, unpredictability and the choice to use the pin configuration as a defence mechanism against the neck area being touched.

We add a third problem, that clustering together all 50-kHz vocalizations as an index of the positively affective state of the rat during heterospecific play as is typically done [24, 168], may be misleading. During conspecific play different types of frequency modulated (FM) 50-kHz calls, as well as 50-kHz flat calls, are emitted in context specific ways [31, 160]. For example, the most frequently emitted 50-kHz calls are not the ones significantly associated with the pin configuration. Therefore, it cannot be assumed that how vocalizations are used in heterospecific and conspecific play are the same. Both may include a certain amount of calling that reflects a common positive affective state, but which calls are used in different phases of the encounter may differ markedly.

In this paper, we address the following question: Are the emitted USV during play with a conspecific similar to those emitted during heterospecific tickling? If 50-kHz calls

primarily arise as an expression of positive affect as hypothesized by Panksepp and Burgdorf [24], we predict that the pattern and type of call emission between conspecific and heterospecific play should not differ substantially. However, if such calling is not a generalized expression of state, reflecting the playful mood of the subjects (see the example of keas above), but rather primarily used for communication [1, 160], we predict conspecific play and heterospecific play should have a distinct pattern of USV emission. This is because demands on communication would differ markedly between the two types of play.

4.3. Materials and methods

4.3.1. Subjects

In total 30 juvenile male Lister Hooded rats (Charles River, Sulzfeld, Germany) arrived at postnatal day 21 ± 2 days in the facility and were group housed in same sex cages (3 or 4 animals per cage) under controlled conditions (i.e., temperature 20-21 °C, 55-65% relative humidity and 12/12h light cycle with lights on at 7.00 a.m.) and ad libitum access to water and chow. The rats were acclimatized to the facility for 5 days upon arrival and were handled at least two days prior to testing. Animals were weighed the day before each test. All experiments were approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD) and the Animal Ethics Committee of Utrecht University (Licence: AVD108002015189 with protocols 189-1-07, 189-1-08 and 189-1-09) and conducted in agreement with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

4.3.2. Experimental procedures

4.3.2.1 Conspecific play procedure

The 30 rats were tested for their peer-peer social play in dyads on postnatal days (PND) 26, 28, 30, 34, 36, 41 and 43. The animals were paired with an unfamiliar partner (i.e., not a cage mate). Trials were performed in a sound attenuated chamber under red light conditions. The testing arena was a Plexiglas cage (40 x 40 x 60 cm; l x w x h) with approximately 2 cm of cellulose fibers (ALCarefresh®). Animals were habituated to the test cage for 10 minutes on PND 22 and 23 with their cage mates. On the test-days, each rat was isolated for 2.5 hours prior to testing, in a Eurostandard type III cage in a room different from the housing room, to increase their motivation to play [173, 174]. From the trials between PND 26-36, the 10 most playful and the 10 least playful dyads were selected, with the number of nape attacks used to determine the magnitude of playfulness.

4.3.2.2 Tickling procedure

From the 30 rats tested for social play, 12 were randomly selected to assess their responses to being tickled by an experimenter. Nine out of these 12 animals were part of the 10 most and least playful dyads. Selected animals were subjected to tickling on PND 35. The testing arena was a Plexiglas cage (Macrolon type 4) that is normally used as a home-cage, with sawdust bedding of approximately 2 cm thick. The bedding consisted of sawdust from all homecages of the tested animals. Animals were habituated to the testing arena for one minute before the tickling procedure started, with the test lasting 3 minutes. During these 3 minutes the animal was subjected to a tickling procedure as described by LaFollette [167] and adapted from Panksepp and Burgdorf [175]. The tickling procedure combined aspects from previously described methodologies to mimic rough and tumble play between the rat and the hand [175, 176]. To more closely resemble conspecific play, both the animal and the hand initiated play but not continuously. After completion of testing the rats were returned to their respective home-cages.

Each session of both types of interaction was recorded using a digital camera (Logitech C922 pro stream webcam, Lausanne, Switzerland). The behaviour of both partners in the rat dyads and of the rat and the human 'tickler' was assessed afterwards to perform detailed scoring by a trained observer using Avidmux software (free online software). Vocalizations were collected using a Pettersson M500-384 USB Ultrasound Microphone (Pettersson Elektronik AB, Sweden) that was suspended roughly 65 cm above the floor of the test-cage.

4.3.3. Behavioural Analyses

4.3.3.1.Conspecific play

Social play in rats involves attack and defence of the nape of the neck, with several options to avoid such contact [78]. One rat can approach and pounce on the other, aiming at the nape, and the recipient can defend itself by evading or turning to face the attacker. The latter option often involves the defender rotating to supine, leading the attacker to stand over it in the 'pin' configuration. During test trials the rats may also explore their surroundings while seemingly ignoring the presence of the partner and periodically, especially when first introduced to the test chamber, approach the partner and sniff it, most often in the anogenital area. So, during the trials the rats can behave non-socially, engage in social investigation and in social play that can involve multiple actions. Given that different USV can be emitted while performing different actions [1, 31], the different actions that could occur during the play sessions were scored (Table 4.1). A random sampling of all 30 rats (total pairs, the 10 highest and lowest playing pairs) were used to assess play.

Table 4.1. Description of conspecific play behaviours scored adapted from [1].

Behaviour	**Description**

Playful	
Nape	When the rat moves towards the nape of its partner's neck with its snout.
Chase	When, following an interaction, one rat chases its fleeing partner.
Pin Active	When one rat stands over its supine partner, attempts to free itself or counterattack. The partner standing on top may move to block the supine rat's manoeuvres.
Pin Passive	When one rat stands over its supine partner, but the supine rat remains relatively immobile.
Mutual Upright (m.u.)	When both rats face one another while rearing on to their hind feet, usually holding each other with their forepaws. From this position, they can sniff or push one another.
Evade	When the recipient of a nape attack protects against contact on its nape by dodging, running or jumping away.
Non-playful	
Sniff	When one rat sniffs the face and flanks of its partner.
Sniff (anogenital)	When one rat sniffs the anogenital area of its partner.
Approach	When one rat moves toward its partner, but without targeting the nape.
Follow	When one rat follows its partner, but unlike chasing, is not preceded by an interaction.
Solo	When the rats are not engaged with each other

4.3.3.2. Heterospecific play

Tickling play involves the experimenter approaching, touching and flipping the rat onto its back and gently rubbing it with their fingers [24]. However, as pointed out by Bombail and colleagues [168], the procedure does not truly reflect the dynamics of conspecific social play. Therefore, as well as following the standard procedure, we incorporated an addition to this hand-rat interaction to make it more flexible and more closely resemble conspecific social play. Thus, the hand could chase the rat and the rat could chase the hand, the hand could contact the rat's nape and the rat could pounce on the hand like it pounces on a partner to contact the nape. In the latter, the rat could nuzzle the wrist as if

it were the nape of another rat. Moreover, as well as tickling its belly when the rat was flipped on its back, when not on its back, it was also tickled on its flanks. As for conspecific play, the behaviour was manually scored according to the behaviours described in Table 4.2.

Table 4.2. Description of the heterospecific play ("tickling") behaviours scored

Behaviour	**Description**
Playful	
Nape	When, following an approach or pounce, the rat moves toward the experimenter's wrist or upper palm and nuzzles these areas with its snout.
Naped	When the experimenter uses their thumb and index finger to rub the nape of the rat's neck and then flips it onto its back.
Follow	When the rat follows the experimenter's hand around the enclosure.
Pin	When the rat stands on the fingers and palm of the experimenter's hand as the experimenter tickles the rat's ventral surface, as the rat lays on its back.
Pinned	When experimenter places their fingers on top of the rat's ventral surface, moving their fingers in a tickling motion.
Evade	When the rat protects against contact on its nape by dodging, running or jumping away.
Tickle Ventral	When, following nape contact, the experimenter tickles the standing rat's ventral surface [24].
Tickle Dorsal	When the experimenter tickles the rat's dorsal surface especially its neck [24].
Tickle Double	When the experimenter tickles the rat on its dorso-lateral surfaces, using both hands.
Non-playful	
Approach	When the rat moves towards the hand, but without any clear indication that the wrist is being targeted.
Solo	When the rat is not interacting with the experimenter's hand.
Sniff	When the rat sniffs the experimenter's hand.
Other	When the rat is not engaging with the experimenter's hand and is exploring the side of the enclosure.

The various behaviours shown in Tables 4.1 and 4.2 were scored manually, so that every second of the first 4 minutes of each conspecific play trial and the 3 minutes of the tickling trial was assigned with a behaviour. The start and stop of each behavioural event was recorded. We chose the first 4 minutes of each conspecific play trial because,

rats, on average, start playing on average 60.86 seconds ± 7.34s (mean ± sem) after a trial begins, so the majority of the play occurs during the last 3 minutes sampled, thus matching the time sampled in heterospecific play. The vocalizations were manually selected and the calls were categorized using previously established spectrographic criteria [26]. The time each call began and ended was then superimposed on the behavioural events to identify the calls and behaviours that co-occurred (see below).

4.3.4. Vocalization analyses

Acoustic data were analysed using Raven Pro 1.4 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). The Raven Pro software generated spectrograms with a 256-sample Hann window from which the experimenter manually selected 50-kHz vocalizations. After manual scoring, the Raven Pro software provided the beginning and end times for each vocalization and the overall number of vocalizations. Based on its frequency over time, each call was categorized according to the schema provided by Wright and colleagues [26], in which 14 distinct categories of 50-kHz calls are recognized (see Figure 1 in [30]). These data were used to compare the overall number of calls, the production of different types of calls, and how different types of calls were associated with different types of behaviours during the two types of play.

4.3.5. Statistical analyses

4.3.5.1. Vocalization counts

During conspecific play interactions, both partners emit USVs, whereas during tickling there is only one rat present to contribute to the number of calls emitted. However, when the rate of calling per unit time was compared between pairs of intact rats with pairs in which one partner was surgically devocalized, there was no statistical difference [1]. Whether one or two rats are calling during conspecific play, the average

amount of calling is the same, therefore, in the present study, the rate per pair was compared to the rate of calling in the tickling condition. Since the trials sampled had different durations the number of calls per minute was used for comparison. Even though some of the same rats were tested in the two play paradigms, we could not discern which partner in the dyad contributed to calls, so it was not possible to compare the same rats in the two conditions. Therefore, an independent t-test, rather than matched-pairs t-test, was used. To evaluate whether the number of calls was correlated with the number of nape attacks (conspecific play) or tickling events (heterospecific play), linear regression analyses were used.

To compare the types of calls produced the percentage of occurrence of each call type was scored for each pair (conspecific play) or individual rat (heterospecific play) to produce the mean for each call type. Call types that occurred < 1%, which included stepdown, step up, multi, inverted u, and complex calls, were grouped together in an 'other' category. To assess the overall pattern, all call types containing a trill component were lumped together and the average use of these was compared between the two types of play using an independent t-test. Similarly, each of the 10 call types compared were tested between the two play types using independent t-tests. The alpha value for significance was set at 0.05. Given that comparing call types involved 10 separate t-tests, a Bonferroni correction was used yielding a revised significance level of 0.005.

4.3.5.2. Vocal-behavioural correlations

To evaluate the associations between all the conspecific and heterospecific interactions and call types, a Monte Carlo shuffling method was used [30]. First, we counted the number of co-occurrences of each vocalization type with each of the coded

behavioural categories. Next, for each animal pair, vocalizations were repeatedly shuffled and the number of behaviour-vocalization co-occurrences computed. A vocalization was counted as occurring during a particular behaviour if the mid-point of the call occurred between the beginning and end of the behaviour. Shuffling was achieved by assigning each vocalization a random time within the duration of the observation periods. Hence, the relative frequency of vocalizations was kept the same for each shuffle. Shuffling was done 10,000 times and the total number of co-occurrences of each vocalization type with each type of behaviour was tabulated (for a graphical illustration of the method, see Figure 2 [141]). Based on the distribution of these random-shuffle counts, we assigned a z-score to the actual number of occurrences. The higher the z-score, the less likely that a specific combination of vocalization and behaviour could have occurred by chance (i.e., a z-value of 1.96 gives a p-value of < 0.05 and a z-value of 2.58 gives a p-value of < 0.01). Shuffling was performed separately for each trial (conspecific and heterospecific) and then the z-scores were averaged across each trial to generate the final, z-score values.

4.3.6. Data Accessibility

All relevant data, codes and materials used in this article can be found here (https://doi.org/10.5061/dryad.fn2z34tvc).

4.4. Results

4.4.1. Vocalization counts

Overall vocal production per minute was significantly higher in the heterospecific ($M = 97.0$; $SD = 64.4$) compared to the conspecific ($M = 53.7$; $SD = 22.7$) context ($t(30) = 2.75$, $p = .01$). No relationship between the number of ultrasonic vocalizations and the number of nape attacks that occurred was found ($F(1,18) = 0.01$, $p = .919$). Similarly, no

relationship between the number of ultrasonic vocalizations and the number of tickles (dorsal, ventral and double tickles) was found ($F(3,8) = 1.05$, $p = .421$).

4.4.2. Vocalization types

The distribution of producing different types of calls was similar in the two forms of play (Figure 1). In both cases, the majority of calls were those that contained trills (mean ± standard deviation: heterospecific play – 76.88% ± 12.01 versus conspecific play - 70.35% ± 10.85), and these percentages did not differ significantly between the two contexts ($t(30) = 1.58$, $p = .124$). Visual inspection of Figure 1 indicates that some calls were used more in one type of play than the other, but pairwise comparisons between the two types of play only revealed one significant difference, more trill with jumps were emitted during heterospecific play ($t(30) = 4.5$, $p < .001$).

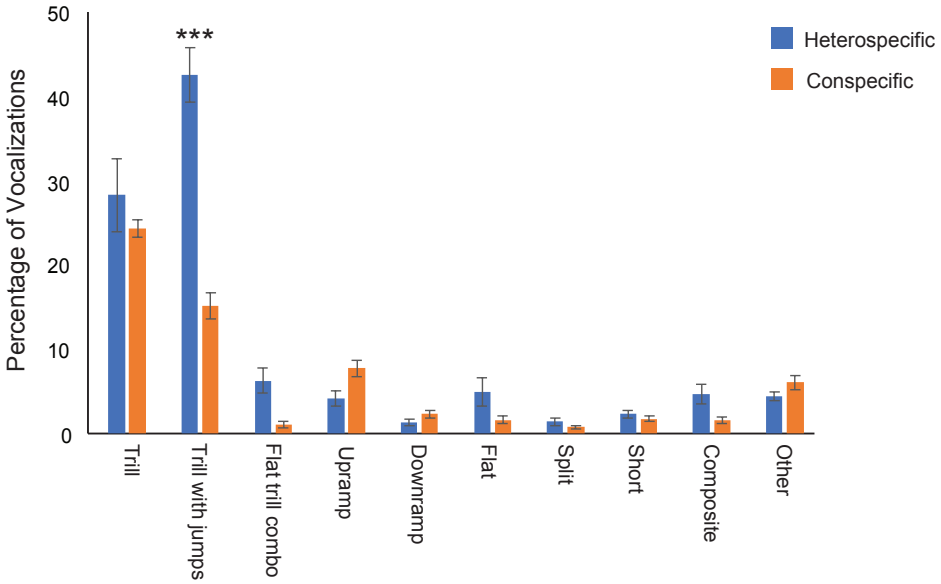

Figure 4.1. The different types of USV emitted are compared between heterospecific and conspecific play as percentages (mean ± SD). ***$p < .001$.

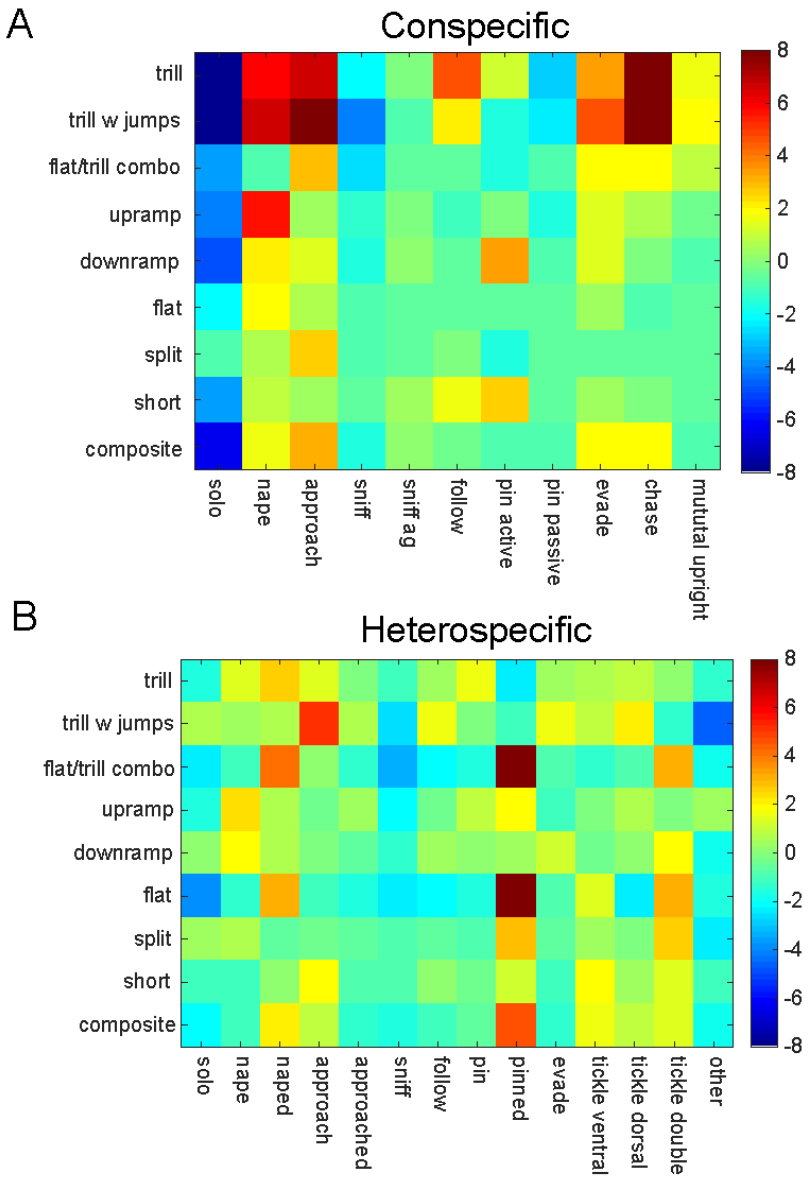

Figure 4.2. The matrix shows call types on one axis and the types of behaviour on the other, with z scores showing the strength of the associations between particular calls and particular behaviours, spanning from deep red for the strongest positive association, green for no association and deep blue for the strongest negative association. A z-value of 1.96 gives a p-value of < 0.05 and a z-value of 2.58 gives a p-value of < 0.01. Because some of the behaviours scored in the two play contexts were different, some of the behaviours in panel A and panel B differ. The description of the relevant behaviours scored can be found in Tables 4.1 and 4.2.

4.4.3. Vocal-behavioural correlates

During conspecific play calls are emitted by both partners. However it cannot be ascertained whether it is the attacking rat, defending rat or both that contribute to

significant associations between specific calls and specific behaviours. In contrast, in heterospecific play the partner producing a call in a particular context is always the rat. Therefore, caution needs to be taken in interpreting differences in association patterns between calls and behaviours. Nonetheless, some key similarities and differences can be unambiguously identified (Figure 4.2)

In both types of play, when the rats engaged in solitary, non-playful behaviour, they were unlikely to emit any calls, as shown by the green (no significant associations) and blue (significant negative associations) boxes. That is, rats are more likely to integrate calling in a patterned manner when interacting with another rat or with an experimenter's hand. For positive associations there were two striking differences between the two types of play. First, in conspecific play, the strongest associations were with approaching, following, chasing and contacting the nape, whereas, in heterospecific play, the strongest associations were when the rat was pinned. Second, most of the strongest associations in conspecific play involved calls with trills, whereas, in heterospecific play, several of the strongest associations involved non-trill calls, including flat calls. Interestingly, far less strong associations with call patterns were found for evading, approaching and nape attacks in heterospecific play compared to the conspecific play context. The opposite was true for being pinned. In summary, while in both forms of play, rats are more likely to produce particular calls, in particular behavioural contexts, the pattern of such calling differs markedly between the two types of play.

4.5. Discussion

The evidence that heterospecific play, in the form of tickling, induces a positive effective state in rats and can have a positive effect on their welfare is very strong [167,

177-179] Indeed, we detected no aversive 22kHz calls [25, 180] in either type of play, indicating both were rewarding to the rats. Our goal in this paper was not to challenge these positive associations between the tickling of rats by humans, but whether, as is often claimed, heterospecific play simulates conspecific play [168]. As 50-kHz calls are emitted during both tickling and playing with another rat [23, 165], this has been taken as evidence of a common positive effective state [177, 178]. Therefore, in the present study we conducted a detailed analysis of the contextual use of the different types of 50-kHz calls [26] in hetero- and conspecific play. Given that heterospecific play aims to mimic the 'tickling' that occurs when rats nuzzle each others' napes following a pounce and each others' ventrums when in the pin configuration [177], it would be predicted that the pattern of calling should be most similar in these contexts.

As previously reported [24], in terms of overall 50-kHz call production, we found that more calls were emitted per unit time during heterospecific play than conspecific play. Also consistent with previous findings [1, 23, 24, 165, 177], it was the frequency modulated 50-kHz calls that were most frequently emitted during both types of play, especially the calls including trills. Even though trill based calls constituted 70-80% of all calls emitted in both types of play, one difference was that there was a significant increase in the production of trill with jumps in heterospecific play (Figure 4.1). Unlike previous findings [23, 29, 178] neither type of play had a positive correlation between the amount of playful contact and the number of calls produced. At present this inconsistency remains to be explained. In terms of the overall pattern of producing calls, our data are consistent with previous findings, and where they were not, we found no differences between the two types of play. It is in how the calls are contextualized that the two types of play differ.

Whether the trial involved interacting with another rat or with an experimenter's hand, when not engaged socially the rats were highly unlikely to emit calls (Figure 4.2), supporting growing evidence that while 50-kHz calls reflect a positive affective state, they are more strongly associated with social stimuli [32, 38, 77]. During conspecific play, rats are most likely to emit 50-kHz calls immediately prior to making playful contact, and by using pairs in which one partner is devocalized, it was shown that the calls are emitted both by the initiator and the receiver [37],with the strongest positive associations being between trill containing calls and approaching, chasing, evading and contacting the nape, with little or no association between such calls and pinning [1, 141]. This is the same pattern as was found in the current study (Figure 4.2A).

The pattern of call emission during heterospecific play differed markedly from that in conspecific play (Figure 4.2B). The strongest positive associations were when the rat was pinned. Indeed, one of the strongest associations was between the flat 50-kHz call and pinning. Moderately strong associations occurred between both flat and trill calls when contacted on the nape by the experimenter, as is the case in conspecific play. However, when one partner is devocalized, so that the emitter can be identified, typically the attacker, not the recipient emits these calls [1]. As is the case with conspecific play, there is a moderate association with the rat attacking the partner's nape, which in the case of the experimenter's hand is the wrist. To further differentiate tickling from the rat's postural configuration, the rat was tickled with one or both hands on the dorsum and the ventrum when standing prone (see Table 4.2). Only tickling the dorsum with both hands produced some significant positive associations, but surprisingly not when tickled on the ventrum, as the hand pinning the rat and tickling the ventrum is thought to be the hallmark of heterospecific play being a simulation of conspecific play [177]. This

suggests that the high production of calls when pinned is not because of the ventrum being rubbed, as rubbing the ventrum in another context does not produce this effect. Rather, it is either being pinned that is crucial or the combination of being tickled when pinned. This is unlike conspecific play in which it is the rat standing over the pinned rat, not the pinned rat, which emits the vast majority of calls [59].

Given the similarity in the pattern of contextual call emission in the present study with that found in [1], in which a different strain of rat was studied in a different laboratory with some environmental differences (e.g., size of housing cage, size of testing cage, number of rats housed together, familiarity of the play partners), it is likely that the general pattern reflects the typical case for conspecific play. While similar to each other, the patterns are highly divergent from that found for heterospecific play (Figure 4.2). There are some similarities, but the difference in which calls are emitted and how strongly they are associated is marked – the pattern associated with nape contact is only partially similar and the pattern for pinning is completely dissimilar. Based on these differences, the present findings support the view that heterospecific play is not a simulation of conspecific play [59, 168], but rather a different kind of play experience the positive aspects of which are not valued to the same extent by different rats [172].

While our data are consistent with the view that hetero- and conspecific play are not viewed by rats as equivalent experiences, some of the differences that emerged require explanation. Most striking was that during heterospecific play calling was around two times more frequent than in conspecific play. Given that the majority of those calls were ones involving trills, it was surprising that there were so few positive associations between trill calls and specific behavioural contexts (Figure 4.2B). This means that many of these calls were emitted more indiscriminately during the test trial, rather than coupled

with particular actions. Feedback from the partner, so that they exchange signals back and forth, has been shown to be important for initiating and sustaining play fighting in several species in which visual signals are used [181-183]. Such signalling feedback is not available to rats interacting with an experimenter's hand, and this may account for the increased calling in heterospecific play.

Devocalized rats are less likely to reciprocate during play, suggesting that some of the 50-kHz calls are used to signal to the partner to ensure continued play [37]. In some situations, it may be that it is the exchanging of calls between partners is critical to communicate effectively and to prevent the encounter from escalating to aggression [1, 31, 141]. Alternatively, since pairs of devocalized rats also play less than vocal pairs [37], it is possible that some calls, such as trills [1], have a tonic function, that of stimulating a playful mood, as some data suggest for some visual signals [161, 162], and has been experimentally demonstrated for an acoustic play signal [163]. When interacting with an experimenter's hand, no auditory signalling is possible, as the hand follows a previously scripted series of actions [177], so from the rat's perspective, the hand is not responding to calls so as to modify its behaviour, as would a conspecific [141]. This could account for the strong positive associations of some calls, mostly non-trill ones, when pinned (Figure 4.2) – signals are repeated over and over because the hand is failing to change its behaviour! The only signalling possible is with itself, that is emitting signals that maintain its own playful mood, as appears to be the case for the emission of some play signals in other species [161, 162]. In rats, trill-based calls are the most frequently emitted calls in both hetero- and conspecific play, and are the likeliest candidate as signals for maintaining the playful mood. The increased emission of calls during heterospecific play, and the less structured emission of these calls with specific actions

(Figure 4.2B), suggests that greater non-associated emission of trills throughout the trial may help sustain the rat's playful mood. Indeed, when playing with a devocalized partner, the vocal partner doubles its production of calls [37], so the tripling of call production in heterospecific play may be way that the rat compensates for the muteness of its 'partner'. That is, 'playing' with a non-communicating hand may stimulate the production of non-directed 'laughter'.

More needs to be known about the signalling and non-signalling functions of USV during playful encounters to fully understand the differences in calling present in hetero- compared to conspecific play. Indeed, the study needs to be replicated with females and other strains of rats to determine the generality of the present findings. Nonetheless, the striking difference between the two types of play in how different calls are inserted into particular actions suggest that for rats, playing with a human hand is not the same as playing with a conspecific.

4.6. Acknowledgments

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Chapter 5: Do 50-kHz USVs impact the behaviour of a conspecific? *

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5.1. Abstract

Communication during play fighting can be crucial in facilitating and sustaining contact. As juvenile rats play mostly in the dark, visual signals are unlikely to fulfill this function. However, during play, rats have a variety of ultrasonic calls that are emitted and there is growing evidence that some of these calls may provide a means of communication. Particular calls are associated with particular actions and in the present paper we show that specific calls by one rat changes the probability of the actions taken by another. We have found that some calls appear to act as a means of sustaining the animals' playful mood and so facilitate the occurrence of play, some calls ensure that the interaction remains playful and so avoid escalation to aggression, and some appear to promote reciprocal exchanges and so ensure that playful contact continues.

5.2. Introduction

The seminal work by Jaak Panksepp and his group showed that when rats anticipate or actually engage in play they emit ultrasonic vocalizations (USVs) [23, 53]. In particular, calls in the 50-kHz range are associated with play. Given that juvenile rats will actively seek out both play and 50-kHz calls [184], and that 50-kHz calls are associated with other appetitive experiences such as mating and eating, Panksepp and colleagues suggested that 50-kHz calls are used to communicate positive affect. Indeed, he suggested that 50-kHz calls emitted during rat "tickling" serve a similar purpose to human laughter [165]. This has opened an important avenue by which to track the affective states of rats [29] and allowed Panksepp and his colleagues to trace the neural circuits underlying positive affect

(e.g. [185, 186]). In this review, we will focus on the evidence suggesting that USVs not only reflect the positive mood associated with play, but also serve to coordinate specific social actions between partners.

Communication during play fighting can be crucial in facilitating contact and sustaining playful wrestling [187]. Given that play fighting and serious fighting co-exist in the same individual at all ages [135, 155, 188], there is the risk that animals may misinterpret the actions of their partner and either stop playing or escalate to aggression, so using signals to communicate how actions should be interpreted has been argued to be critical, especially if the context is ambiguous [189-192]. Considerable empirical evidence has been amassed showing that such signals are strategically emitted to facilitate play. For example, dogs are more likely to perform a play bow toward their partner immediately before lunging to bite [193]. Similarly, if partners synchronize their play bows, they are able to sustain longer lasting playful wrestles [183]. Other visual gestures, such as the primate open mouth play face [194] have also been shown to serve these diverse functions during social play [187].

Given the important role of rats in the study of the neurobiology of social play [113, 195], extending the study of play signals to this species is potentially valuable for exploring the neuroscience underlying the communication processes during play. Rats compete to contact the nape of the neck during play fighting. [58, 155]. Importantly, as is the case for play fighting in many species [157], the play of rats involves role reversals, in which the play partners alternate roles as attacker and defender. Across a wide variety of strains of rats, about 30% of play fights result in role reversals [196]. To ensure such reciprocal exchanges, the animals follow rules of engagement that allow partners to gain and relinquish the advantage during encounters [156]. This coordination presumably requires

some form of signaling to avoid the many situations in which misinterpretations might occur.

Rats have various body postures that appear to function as communication signals during sexual and aggressive interactions [197, 198], with at least some of these being reported in play fighting [135, 199]. Specific to play, rats often open their mouths [165] and hop vertically upwards [138] - gestures that could be useful as play signals. Further, during tickling it has been demonstrated that the rats' ear color and angle could also predict a positive affect [200]. However, as rats can play in complete darkness [78], such visual signals are unlikely to be practical for communication. The vertical hopping makes a distinctive noise that could be used by a rat to assess the playful state of its partner. Even so, such hops would be unlikely to signal to the partner that a particular action is to follow, as the hops can occur when the rat is both moving towards or away from the partner, and even when not involved in social interactions. This is unlike the case in dogs, in which bowing in front of one's partner immediately before lunging to bite provides clear information about the forthcoming action [193]. An alternative source of potential communication signals is the ultrasonic calls that are emitted in many social interactions [23, 40, 69, 201].

5.3. USVs as play signals

As both 50-kHz calls [29] and play [113] are associated with positive affective states, the frequent emission of calls in this bandwidth during play [23] makes them prime candidates for being potential signals during play [54]. What are called 50-kHz calls can range from 30-80 kHz [57]. Although there are different estimates as to the number of distinct types of calls, there is general agreement that there are multiple types [20, 22, 23, 26]. For example, it is agreed that those that involve some degree of frequency modulation

(FM) are different from those that are flat [23]. Most of the discord concerns the subdivision of the FM calls. In our studies, we have used the framework proposed by Wright et al. (2010), in which 13 distinct FM 50-kHz calls are recognized (Figure 5.1).

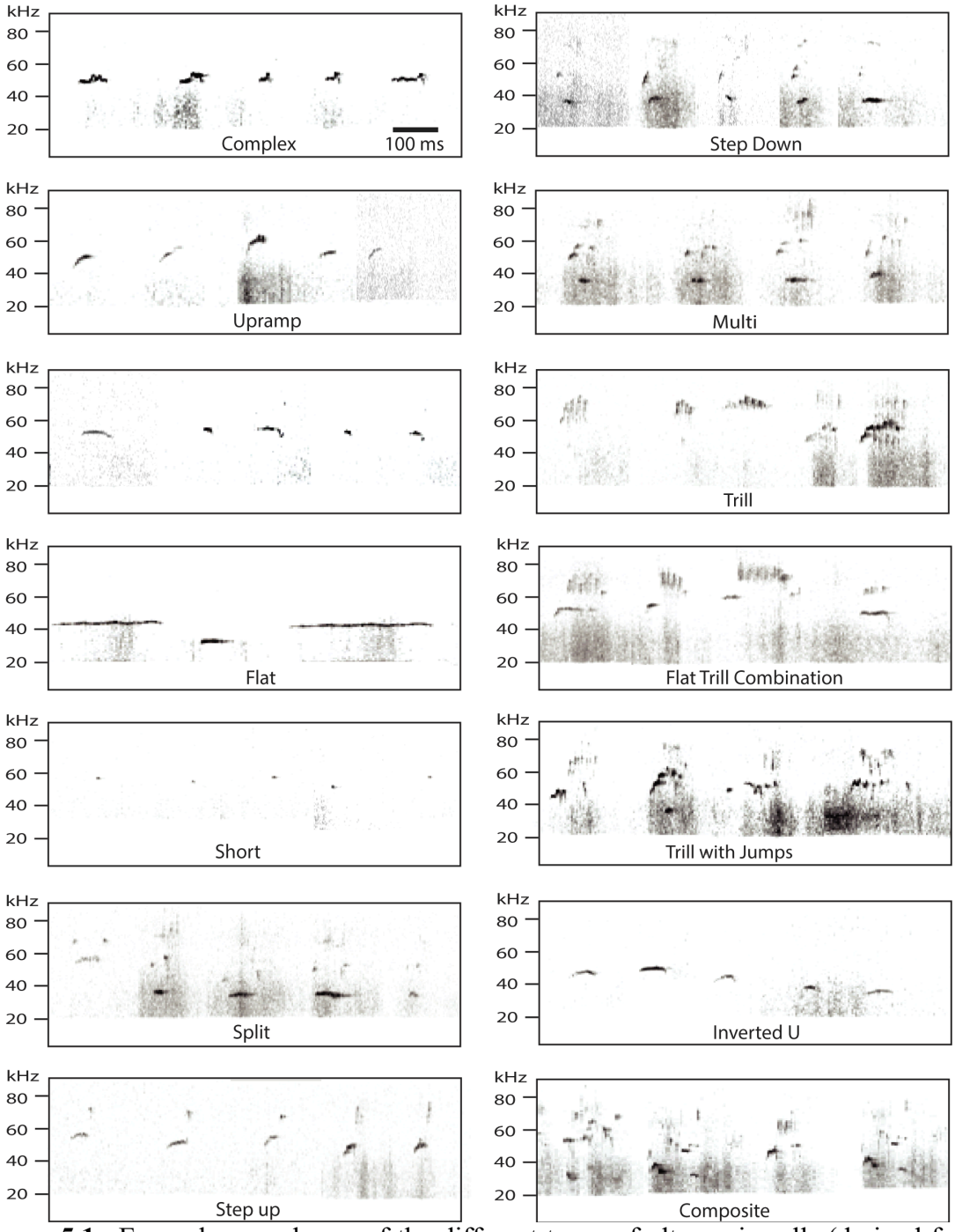

Figure 5.1. Examples are shown of the different types of ultrasonic calls (derived from Wright et al., 2010) that are used in the present study (reprinted with permission from Burke et al. (2017b)).

Rats are most likely to emit calls immediately prior to making playful contact [54] and these pre-contact calls are emitted both by the initiator and recipient of the contact [52]. This suggests that calling may facilitate playful contact as has been shown for many species using visual signals [187]. When rats are unable to hear, the frequency of close quarter wrestling during play fighting is diminished [58]. Moreover, pairs of rats that are unable to vocalize not only play less frequently than do vocal pairs, but also, and most critically, the incidence of role reversals is more than halved [52]. Thus, there is evidence that calls facilitate playful contact and regulate critical moments during encounters [202]. A key question that arises from these findings concerns which of the many calls are crucial for communication, and what they may signal.

5.4. What calls are associated with what behavior?

Central to determining whether particular calls are important for different communication functions during play is whether particular calls are associated with certain overt actions by the partners. When we first attempted such an analysis, we encountered a problem. The 13 different calls vary dramatically in their emission rates during play – 70-80% of all calls emitted are trills, with some of the rare ones accounting for only 1% [54]. Similarly, the actions performed during play (Table 4.1) can also vary markedly in occurrence [81]. Consequently, evaluating the possible association between rare calls and rare behavioral actions becomes a statistical challenge. In one study, we looked for associations between specific calls and the onset or offset of a play bout. Once corrected for multiple comparisons, the seven significant associations that we found in that study were reduced to one [54]. Rather than focus narrowly on calls tied to a specific behavior, in our next study, we cast our net more broadly, examining possible associations between

all calls and all behaviors within a given context using a Monte Carlo shuffling technique to determine chance levels of co-occurrence of calls and vocalizations. [30].

Table 5.1. Description of the social behaviors scored (reprinted from Burke et al 2018).

Behavior	Description
Nape	By slowly approaching or by pouncing, one rat moves towards the nape of its partner's neck with its snout.
Chase	Following an interaction, one of the animals chases its fleeing partner.
Pin Active	One animal stands over its supine partner, which by squirming, pushing with its forepaws and kicking with its hind feet, attempts to free itself or attack its partner. Conversely, the partner standing on top moves to block the supine animal's maneuvers.
Pin Passive	One animal stands over its supine partner, but the supine animal remains relatively immobile.
Sniff	One animal sniffs the face and flanks of its partner.
Sniff (Genital)	One animal sniffs the anogenital area of its partner.
Evade	The recipient of a nape attack protects against contact on its nape by dodging, running or jumping away.
Approach	One animal moves towards its partner, but without any clear indication that the nape is being targeted.
Follow	One animal moves in tandem or directly follows its partner. Unlike chasing, such following need not be preceded by an interaction.
Mutual Upright	Both rats face one another while rearing up on their hind feet, usually holding one another with their forepaws. From this position, they can sniff one another or actively push one another.

The video and audio files from each experimental session are synchronized so that behaviors and calls can be temporally compared (Figure 5.2 A). Frequently occurring calls, such as trills, may simply overlap many different behaviors and so appear more commonly associated with a variety of behavioral actions. For example, a count of 24 trill calls occurring during nape attacks may not be more frequent than that expected by chance, whereas six occurrences of a more infrequent call, such as the step up, may be. In the Monte Carlo method, the behaviors are left in the sequence in which they were scored (Figure 5.2A), but the times of occurrence of each vocalization are shuffled 10,000 times (Figure

5.2B). Each random shuffle is then compared to the behavioral events, generating a probability curve (Figure 5.2C) for each call and behavioral action. We can then take our arbitrary count of 24 trill calls emitted during nape attacks and compare that number to the probability curve generated by the shuffling. The further out on a tail of that distribution that raw score falls, the more likely that the association is greater than expected by chance (Figure 5.2C). The distance from the mean of the distribution can be assigned a z-score, with $z \geq \pm 1.96$ indicating that the chance of that association occurring by chance would have a probability of ≤ 0.05.

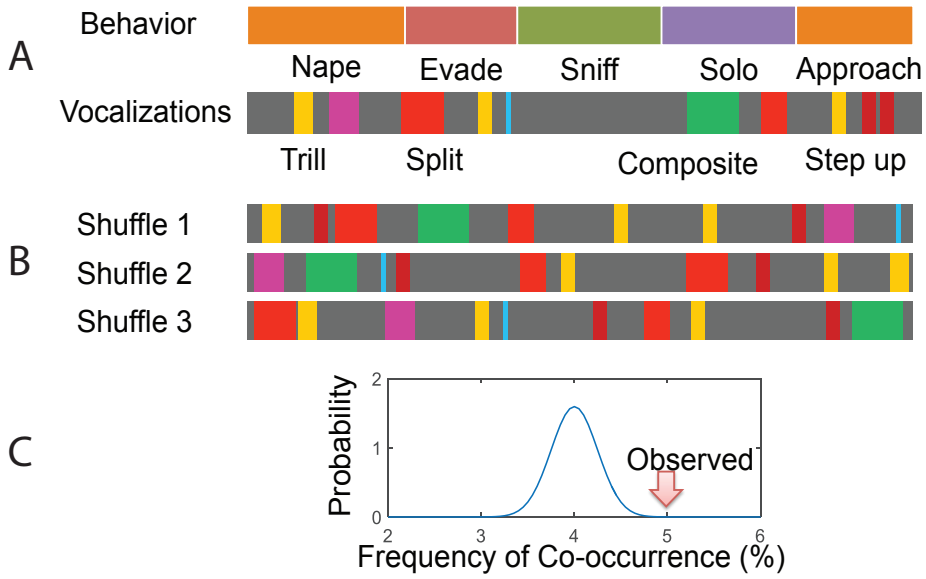

Figure 5.2. Visual representation of the Monte Carlo shuffling technique used to identify significant associations between behaviors and vocalizations.

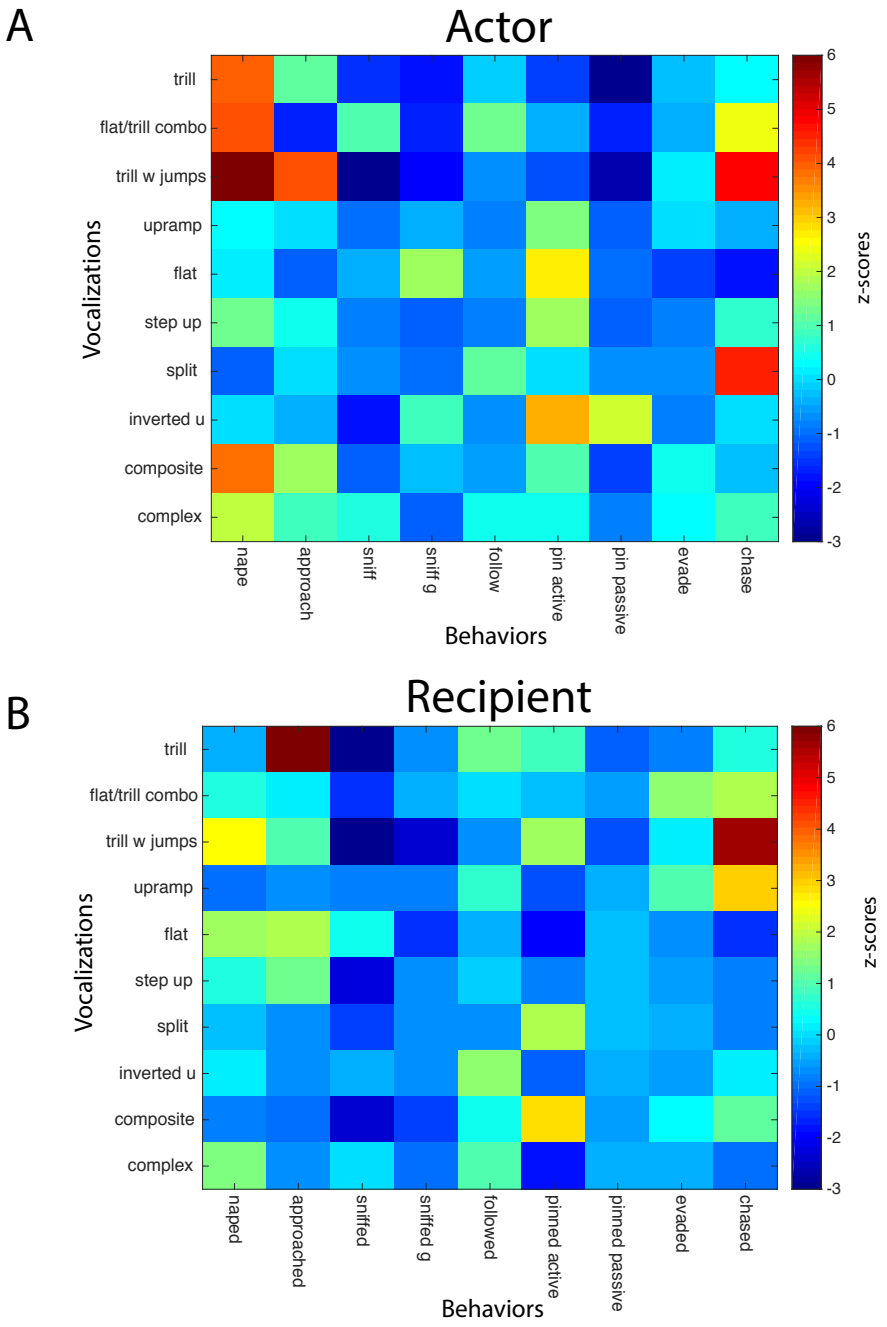

Figure 5.3. The matrix shows call types on one axis and the types of behavior on the other, with z-scores showing the strength of the associations between particular calls and particular behaviors, spanning from deep red for the strongest and deep blue for the weakest. This matrix shows the associations between calls and behaviors by actors (A) and recipients (B) (see text). The data are derived from pairs in which one partner was devocalized (reprinted with permission from Burke et al., 2018).

The data derived from this method shows that an animal is highly likely to utter particular calls when it performs particular actions and very unlikely to emit calls when engaged in other actions [1, 31]. These associations are represented by a color matrix showing calls on one axis and behavior on the other, with deep red indicating a highly positive association and dark blue indicating a highly negative association (Figure 5.3). When both partners are able to vocalize, it is not possible to determine which partner is emitting a particular call in a given context, therefore, we analyzed pairs of rats in which one was devocalized [52]. In this way, the calls by the intact rat could be matched to its behavior. In Figure 5.3A, the calls made by the rat performing behavioral actions are shown and in Figure 5.3B, the calls made by the rat when its partner performs the actions are shown. For example, a rat is highly likely to emit trills with jumps when approaching its partner (Figure 5.3A). When being chased, a rat is highly likely to emit trills (Figure 5.3B).

In general, calls are significantly less likely to be emitted when the rat is engaging in non-social behavior than when it is engaging in social behavior. In turn, calls are less likely to be emitted during social investigation but are highly likely to be emitted when engaged in play with its partner [1]. That is, calls are more strongly associated with actions involving playful contact than with actions, including social ones, not involving play. If particular calls are associated with particular actions by a partner, then the other animal could use those calls as an indicator of what will happen next and so alter its own behavior accordingly [83].

5.5. Communicating play via USVs

The key criterion for determining whether communication has occurred is that a presumptive signal emitted by one animal changes the probability of response in a recipient [203]. Some USVs have been associated with communication, as is evidenced by rats approaching speakers broadcasting those calls [15, 87], and by dams approaching the calls emitted by pups [204]. USVs have been shown to be associated with altering the behavior of conspecifics in non-playful contexts (e.g., [21, 205), and there is an

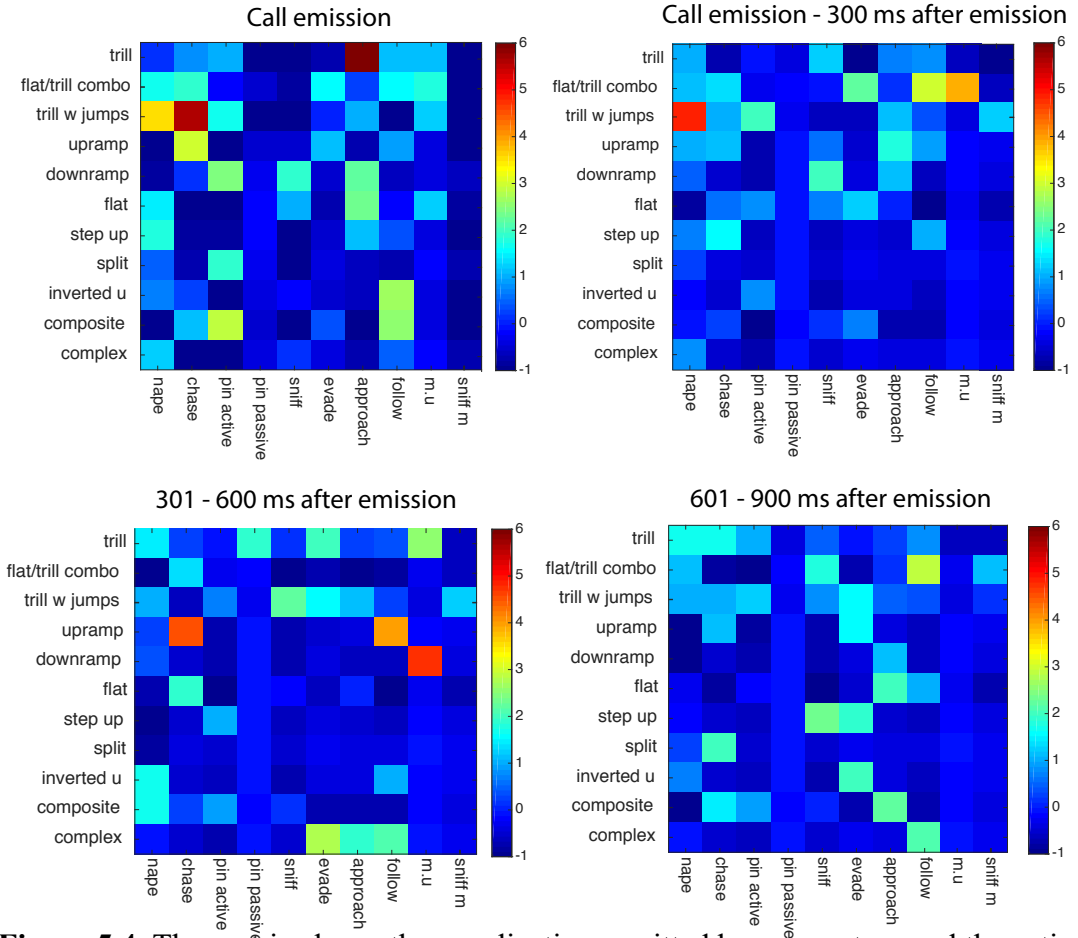

Figure 5.4. The matrix shows the vocalizations emitted by one partner and the actions performed by the recipient across four time bins: the time when the call is being emitted (call emission), from the end of call emission to 300 ms after the call, from 301-600 ms after the call and finally, from 601-900 ms after the call. The data are derived from pairs in which one partner was devocalized. M.U denotes mutual uprights and sniff m denotes mutual sniffing (i.e, the pair sniffs each other)

indication that this may also be the case during play [54]. To test this possibility more robustly, we used the Monte Carlo shuffling method to re-analyze the data presented in Burke et al. (2018) [1], to evaluate whether the call of one rat was correlated to the subsequent behavior of its partner. Again, by using pairs in which only one partner could vocalize, it was possible to identify the rat that emitted the calls and track the behavior of the recipient. Also, since signals can have both immediate and long lasting influences on the behavior of recipients [206], the possible associations between the calls of one animal and the behavior of the other was analyzed at different time intervals from the time at which the call was emitted.

The behavior of the recipient was scored at the time the call was emitted and then, subsequently, at 300 ms intervals. For further details on the use of such time bins, see Burke et al. (2018)[1]. As shown in Figure 5.4, particular calls by one animal are strongly correlated with the subsequent actions of its partner. Overall, the color plots at different times post-call indicate that there are specific actions by the recipient of particular calls and these associations change over time. That is, calls by one rat do indeed influence the subsequent behavior of its partner. To simplify the information from Figure 4 and so more readily illustrate the pattern of communication over time, only call-behavior associations with z-scores $\geq$ 2.58 were plotted, with the X-axis showing the behaviors and the Y-axis showing the time intervals. This simplified diagram shows that the number of associations between particular calls and particular behaviors decrease with time post call emission, so that no associations are present after 600 ms (Figure 5.5). In addition, it can be seen that particular calls are associated with particular behaviors and that these have limited temporal associations. For example, while the recipient is likely to start chasing the caller immediately after it emits the trill with jump calls, it attacks its partner's nape 300 ms after

the same call. Thus, while particular calls may have signaling value, how they are interpreted must also depend on the context in which they are used.

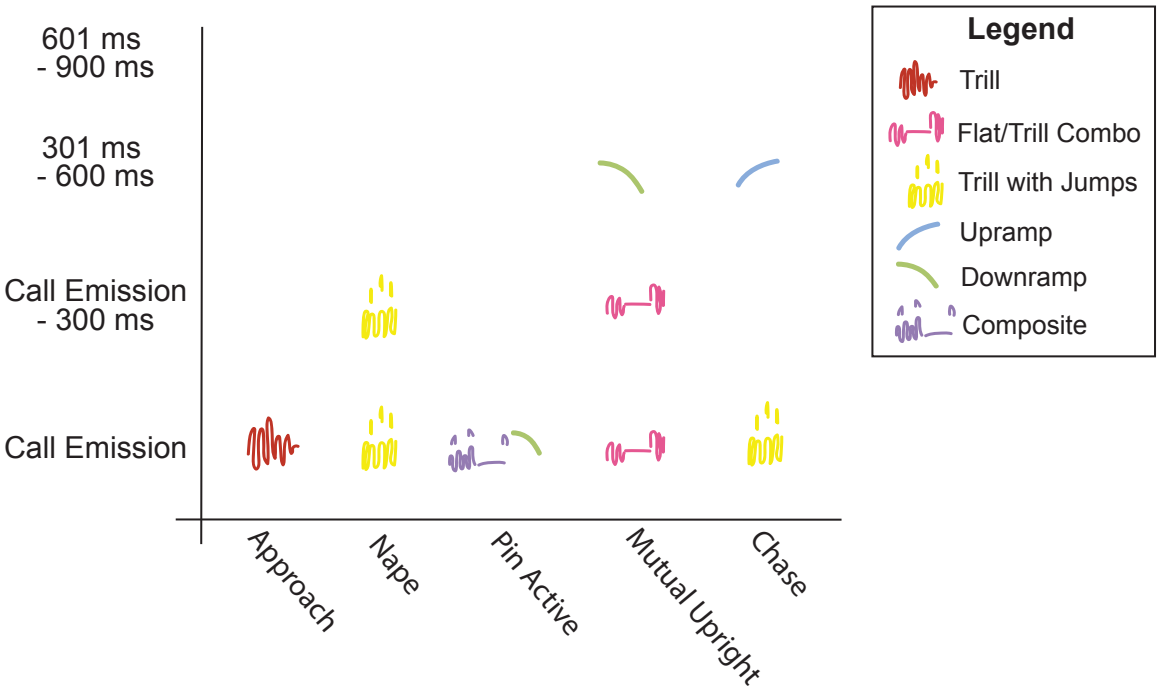

Figure 5.5. A simplified representation illustrates the temporal changes in the associations between calls by one animal and the behavior of the recipient, as represented in the matrices shown in Figure 4. Note that for this figure, a more stringent cut-off value of $z \geq 2.58$ was used.

Not only do these findings provide strong support for the communicative function of 50-kHz calls, but they also provide further insight into the variety of calls that are emitted. If all FM 50-kHz calls were simply variations of a common affective state, then it would be expected that all the calls would be just as likely to influence the partner's behavior in a similar manner. Our findings show that this is not the case; different calls alter the probability of different behaviors, suggesting that at least some of the calls are functioning to communicate specific information to the partner. This would suggest that there must be neural circuits that influence which calls are emitted in which contexts by the performer

(Figure 5.3), but also circuits that can interpret specific calls in those situations (Figures 5.4 and 5.5). This possibility does not invalidate the well-documented association of FM 50-kHz calls with positive affective states [29], but it does add a layer of complexity to the control mechanisms involved. For the calls to be used for communication, the neural circuitry involved in their production [25] needs to be modulated by some additional neural circuits to emit particular calls in particular contexts.

There is an alternative possibility that needs to be discounted before it can be concluded that it is the calling that alters the behavior of the recipient. That is, the behavior performed when the call is uttered may be the proximal cause of the change in the behavioral actions of the partner. Either the behavioral action itself may constrain the partner to behave in a specific [207] way or the action, rather than the vocalization, could provide a communication signal to the partner [208]. That calls can influence the recipient's behavior before playful contact is made [54] and can have continuing effects well after contact is terminated (present study) would suggest that the calls themselves may have some communicatory function of their own. Nonetheless, the relative importance of calls versus behavioral actions in altering the behavior of partners needs to be dissociated to understand fully the unique role of calling. Even so, the evidence to date does provide clues as to how calls may be used for communication during the play fighting of rats.

5.6. The functions of signaling during play

Some of the strongest associations between behavior and calls were for some of the least frequent calls [1, 31, 141]. Trills, which are the most frequently emitted calls [26, 54], are not only associated with approaching and contacting the nape [1], but also with locomotion in general [30]. It is possible that some of the more rarely emitted calls are critical to coordinate joint actions between partners, such as role reversals which are most

likely to occur when the pair are actively wrestling during a pin or during mutual uprights (see Table 5.1). In contrast, the more commonly emitted calls such as trills may have a more general mood enhancing function during play. There is a precedent for play signals having such multiple functions.

Keas, a parrot from New Zealand, emit an audible warble call when playing [209, 210]. Exposing keas to playbacks of warbles increases the recipients' engagement in play [163]. Importantly, both object and social play is increased, suggesting that the warble is elevating the recipients' playful mood, rather than inducing them to perform specific playful actions. Similarly, for some species of primates, the performance of play signals appears to help sustain the playful mood of the performer and/or recipient, and so facilitates continued playing [161, 162]. In other cases, particular signals and how they are coordinated with a partner appear to have a specific effect on the interaction. For example, in several species of mammals, close temporal synchrony of signaling and signal matching increases the likelihood of play fighting, the duration of playful contact and the degree of reciprocity in the playful wrestling (e.g., [162, 181, 182, 183]). Like these other species, the vocalizations used by rats during play could function as play mood enhancers and as directed signals that ensure that the animals coordinate their playful actions. Discerning which calls may have particular functions is a challenge yet to be resolved.

In order to determine which partner emits the call during play fighting in rats, we opted to test pairs in which one partner was devocalized and compared these patterns to those of pairs in which both could vocalize [37, 52]. Overall, the patterns of call and behavior associations are similar, but there are some minor differences that could be very important [1, 31]. In adult rats during interactions in which the sequence appears to deviate from the typical pattern, such as when play begins to escalate to aggression, emission of

particular calls, such as a flat combined with a trill appears to be essential. In intact pairs, in which both partners could call, the flat-trill combination call was often associated with a mutual upright posture. In pairs with a devocalized rat, however, this call was associated with other behaviors such as sniffing and pinning. Because the mutual upright posture often leads to a wrestling bout and, in some cases, one rat biting the other, we have suggested that the flat-trill call during a mutual upright posture may be necessary to mitigate subsequent aggression. Further, because the call is only made during the mutual upright posture when both rats are intact, it suggests that some kind of reciprocal calling is necessary to provide the right context for this call to be used [31]. Similar to the pattern seen in some species in which visual signals are coordinated between partners (e.g., [182, 183]), the inability of one rat to call appropriately may deprive the pair of an important mode of communication – reciprocal calling.

To explore this potential source of information transfer, techniques that can track the calls and behavior of both partners simultaneously are needed [59]. Recent methods using array microphones have promise but so far cannot disambiguate calls when the partners overlap in space, which is a common situation during play [211, 212]. But such a solution would exacerbate another current problem in scoring USVs - the sheer number and variety of calls emitted is daunting to score manually, especially when one is trying to match these to the animals' overt behavior. A possible solution would be to combine programs that can automate vocal scoring, such as Deep Squeak [213], and behavioral coding, such as Noldus Ethovision [214]. However, there is as yet no proven substitute for detailed manual scoring. Another problem is that we have focused on calls that have a distinguishable sonographic structure (see Figure 5.1), but calls also vary in their rate of production, frequency of emission, amplitude modulation and duration [215]. For example,

during play fighting between unfamiliar adult males, flat calls are emitted at around 50-60 kHz, but as the encounter escalates to aggression, the frequency of these calls is gradually lowered to around 30 kHz, and their duration increases [31]. When all these factors are considered, the potential information in the thousands of calls emitted during a standard 10 min play trial is staggering.

Even if we could measure all this variation, it is unknown what recipients recognize. Playback studies have shown that rats will approach some 50-kHz calls [22, 184], and that altering specific acoustic features can change the likelihood and latency of the approach. This means that the rats can distinguish some spectral properties of calls, but the entire range of variation possible has not been tested. More work is required to determine which call properties serve a communicative function and which call categories are important to the animal, and so the types of calls and call properties that we should be scoring.

5.7. Conclusion

Despite all the unknowns, our work has shown that 50-kHz calls are not randomly emitted but are produced when the performer is engaged in particular actions [1, 31], with some of those calls likely having highly specific influences on the subsequent behavior of the recipient (new data presented in this review). Together, these findings support the possibility that some calls in some situations function as a means of communication – that the calls act as signals that facilitate the occurrence of play, ensure that it remains playful and that it continues. The production mechanisms for both the affective and aversive calls are organized subcortically [15]. However, given that at least some calls can be modulated contextually, which seems to imply that they are used tactically to influence their partner's behavior (see Figure 5.4 and 5.5), it is possible that the prefrontal cortex may be important for making decisions on the context and timing of calls. In support of this possibility, social

isolation during the juvenile period decreases the ability to respond to particular USVs in contextually appropriate ways [216]. Interestingly, that same deprivation leads to altered organization of the prefrontal cortex and diminished executive functions with associated impoverishment in social skills [150, 151, 217, 218]. Recent evidence also points to the importance of the insula, which has strong reciprocal connectivity with prefrontal cortex, in social decision making [219]. It is possible that not only are calls important for communication during play, but also that play provides experiences that refine the development of the cortical mechanisms that improve the rats' ability to use different USVs in a contextually appropriate manner [83].

5.8. Acknowledgments

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Chapter 6: Discussion

6.1. General Discussion

The purpose of this thesis was to test the social theory of rat vocalization. The four experiments conducted support the theory. Below, I will discuss the main findings and their significance with respect to the question of the evolutionary value of vocalization.

6.1.1. Summary of Thesis Objectives

The first experiment explored the different vocal responses to social and food rewards. I found that the play reward group was the only group to produce significant increases in 50-kHz USVs over the 7 days of testing. The food condition and social control group did not show this increase. There was also a difference in call emission between conditions; the food conditions (both control and reward) emitted significantly fewer calls then the play condition. The main difference in the emission of calls in the play and food groups was in trills and trills with jumps. These are the same two calls that correlate with the expectation of social reward. This adds to the evidence that these calls have a social role.

The second experiment explored whether 50-kHz social anticipatory calls differed in a depressed phenotype. I demonstrated that, although both strains learned to anticipate the arrival of a social partner, the WKY rats (depressed phenotype) were less active, vocalized less, and used significantly fewer types of 50-kHz calls in comparison to the WI rats. Further, this difference between the strains continued into adulthood, with the WKY rats demonstrating a reduced motivation to play. This study provides evidence that

vocalizations represent an affective state, as. In the depressed state, in which reduced socialization would be expected, there was a poverty of social calls.

The third experiment evaluated whether the emission of 50-kHz USVs were the same in both tickling and social play. I found that, although 50-kHz USVs were emitted in both ticking and conspecific play, the contextual use of the calls was markedly different. Tickling induced double the calling in comparison to conspecific play, but these calls were used indiscriminately. How? Further, in both contexts, non-social periods were negatively associated with calls, indicating that there is a tight linkage between USVs and social interaction.

The fourth experiment examined whether 50-kHz USVs had an impact on the behaviour of a conspecific. I found that the emission of specific calls has an impact on the behaviour of the conspecific. Further, there is a temporal association between the calls and behaviour, with some calls having long-term effects and some calls having short-term effects. This finding provides evidence for social communication as the signals of one rat influence the behaviour of the conspecific.

6.1.2 Comparisons Across Experiments

The relation between UVS and social behaviour across experiments showed a number of consistencies. USVs were not random, they occurred in a social context, and only certain calls predominated. I will discuss each of these points in turn.

6.1.2.1. Calling is not random

The first consistent finding between the experiments is that calls were linked to behaviour and thus, not emitted randomly. All experiments showed USVs were predominantly associated with active or social behaviours. In the first experiment, USVs were emitted in both contexts, anticipation of food and play, however, most vocal-behavioural associations were related to the play context, with the play reward group having the most vocal-behavioural pairings. In the second experiment, calls were paired with behaviour while both strains of rats (WI and WKY) anticipated the arrival of a partner. In the third experiment, during both tickling and play, the rats again paired calls with behaviour, and a negative association existed between non-social interactions and USVs. Finally, in the last experiment, data were presented from vocal-devocalized pairings, showing that again, calls were paired with behaviour, but these associations also have a temporal relationship.

6.1.2.2. Context of Calls

The experiments presented in this thesis also demonstrate that, in different ages and strains, the context appears critical, especially in social contexts, when and if calling will occur. In the first experiment, I demonstrated that USVs were intrinsically linked to the social context, specifically receiving a social partner to play. Both the control group and play group paired trill type USVs with behaviour on both day 1 and 7 of testing. Interestingly, the food condition started with trill associations with walking on day 1, but lost the associations on day 7. It is likely that in their natural habitat rats do not emit calls to obtain food whereas they do so in social contexts. Thus, these calls, especially the trill type calls, are paired contextually in social settings. In the second experiment, I saw that both phenotypes of rats (the depressed and control) showed anticipation of social reward

by increasing their USVs from day 1 to 7, however, the depressed group, showed an impoverished pattern of vocal-behavioural correlates, and reduced variety of calls. This is interesting because this reduction mimics the play results, with the WKY playing less and using less aggressive forms of attack. This again points to the context in which the animals are anticipating alters the vocal-behavioural correlates.

In the last two experiments, examining play itself, not anticipation, I again saw that context alters the behavioural-vocal correlates. The third experiment shows that as in the second experiment, although social contexts elicit high levels of USVs and create predictable vocal-behavioural correlates, the context in which the calls are being used changes these correlates. In this case, comparing heterospecific (tickling) and conspecific play means that different calls are paired with different behaviours in these two contexts, and although both are social, they are clearly not the same to the rat.

6.1.2.3. Trill calls and social context

In all the experiments, during social contexts, trill calls had significant behavioural associations. In the first experiment, on day 1, all groups showed a trill – walk association, however, only the social condition kept this association into day 7. Further, the social reward group had associations with trill type calls and running, and flat type calls and jumps. Clearly, trills appear important for the impending arrival of a partner. In the depression experiment, again in anticipation of play, both groups showed the trill- walk association on day 7 at both age groups. However, the WI controls not only made more types of calls, but they also had more behavioural associations. In the play contexts, trill calls are usually associated with nape attacks, as seen by the conspecific play condition, interestingly, although plenty of trill calls were produced by the

heterospecific play group, the trills were not significantly paired in this group, thus, potentially the trill and nape association may be specifically for conspecific play. Finally, it appears that trill type calls could be used to initiate interactions, as they appear to be the precursor to many types of play interactions.

6.1.3. Evaluating the Affective State Theory

6.1.3.1. Evidence from social and non-social rewards

In this study, we used anticipation of play and food to assess whether positive rewards elicit the same 50-kHz calls. It was previously shown by two recent studies that examined vocalizations elicited in the presence of either a female or food, that the female elicited significantly more trill type calls [76, 77]. The affective state theory predicts that if a particular 50 kHz call communicates positive affect, we would expect to see elevated rates of this call type during anticipation of both food and play. A potential candidate for this call would be trills, as previously discussed, they are the predominant type of call in the anticipation of a variety of rewards [26]. This was not the case, the only group that had a significant increase in calls was the social reward group. Further, only the social conditions had vocal-behavioural correlates after training, if the calls were simply made to reflect positive affect, then we would expect the vocal-behavioural correlates to be the same between conditions, which was not the case.

Although this study does provide convincing evidence against the affective state theory, we must consider some potential pitfalls of the experiment. Due to the ages of the animals, it may not be possible to do a strict comparison between food and social reward because food deprivation studies work better with adults due to the ability to do a more drastic food restriction paradigm, and play predominates in the juvenile period. Further,

the study did not show an increase in 50-kHz USVs over days of testing in the food condition, as is reported in some other work [50, 66, 68, 76]. Therefore, the animals may have lacked the proper motivation to assess food deprivation. However, there is still compelling evidence that USVs are socially linked and are not simply used as an indicator of affective state.

6.1.3.2. Evidence from depressive phenotypes

The affective state theory would predict that, in a depressed phenotype, like WKY rats, because of the generalized reduction in positive affect, 50-kHz USVs should be reduced in the anticipatory period. However, it does not predict that different calls would be emitted, or different behavioural-vocal correlates would exist, as there should be simply a global reduction in calls and associations. Although the WKY group did vocalize less, it was not simply an overall reduction in calls, the types of calls were different and how the calls were used also differed. This indicates that while the number of USVs could predict affect state, how the calls are used suggests social communication, with the call-behaviour associations reflecting altered play behaviour.

6.1.3.3. Evidence from heterospecific and conspecific play

Because both conspecific and heterospecific play are positive experiences, the affective state theory again predicts similar call production and vocal-behavioural correlates. However, I again found that the two conditions elicit different numbers and types of calls, and with heterospecific play, the rats significantly reduce their vocal-behavioural correlates. First, this indicates that conspecific and heterospecific play, although both forms of play, are clearly not perceived as being the same by the rats. Second, the conspecific condition used vocal-behavioural correlates as previously

described [1, 31], while in the heterospecific condition few such associations were present, indicating that while playing with another rat vocal-behavioural correlates are important, this is not the case with a human hand.

6.1.3.4. Evidence from the behavioural effects of USVs

The last piece of evidence against the affective state theory is that the vocalizations emitted by one animal changes the likelihood of the behaviour being performed by the partner. The affective state theory would not predict this, as the emission of calls should just convey a positive mood [29], however, the calls appear to not only change the behaviour of the partner, but also have different temporal effects. This is consistent with findings that particular calls may facilitate role reversals during the play by juveniles [1, 37] and others may be critical to avoid play fighting escalating to serious fighting in adult males [31].

6.1.4. Neural Implications

Vocal emission is regulated by the cholinergic system and the mesolimbic dopaminergic system [15]. The cholinergic system originates from the laterodorsal tegmental nucleus and travels to the medial regions of the diencephalon, basal forebrain, and lateral septum. Activation of the cholinergic system induces defensive behavior and production of 22-kHz vocalizations [34, 220]. Pharmacologically, giving cholinomimetics causes the production 22- kHz calls, while antagonism of the system decreases the number 22-kHz calls [34]. The mesolimbic dopaminergic system originates in the ventral tegmental area and travels to the ventral striatum, and in contrast to the cholinergic system, activation of the dopaminergic system causes increased locomotor activity, exploration, and the production of 50-kHz calls [49, 221, 222].

The two distinct pathways are generally assumed to be in support of the affective state theory of vocalizations, with separate systems for positive and negative affect. However, as a potential pathway for social communication, the striatum and the prefrontal cortex that are part of the dopaminergic system, have implications for understanding communication during play.

The prefrontal cortex is involved in regulating executive functions, and these skills are important for regulating play. Lesion studies of the medial prefrontal cortex (mPFC) show that rats are limited to simpler patterns of playing [223], which appears to be due to a reduced ability to coordinate one's movements with those of the partner [133]. This provides evidence that the mPFC is used to fine tune behaviour during play, and is, in turn, influenced by the experience of play in the juvenile period, pruning the dendritic morphology of mPFC neurons [224]. Further, the mPFC appears to be critical for emotional learning, that is important for social play [86]. There is also unpublished evidence from the Pellis/Euston lab that show that mPFC lesioned rats lose the ability to contextualize their vocalizations. Thus, it is likely that play and vocal regulation during play both involve control by the mPFC.

The striatum controls voluntary motor output based on emotional and cognitive information, which again should have a key role in play regulation. Indeed, neonatal lesioning of striatal DA system results in animals showing truncated play bouts and sequences [225]. The DA producing neurons in the ventral tegmental area (VTA) and their projection targets, including the mPFC, amygdala (AMY) and nucleus accumbens (NAc), all of which are heavily implicated in motivation and emotion related processing [226]. Again, because the striatum is implicated in vocal production it is likely that this area would also be involved in the control of vocalizations during play. The methods I

have refined in this thesis to analyze the interface between actions performed during play and the USVs emitted would be helpful in further exploring how these neural mechanisms are used to ensure effective communication during play.

6.1.5. Caveats

Although the experiments in this thesis provide convincing evidence in favour of a social communication theory of USVs, there are some caveats related to experimental design and results that need to be considered.

Experiment 4, which posits communication based on behavioural modification by the receiver, was completed with pairs of rats in which one could vocalize and the other could not. The purpose of this was, of course, to allow me to be able to identify which animal produced the calls and so make clear what behaviours were correlated with which calls. When two vocal animals are together, it is not possible to distinguish which of the two animals are calling. So, although social communication is the focus of this thesis, the absence of actual socialization between two normal animals is a major weakness of this thesis. There has been recent work using array microphones to localize the calls of individual free moving mice [227]. This set up would allow for a better understanding of the social communication when multiple rats interact.

The primary method used in experiments 1 and 2 was anticipation behaviours, where the rats await reward in a plexiglass box. An alternative to this method, for the social condition, would be to separate the rats using a barrier and then lift the barrier after the allotted time. However, the presence of another animal (smells, calls and behaviour) would likely alter the behaviour of the target rat. For simplicity, I decided to use the

methods described above, however, this design provides an exciting avenue for future studies.

Although the strain and age of the rats differed between experiments, none of the data in this thesis deals with female rats. This is problematic as the conclusions of the theory should be generalizable to all rats. It is always possible that females would not perform in the same manner as male rats. Nevertheless, there is evidence that females also use ultrasonic calls as a form of communication during mating and play [228-230]. Follow up studies need to be done in order to discover if the social communication theory applies to females in the same way I have demonstrated to be the case for males. Further, from vocal profile analysis, although each experiment had crossover between the results, it is obvious that some differences exist. A detailed strain comparison needs to be done in order to distinguish if these differences are derived from strain differences or are the effect of the experimental manipulation.

6.2. Conclusion

The purpose of this thesis was to test the social communication theory of rat vocalizations. Four experiments tested different aspects of the theory and provided evidence that indeed, although the calls relate to affective state, it appears that they also serve a socio-communicative function. The implications for such a finding are twofold, first, this gives new insight into why these USVs are produced, thus providing new, testable avenues for deciphering what individual calls communicate. Second, this gives a new model of vocal animal communication in which pharmaceutical treatments can be tested. For example, in experiment 2, the findings that anticipation of play creates quantifiable differences between groups allows for the use of pharmaceuticals to alleviate

the symptoms associated with depression. This model is significantly closer to the human experience compared to that of other depression tests. Demonstrating that rats have an effective vocal communication system means that rats can be used as social models of other human language and social disorders like autism. Overall, USVs being used for social communication unearths many more questions to answer and a new problem, to understand what the rats are saying.

6.3 References

1. Burke, C.J., et al., *Do juvenile rats use specific ultrasonic calls to coordinate their social play?* Animal Behaviour, 2018. **140**: p. 81-92.
2. Anderson, J.W., *The Production of Ultrasonic Sounds by Laboratory Rats and Other Mammals.* Science, 1954. **119**(3101): p. 808-809.
3. Rosenzweig, M.R., D.A. Riley, and D. Krech, *Evidence for Echolocation in the Rat.* Science (American Association for the Advancement of Science), 1955. **121**(3147): p. 600-600.
4. Sales, G. and D. Pye, *Ultrasonic communication by animals*. 1974: Chapman and Hall.
5. Okon, E.E., *The effect of environmental temperature on the production of ultrasounds by isolated non-handled albino mouse pups.* Journal of Zoology, 1970. **162**(1): p. 71-83.
6. Noirot, E., *The onset of maternal behavior in rats, hamsters, and mice: A selective review*, in *Advances in the study of behavior: IV*. 1972, Academic Press: Oxford, England. p. xiv, 217-xiv, 217.
7. Allin, J.T. and E.M. Banks, *Functional aspects of ultrasound production by infant albino rats (Rattus norvegicus).* Animal Behaviour, 1972. **20**(1): p. 175-185.
8. Blumberg, M.S., I.V. Efimova, and J.R. Alberts, *Thermogenesis during ultrasonic vocalization by rat pups isolated in a warm environment: A thermographic analysis.* Developmental Psychobiology, 1992. **25**(7): p. 497-510.
9. Shair, H.N., *Factors affecting emission of infantile vocalizations and vocal expression of emotional states*, in *Handbook of ultrasonic vocalization: A window into the emotional brain.* 2018, Elsevier Academic Press: San Diego, CA, US. p. 157-168.
10. Boulanger-Bertolus, J., et al., *Understanding pup affective state through ethologically significant ultrasonic vocalization frequency.* Scientific reports, 2017. **7**(1): p. 1-12.
11. Brudzynski, S.M., P. Kehoe, and M. Callahan, *Sonographic structure of isolation-induced ultrasonic calls of rat pups.* Developmental Psychobiology, 1999. **34**(3): p. 195-204.
12. Nitschke, W., *Acoustic behavior in the rat: research, theory, and applications*. 1982: Greenwood.
13. Borszcz, G.S., *Contribution of the ventromedial hypothalamus to generation of the affective dimension of pain.* Pain, 2006. **123**(1-2): p. 155-168.
14. Blanchard, R.J., K.J. Flannelly, and D.C. Blanchard, *Defensive Behaviors of Laboratory and Wild Rattus norvegicus.* Journal of comparative psychology (1983), 1986. **100**(2): p. 101-107.
15. Brudzynski, S.M., *Communication of adult rats by ultrasonic vocalization: biological, sociobiological, and neuroscience approaches.* ILAR J, 2009. **50**(1): p. 43-50.
16. Sanders, I., et al. *The mechanism of ultrasonic vocalization in the rat*. in *Soc. Neurosci. Abstr*. 2001.
17. Mahrt, E., et al., *Mice produce ultrasonic vocalizations by intra-laryngeal planar impinging jets.* Current biology, 2016. **26**(19): p. R880-R881.

18. Brudzynski, S.M., et al., *Analysis of 22 kHz ultrasonic vocalization in laboratory rats: long and short calls.* Physiol Behav, 1993. **54**(2): p. 215-21.
19. Brudzynski, S.M. and G. Holland, *Acoustic characteristics of air puff-induced 22-kHz alarm calls in direct recordings.* Neuroscience & Biobehavioral Reviews, 2005. **29**(8): p. 1169-1180.
20. Burgdorf, J., et al., *Neurobiology of 50-kHz ultrasonic vocalizations in rats: electrode mapping, lesion, and pharmacology studies.* Behav Brain Res, 2007. **182**(2): p. 274-83.
21. Wöhr, M., et al., *Effects of experience and context on 50-kHz vocalizations in rats.* Physiol Behav, 2008. **93**(4-5): p. 766-76.
22. Wohr, M., et al., *Effects of experience and context on 50-kHz vocalizations in rats.* Physiol Behav, 2008. **93**(4-5): p. 766-76.
23. Burgdorf, J., et al., *Ultrasonic vocalizations of rats (Rattus norvegicus) during mating, play, and aggression: Behavioral concomitants, relationship to reward, and self-administration of playback.* J Comp Psychol, 2008. **122**(4): p. 357-67.
24. Panksepp, J. and J. Burgdorf, *50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables.* Behav Brain Res, 2000. **115**(1): p. 25-38.
25. Brudzynski, S.M., *Ethotransmission: communication of emotional states through ultrasonic vocalization in rats.* Curr Opin Neurobiol, 2013. **23**(3): p. 310-7.
26. Wright, J.C. Gourdon, and P.B. Clarke, *Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context.* Psychopharmacology (Berl), 2010. **211**(1): p. 1-13.
27. Brudzynski, S.M. and J.S. Burgdorf, *Recent Studies of Rat Ultrasonic Vocalizations—Editorial.* Brain Sciences, 2021. **11**(11): p. 1390.
28. Blumberg, M.S., *Rodent ultrasonic short calls: locomotion, biomechanics, and communication.* J Comp Psychol, 1992. **106**(4): p. 360-5.
29. Knutson, B., J. Burgdorf, and J. Panksepp, *Ultrasonic vocalizations as indices of affective states in rats.* Psychol Bull, 2002. **128**(6): p. 961-77.
30. Burke, C.J., et al., *Specific 50-kHz vocalizations are tightly linked to particular types of behavior in juvenile rats anticipating play.* PLoS One, 2017. **12**(5): p. e0175841.
31. Burke, C.J., et al., *Avoiding escalation from play to aggression in adult male rats: The role of ultrasonic calls.* Behav Processes, 2017. **144**: p. 72-81.
32. Burke, C.J., et al., *Rat 50 kHz Trill Calls Are Tied to the Expectation of Social Interaction.* Brain Sci, 2021. **11**(9).
33. Blumberg, M.S. and G. Sokoloff, *Do infant rats cry?* Psychological review, 2001. **108**(1): p. 83.
34. Brudzynski, S.M., *Pharmacological and behavioral characteristics of 22 kHz alarm calls in rats.* Neuroscience & Biobehavioral Reviews, 2001. **25**(7-8): p. 611-617.
35. Alves, J.A., B.C. Boerner, and D.A. Laplagne, *Flexible coupling of respiration and vocalizations with locomotion and head movements in the freely behaving rat.* Neural Plasticity, 2016. **2016**.

36. Riede, T., *Stereotypic laryngeal and respiratory motor patterns generate different call types in rat ultrasound vocalization.* J Exp Zool A Ecol Genet Physiol, 2013. **319**(4): p. 213-24.
37. Kisko, T.M., D.R. Euston, and S.M. Pellis, *Are 50-khz calls used as play signals in the playful interactions of rats? III. The effects of devocalization on play with unfamiliar partners as juveniles and as adults.* Behav Processes, 2015. **113**: p. 113-21.
38. Laplagne, D.A. and M. Elias Costa, *Rats Synchronize Locomotion with Ultrasonic Vocalizations at the Subsecond Time Scale.* Front Behav Neurosci, 2016. **10**: p. 184.
39. Panksepp, J., et al., *Regional brain cholecystokinin changes as a function of friendly and aggressive social interactions in rats.* Brain Res, 2004. **1025**(1-2): p. 75-84.
40. Thomas, D.A., L.K. Takahashi, and R.J. Barfield, *Analysis of ultrasonic vocalizations emitted by intruders during aggressive encounters among rats (Rattus norvegicus).* J Comp Psychol, 1983. **97**(3): p. 201-6.
41. Inagaki, H., et al., *The influence of social environmental condition on the production of stress-induced 22 kHz calls in adult male Wistar rats.* Physiology & behavior, 2005. **84**(1): p. 17-22.
42. Barfield, R.J. and L.A. Geyer, *Sexual behavior: ultrasonic postejaculatory song of the male rat.* Science, 1972. **176**(4041): p. 1349-1350.
43. McIntosh, T.K., R.J. Barfield, and D. Thomas, *Electrophysiological and ultrasonic correlates of reproductive behavior in the male rat.* Behavioral neuroscience, 1984. **98**(6): p. 1100.
44. Blanchard, D.C., R.J. Blanchard, and G. Griebel, *Defensive responses to predator threat in the rat and mouse.* Current protocols in neuroscience, 2005. **30**(1): p. 8.19. 1-8.19. 20.
45. Brudzynski, S.M. and D. Ociepa, *Ultrasonic vocalization of laboratory rats in response to handling and touch.* Physiology & behavior, 1992. **52**(4): p. 655-660.
46. Kikusui, T., et al., *Conditioned fear-related ultrasonic vocalizations are emitted as an emotional response.* Journal of veterinary medical science, 2003. **65**(12): p. 1299-1305.
47. Simmons, S.J., D.J. Barker, and M.O. West, *Ultrasonic vocalizations capture opposing affective states during drug self-administration: revisiting the opponent-process model of addiction*, in *Handbook of Behavioral Neuroscience*. 2018, Elsevier. p. 389-399.
48. Knutson, B., J. Burgdorf, and J. Panksepp, *High-frequency ultrasonic vocalizations index conditioned pharmacological reward in rats.* Physiol Behav, 1999. **66**(4): p. 639-43.
49. Burgdorf, J., et al., *Nucleus accumbens amphetamine microinjections unconditionally elicit 50-kHz ultrasonic vocalizations in rats.* Behav Neurosci, 2001. **115**(4): p. 940-4.
50. Burgdorf, J., B. Knutson, and J. Panksepp, *Anticipation of rewarding electrical brain stimulation evokes ultrasonic vocalization in rats.* Behav Neurosci, 2000. **114**(2): p. 320-7.

51. Bialy, M., M. Rydz, and L. Kaczmarek, *Precontact 50-kHz vocalizations in male rats during acquisition of sexual experience.* Behav Neurosci, 2000. **114**(5): p. 983-90.
52. Kisko, T.M., et al., *Are 50-kHz calls used as play signals in the playful interactions of rats? II. Evidence from the effects of devocalization.* Behav Processes, 2015. **111**: p. 25-33.
53. Knutson, B., J. Burgdorf, and J. Panksepp, *Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats.* J Comp Psychol, 1998. **112**(1): p. 65-73.
54. Himmler, B.T., et al., *Are 50-kHz calls used as play signals in the playful interactions of rats? I. Evidence from the timing and context of their use.* Behav Processes, 2014. **106**: p. 60-6.
55. Burgdorf, J. and J. Panksepp, *Tickling induces reward in adolescent rats.* Physiol Behav, 2001. **72**(1-2): p. 167-73.
56. Thomas, D.A.H., S. Beth ;Barfield, Ronald J. , *Male-produced ultrasonic vocalizations and mating patterns in female rats.* Journal of comparative & physiological psychology, 1982. **96**(5): p. 807-815.
57. Wöhr, M. and R.K. Schwarting, *Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior?* PLoS One, 2007. **2**(12): p. e1365.
58. Siviy, S.M. and J. Panksepp, *Sensory modulation of juvenile play in rats.* Dev Psychobiol, 1987. **20**(1): p. 39-55.
59. Kisko, T.M., et al., *From Play to Aggression: High-Frequency 50-kHz Ultrasonic Vocalizations as Play and Appeasement Signals in Rats.* Curr Top Behav Neurosci, 2017. **30**: p. 91-108.
60. Riebel, K., et al., *New insights from female bird song: towards an integrated approach to studying male and female communication roles.* Biology letters (2005), 2019. **15**(4): p. 20190059-20190059.
61. Slater, P.J., *Bird song: acoustic communication in birds.* Science (American Association for the Advancement of Science), 1983. **222**(4620): p. 155-156.
62. Logue, D.M. and M.L. Hall, *Migration and the evolution of duetting in songbirds.* Proceedings of the Royal Society. B, Biological sciences, 2014. **281**(1782): p. 20140103-20140103.
63. Fischer, J. and T. Price, *Meaning, intention, and inference in primate vocal communication.* Neuroscience and biobehavioral reviews, 2017. **82**: p. 22-31.
64. Winkler, S.L. and G.A. Bryant, *Play vocalisations and human laughter: a comparative review.* Bioacoustics (Berkhamsted), 2021. **30**(5): p. 499-526.
65. Wintink, A.J. and S.M. Brudzynski, *The related roles of dopamine and glutamate in the initiation of 50-kHz ultrasonic calls in adult rats.* Pharmacol Biochem Behav, 2001. **70**(2-3): p. 317-23.
66. Brenes, J.C. and R.K. Schwarting, *Individual differences in anticipatory activity to food rewards predict cue-induced appetitive 50-kHz calls in rats.* Physiol Behav, 2015. **149**: p. 107-18.
67. Buck, C.L., et al., *Dopamine D1 and μ-opioid receptor antagonism blocks anticipatory 50 kHz ultrasonic vocalizations induced by palatable food cues in Wistar rats.* Psychopharmacology (Berl), 2014. **231**(5): p. 929-37.
68. Opiol, H., et al., *Ultrasonic vocalizations in rats anticipating circadian feeding schedules.* Behav Brain Res, 2015. **284**: p. 42-50.

69. Thomas, D.A. and R.J. Barfield, *Ultrasonic vocalization of the female rat (Rattus norvegicus) during mating.* Animal Behaviour, 1985. **33**(3): p. 720-725.
70. Börner, A., et al., *Ultrasonic vocalizations of female Norway rats (Rattus norvegicus) in response to social partners.* J Comp Psychol, 2016. **130**(1): p. 76-80.
71. Willey, A.R. and L.P. Spear, *Development of anticipatory 50 kHz USV production to a social stimuli in adolescent and adult male Sprague-Dawley rats.* Behav Brain Res, 2012. **226**(2): p. 613-8.
72. Brudzynski, S.M. and A. Pniak, *Social contacts and production of 50-kHz short ultrasonic calls in adult rats.* J Comp Psychol, 2002. **116**(1): p. 73-82.
73. Blanchard, R.J., et al., *High-frequency (35-70 kHz) ultrasonic vocalizations in rats confronted with anesthetized conspecifics: effects of gepirone, ethanol, and diazepam.* Pharmacol Biochem Behav, 1993. **44**(2): p. 313-9.
74. Wright, J.M., J.C. Gourdon, and P.B. Clarke, *Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context.* Psychopharmacology (Berl), 2010. **211**(1): p. 1-13.
75. Takahashi, N., M. Kashino, and N. Hironaka, *Structure of rat ultrasonic vocalizations and its relevance to behavior.* PLoS One, 2010. **5**(11): p. e14115.
76. Willey, A.R. and L.P. Spear, *The effects of pre-test social deprivation on a natural reward incentive test and concomitant 50 kHz ultrasonic vocalization production in adolescent and adult male Sprague-Dawley rats.* Behav Brain Res, 2013. **245**: p. 107-12.
77. Mulvihill, K.G. and S.M. Brudzynski, *Non-pharmacological induction of rat 50 kHz ultrasonic vocalization: Social and non-social contexts differentially induce 50 kHz call subtypes.* Physiol Behav, 2018. **196**: p. 200-207.
78. Himmler, B.T., V.C. Pellis, and S.M. Pellis, *Peering into the dynamics of social interactions: measuring play fighting in rats.* J Vis Exp, 2013(71): p. e4288.
79. Niesink, R.J. and J.M. Van Ree, *Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats.* Neuropharmacology, 1989. **28**(4): p. 411-8.
80. Panksepp, J. and W.W. Beatty, *Social deprivation and play in rats.* Behav Neural Biol, 1980. **30**(2): p. 197-206.
81. Pellis, S.M. and V.C. Pellis, *Differential rates of attack, defense, and counterattack during the developmental decrease in play fighting by male and female rats.* Dev Psychobiol, 1990. **23**(3): p. 215-31.
82. Lengwiler, Y., *Gini coefficient and the Lorentz curve.* MATLAB Central File Exchange, 2021.
83. Pellis, S.M., et al., *50-kHz vocalizations, play and the development of social competence*, in *Handbook of behavioral neuroscience*. 2018, Elsevier. p. 117-126.
84. Brenes, J.C. and R.K. Schwarting, *Attribution and expression of incentive salience are differentially signaled by ultrasonic vocalizations in rats.* PLoS One, 2014. **9**(7): p. e102414.
85. Barfield, R.J. and D.A. Thomas, *The role of ultrasonic vocalizations in the regulation of reproduction in rats.* Ann N Y Acad Sci, 1986. **474**: p. 33-43.

86. Burgdorf, J., J. Panksepp, and J.R. Moskal, *Frequency-modulated 50 kHz ultrasonic vocalizations: a tool for uncovering the molecular substrates of positive affect.* Neurosci Biobehav Rev, 2011. **35**(9): p. 1831-6.
87. Wöhr, M., *Ultrasonic communication in rats: appetitive 50-kHz ultrasonic vocalizations as social contact calls.* Behavioral Ecology and Sociobiology, 2018. **72**(1): p. 1-12.
88. Simola, N. and G. Costa, *Emission of categorized 50-kHz ultrasonic vocalizations in rats repeatedly treated with amphetamine or apomorphine: Possible relevance to drug-induced modifications in the emotional state.* Behav Brain Res, 2018. **347**: p. 88-98.
89. Tripi, J.A., M.L. Dent, and P.J. Meyer, *Individual differences in food cue responsivity are associated with acute and repeated cocaine-induced vocalizations, but not cue-induced vocalizations.* Psychopharmacology (Berl), 2017. **234**(3): p. 437-446.
90. Stark, R.A. and S.M. Pellis, *Using the 'stranger test' to assess social competency in adult female Long Evans rats reared with a Fischer 344 partner.* Behav Processes, 2021. **192**: p. 104492.
91. Gzielo, K., et al., *Valproic acid exposure impairs ultrasonic communication in infant, adolescent and adult rats.* Eur Neuropsychopharmacol, 2020. **41**: p. 52-62.
92. Kisko, T.M., R.K.W. Schwarting, and M. Wöhr, *Sex differences in the acoustic features of social play-induced 50-kHz ultrasonic vocalizations: A detailed spectrographic analysis in wild-type Sprague-Dawley and Cacna1c haploinsufficient rats.* Dev Psychobiol, 2021. **63**(2): p. 262-276.
93. Pellis, S.M., et al., *Multiple differences in the play fighting of male and female rats. Implications for the causes and functions of play.* Neurosci Biobehav Rev, 1997. **21**(1): p. 105-20.
94. Pellis, S.M. and V.C. Pellis, *The prejuvenile onset of play fighting in laboratory rats (Rattus norvegicus).* Dev Psychobiol, 1997. **31**(3): p. 193-205.
95. Ma, S.T., et al., *Repeated intravenous cocaine experience: development and escalation of pre-drug anticipatory 50-kHz ultrasonic vocalizations in rats.* Behav Brain Res, 2010. **212**(1): p. 109-14.
96. Mu, P., et al., *Repeated cocaine exposure induces sensitization of ultrasonic vocalization in rats.* Neurosci Lett, 2009. **453**(1): p. 31-5.
97. Simola, N., et al., *Direct and long-lasting effects elicited by repeated drug administration on 50-kHz ultrasonic vocalizations are regulated differently: implications for the study of the affective properties of drugs of abuse.* Int J Neuropsychopharmacol, 2014. **17**(3): p. 429-41.
98. Simola, N., et al., *Pharmacological characterization of 50-kHz ultrasonic vocalizations in rats: comparison of the effects of different psychoactive drugs and relevance in drug-induced reward.* Neuropharmacology, 2012. **63**(2): p. 224-34.
99. Willadsen, M., et al., *Effects of anxiogenic drugs on the emission of 22- and 50-kHz ultrasonic vocalizations in adult rats.* Psychopharmacology (Berl), 2018. **235**(8): p. 2435-2445.
100. Auger, A.P. and K.M. Olesen, *Brain sex differences and the organisation of juvenile social play behaviour.* J Neuroendocrinol, 2009. **21**(6): p. 519-25.

101. Stark, R.A.M., R. Ramkumar, and S.M. Pellis, *Deficient play-derived experiences in juvenile Long Evans rats reared with a Fischer 344 partner: A deficiency shared by both sexes.* International Journal of Comparative Psychology, 2021. **34**.
102. Gerhard, D.M., E.S. Wohleb, and R.S. Duman, *Emerging treatment mechanisms for depression: focus on glutamate and synaptic plasticity.* Drug Discov Today, 2016. **21**(3): p. 454-64.
103. Aleksandrova, L.R., Y.T. Wang, and A.G. Phillips, *Evaluation of the Wistar-Kyoto rat model of depression and the role of synaptic plasticity in depression and antidepressant response.* Neurosci Biobehav Rev, 2019. **105**: p. 1-23.
104. Okamoto, K. and K. Aoki, *Development of a strain of spontaneously hypertensive rats.* Jpn Circ J, 1963. **27**: p. 282-93.
105. Rittenhouse, P.A., et al., *Amplified behavioral and endocrine responses to forced swim stress in the Wistar-Kyoto rat.* Psychoneuroendocrinology, 2002. **27**(3): p. 303-18.
106. Nam, H., et al., *Learned helplessness and social avoidance in the Wistar-Kyoto rat.* Front Behav Neurosci, 2014. **8**: p. 109.
107. Pare, W.P., *Open field, learned helplessness, conditioned defensive burying, and forced-swim tests in WKY rats.* Physiol Behav, 1994. **55**(3): p. 433-9.
108. Pare, W.P. and E. Redei, *Sex differences and stress response of WKY rats.* Physiol Behav, 1993. **54**(6): p. 1179-85.
109. Lahmame, A., et al., *Are Wistar-Kyoto rats a genetic animal model of depression resistant to antidepressants?* Eur J Pharmacol, 1997. **337**(2-3): p. 115-23.
110. Marti, J. and A. Armario, *Forced swimming behavior is not related to the corticosterone levels achieved in the test: a study with four inbred rat strains.* Physiology & behavior, 1996. **59**(2): p. 369.
111. Lopez-Rubalcava, C. and I. Lucki, *Strain differences in the behavioral effects of antidepressant drugs in the rat forced swimming test.* Neuropsychopharmacology, 2000. **22**(2): p. 191-9.
112. Belujon, P. and A.A. Grace, *Restoring mood balance in depression: ketamine reverses deficit in dopamine-dependent synaptic plasticity.* Biol Psychiatry, 2014. **76**(12): p. 927-36.
113. Vanderschuren, L.J., E.J. Achterberg, and V. Trezza, *The neurobiology of social play and its rewarding value in rats.* Neurosci Biobehav Rev, 2016. **70**: p. 86-105.
114. Malkesman, O., et al., *Two different putative genetic animal models of childhood depression.* Biol Psychiatry, 2006. **59**(1): p. 17-23.
115. Meaney, M.J. and J. Stewart, *A descriptive study of social development in the rat (Rattus norvegicus).* Animal Behaviour, 1981. **29**(1): p. 34-45.
116. Panksepp, J., *The ontogeny of play in rats.* Dev Psychobiol, 1981. **14**(4): p. 327-32.
117. Browne, C.A., D.S. van Nest, and I. Lucki, *Antidepressant-like effects of buprenorphine in rats are strain dependent.* Behav Brain Res, 2015. **278**: p. 385-92.
118. Carnevali, L., et al., *Antidepressant-like activity and cardioprotective effects of fatty acid amide hydrolase inhibitor URB694 in socially stressed Wistar Kyoto rats.* Eur Neuropsychopharmacol, 2015. **25**(11): p. 2157-69.
119. Hurley, L.L., et al., *Antidepressant effects of resveratrol in an animal model of depression.* Behav Brain Res, 2014. **268**: p. 1-7.

120. Pellis, S.M. and V.C. Pellis, *Play-fighting differs from serious fighting in both target of attack and tactics of fighting in the laboratory rat Rattus norvegicus.* Aggressive Behavior, 1987. **13**(4): p. 227-242.
121. Pellis, S.M. and M.M. McKenna, *Intrinsic and extrinsic influences on play fighting in rats: effects of dominance, partner's playfulness, temperament and neonatal exposure to testosterone propionate.* Behav Brain Res, 1992. **50**(1-2): p. 135-45.
122. Siviy, S.M., L.M. Deron, and C.R. Kasten, *Serotonin, motivation, and playfulness in the juvenile rat.* Dev Cogn Neurosci, 2011. **1**(4): p. 606-16.
123. Ikemoto, S. and J. Panksepp, *The effects of early social isolation on the motivation for social play in juvenile rats.* Dev Psychobiol, 1992. **25**(4): p. 261-74.
124. Pellis, S.M., V.C. Pellis, and M.M. McKenna, *Feminine dimension in the play fighting of rats (Rattus norvegicus) and its defeminization neonatally by androgens.* J Comp Psychol, 1994. **108**(1): p. 68-73.
125. Peartree, N.A., et al., *Limited physical contact through a mesh barrier is sufficient for social reward-conditioned place preference in adolescent male rats.* Physiol Behav, 2012. **105**(3): p. 749-56.
126. Thiel, K.J., A.C. Okun, and J.L. Neisewander, *Social reward-conditioned place preference: a model revealing an interaction between cocaine and social context rewards in rats.* Drug Alcohol Depend, 2008. **96**(3): p. 202-12.
127. Trezza, V., R. Damsteegt, and L.J. Vanderschuren, *Conditioned place preference induced by social play behavior: parametrics, extinction, reinstatement and disruption by methylphenidate.* Eur Neuropsychopharmacol, 2009. **19**(9): p. 659-69.
128. Achterberg, E.J.M. and L.J.M.J. Vanderschuren, *Treatment with low doses of nicotine but not alcohol affects social play reward in rats.* International Journal of Play: The Neuroscience of Play: Honoring the Life's Work of Jaak Panksepp, 2020. **9**(1): p. 39-57.
129. Girard, J.M., et al., *Nonverbal Social Withdrawal in Depression: Evidence from manual and automatic analysis.* Image Vis Comput, 2014. **32**(10): p. 641-647.
130. Gullone, E., T.H. Ollendick, and N.J. King, *The Role of Attachment Representation in the Relationship Between Depressive Symptomatology and Social Withdrawal in Middle Childhood.* Journal of Child and Family Studies, 2006. **15**(3): p. 263-277.
131. Sloman, L., P. Gilbert, and G. Hasey, *Evolved mechanisms in depression: the role and interaction of attachment and social rank in depression.* J Affect Disord, 2003. **74**(2): p. 107-21.
132. Thor, D.H. and W.R. Holloway, Jr., *Social play in juvenile rats: a decade of methodological and experimental research.* Neurosci Biobehav Rev, 1984. **8**(4): p. 455-64.
133. Himmler, S.M., et al., *Domestication and diversification: a comparative analysis of the play fighting of the Brown Norway, Sprague-Dawley, and Wistar laboratory strains of (Rattus norvegicus).* J Comp Psychol, 2014. **128**(3): p. 318-27.

134. Pellis, S.M. and V.C. Pellis, *Play fighting of rats in comparative perspective: a schema for neurobehavioral analyses.* Neurosci Biobehav Rev, 1998. **23**(1): p. 87-101.
135. Takahashi, L.K. and R.K. Lore, *Play fighting and the development of agonistic behavior in male and female rats.* Aggressive Behavior, 1983. **9**(3): p. 217-227.
136. Blanchard, R.J. and D.C. Blanchard, *Aggressive behavior in the rat.* Behav Biol, 1977. **21**(2): p. 197-224.
137. Muir, G., *Locomotion*, in *The behavior of the laboratory rat: a handbook with tests*, K.B. Whishaw IQ, Editor. 2005, Oxford University Press: Oxford; New York. p. 150–161.
138. Pellis, S.M. and V.C. Pellis, *Locomotor-rotational movements in the ontogeny and play of the laboratory rat Rattus norvegicus.* Dev Psychobiol, 1983. **16**(4): p. 269-86.
139. Gorzalka, B.B. and L.A. Hanson, *Sexual behavior and wet dog shakes in the male rat: regulation by corticosterone.* Behav Brain Res, 1998. **97**(1-2): p. 143-51.
140. Aldridge, J.W., *Grooming*, in *The behavior of the laboratory rat: a handbook with tests*, K.B. Whishaw IQ, Editor. 2005, Oxford University Press: Oxford; New York. p. 141–149.
141. Burke, C.J., D.R. Euston, and S.M. Pellis, *What do you hear, what do you say? Ultrasonic calls as signals during play fighting in rats.* International Journal of Play: The Neuroscience of Play: Honoring the Life's Work of Jaak Panksepp, 2020. **9**(1): p. 92-107.
142. Siviy, S.M., C.N. Baliko, and K.S. Bowers, *Rough-and-tumble play behavior in Fischer-344 and buffalo rats: effects of social isolation.* Physiol Behav, 1997. **61**(4): p. 597-602.
143. Siviy, S.M., et al., *The relative playfulness of juvenile Lewis and Fischer-344 rats.* Physiol Behav, 2003. **80**(2-3): p. 385-94.
144. Reinhart, C.J., S.M. Pellis, and D.C. McIntyre, *Development of play fighting in kindling-prone (FAST) and kindling-resistant (SLOW) rats: how does the retention of phenotypic juvenility affect the complexity of play?* Dev Psychobiol, 2004. **45**(2): p. 83-92.
145. Siviy, S.M., et al., *Dysfunctional play and dopamine physiology in the Fischer 344 rat.* Behav Brain Res, 2011. **220**(2): p. 294-304.
146. Fortress, A.M., I.M. Smith, and K.C.H. Pang, *Ketamine facilitates extinction of avoidance behavior and enhances synaptic plasticity in a rat model of anxiety vulnerability: Implications for the pathophysiology and treatment of anxiety disorders.* Neuropharmacology, 2018. **137**: p. 372-381.
147. van der Harst, J.E., et al., *Access to enriched housing is rewarding to rats as reflected by their anticipatory behaviour.* Animal Behaviour, 2003. **66**(3): p. 493-504.
148. Ferguson, S.A. and A.M. Cada, *A longitudinal study of short- and long-term activity levels in male and female spontaneously hypertensive, Wistar-Kyoto, and Sprague-Dawley rats.* Behav Neurosci, 2003. **117**(2): p. 271-82.
149. Rao, R.M. and M. Sadananda, *Strain-and context-based 50 kHz ultrasonic vocalizations and anxiety behaviour in the Wistar-Kyoto rat.* J Biosci, 2015. **40**(3): p. 561-70.

150. Pellis, S.M., V.C. Pellis, and B.T. Himmler, *How play makes for a more adaptable brain: a comparative and neural perspective.* American journal of play, 2014. **7**(1): p. 73.
151. Vanderschuren, L.J. and V. Trezza, *What the laboratory rat has taught us about social play behavior: role in behavioral development and neural mechanisms.* Curr Top Behav Neurosci, 2014. **16**: p. 189-212.
152. Baarendse, P.J., et al., *Early social experience is critical for the development of cognitive control and dopamine modulation of prefrontal cortex function.* Neuropsychopharmacology, 2013. **38**(8): p. 1485-94.
153. Burghardt, G.M., *The genesis of animal play: Testing the limits*. 2005: MIT press.
154. Pellis, S. and V. Pellis, *The playful brain: venturing to the limits of neuroscience*. 2013: Simon and Schuster.
155. Pellis, S.M. and V.C. Pellis, *Play-fighting differs from serious fighting in both target of attack and tactics of fighting in the laboratory rat Rattus norvegicus.* Aggressive Behavior, 1987. **13**(4): p. 227-242.
156. Pellis, S.M. and V.C. Pellis, *What is play fighting and what is it good for?* Learn Behav, 2017. **45**(4): p. 355-366.
157. Palagi, E., et al., *Fair play and its connection with social tolerance, reciprocity and the ethology of peace.* Behaviour, 2016. **153**(9-11): p. 1195-1216.
158. Hole, G., Dorothy F, *Play in rodents*, in *Play in animals and humans*, P.K. Smith, Editor. 1984, Blackwell.
159. Wohr, M. and R.K. Schwarting, *Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation.* Cell Tissue Res, 2013. **354**(1): p. 81-97.
160. Burke, C.J., D.R. Euston, and S.M. Pellis, *What do you hear, what do you say? Ultrasonic calls as signals during play fighting in rats.* International Journal of Play, 2020. **9**(1): p. 92-107.
161. Pellis, S.M., et al., *The use of the bared-teeth display during play fighting in Tonkean macaques (Macaca tonkeana): sometimes it is all about oneself.* J Comp Psychol, 2011. **125**(4): p. 393-403.
162. Scopa, C. and E. Palagi, *Mimic me while playing! Social tolerance and rapid facial mimicry in macaques (Macaca tonkeana and Macaca fuscata).* J Comp Psychol, 2016. **130**(2): p. 153-61.
163. Schwing, R., et al., *Positive emotional contagion in a New Zealand parrot.* Curr Biol, 2017. **27**(6): p. R213-R214.
164. Provine, R.R., *Laughter: A scientific investigation*. 2001: Penguin.
165. Panksepp, J. and J. Burgdorf, *"Laughing" rats and the evolutionary antecedents of human joy?* Physiol Behav, 2003. **79**(3): p. 533-47.
166. Panksepp, J., *Neuroevolutionary sources of laughter and social joy: modeling primal human laughter in laboratory rats.* Behav Brain Res, 2007. **182**(2): p. 231-44.
167. LaFollette, M.R., et al., *Rat tickling: A systematic review of applications, outcomes, and moderators.* PLoS One, 2017. **12**(4): p. e0175320.
168. Bombail, V., et al., *Crying With Laughter: Adapting the Tickling Protocol to Address Individual Differences Among Rats in Their Response to Playful Handling.* Front Vet Sci, 2021. **8**: p. 677872.

169. Yamamuro, T., et al., *Tickling stimulation causes the up-regulation of the kallikrein family in the submandibular gland of the rat.* Behav Brain Res, 2013. **236**(1): p. 236-243.
170. Wöhr, M., et al., *New insights into the relationship of neurogenesis and affect: tickling induces hippocampal cell proliferation in rats emitting appetitive 50-kHz ultrasonic vocalizations.* Neuroscience, 2009. **163**(4): p. 1024-30.
171. Ishiyama, S. and M. Brecht, *Neural correlates of ticklishness in the rat somatosensory cortex.* Science, 2016. **354**(6313): p. 757-760.
172. Melotti, L., et al., *Play in rats: association across contexts and types, and analysis of structure.* Animal Behavior and Cognition, 2014. **1**(4): p. 489-501.
173. Achterberg, E.J., V. Trezza, and L.J. Vanderschuren, *Glucocorticoid receptor antagonism disrupts the reconsolidation of social reward-related memories in rats.* Behav Pharmacol, 2014. **25**(3): p. 216-25.
174. Achterberg, E.J., et al., *Contrasting Roles of Dopamine and Noradrenaline in the Motivational Properties of Social Play Behavior in Rats.* Neuropsychopharmacology, 2016. **41**(3): p. 858-68.
175. Panksepp, J. and J. Burgdorf, *20 Laughing Rats? Playful Tickling Arouses High-Frequency Ultrasonic.* Toward a science of consciousness III: the third Tucson discussions and debates, 1999. **3**: p. 231.
176. Schwarting, R.K., N. Jegan, and M. Wöhr, *Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats.* Behav Brain Res, 2007. **182**(2): p. 208-22.
177. Cloutier, S., et al., *Tickling, a Technique for Inducing Positive Affect When Handling Rats.* J Vis Exp, 2018(135).
178. Hinchcliffe, J.K., M. Mendl, and E.S.J. Robinson, *Rat 50 kHz calls reflect graded tickling-induced positive emotion.* Curr Biol, 2020. **30**(18): p. R1034-R1035.
179. LaFollette, M.R., et al., *A happier rat pack: the impacts of tickling pet store rats on human-animal interactions and rat welfare.* Applied Animal Behaviour Science, 2018. **203**: p. 92-102.
180. Wöhr, M. and R.K. Schwarting, *Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation.* Cell Tissue Res, 2013. **354**(1): p. 81-97.
181. Mancini, G., P.F. Ferrari, and E. Palagi, *Rapid facial mimicry in geladas.* Sci Rep, 2013. **3**: p. 1527.
182. Palagi, E., et al., *Sharing playful mood: rapid facial mimicry in Suricata suricatta.* Anim Cogn, 2019. **22**(5): p. 719-732.
183. Palagi, E., V. Nicotra, and G. Cordoni, *Rapid mimicry and emotional contagion in domestic dogs.* R Soc Open Sci, 2015. **2**(12): p. 150505.
184. Wohr, M. and R.K. Schwarting, *Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior?* PLoS One, 2007. **2**(12): p. e1365.
185. Burgdorf, J., et al., *Uncovering the molecular basis of positive affect using rough-and-tumble play in rats: a role for insulin-like growth factor I.* Neuroscience, 2010. **168**(3): p. 769-77.
186. Burgdorf, J., et al., *Rats selectively bred for low levels of play-induced 50 kHz vocalizations as a model for autism spectrum disorders: a role for NMDA receptors.* Behavioural brain research, 2013. **251**: p. 18-24.

187. Palagi, E., et al., *Rough-and-tumble play as a window on animal communication.* Biol Rev Camb Philos Soc, 2016. **91**(2): p. 311-27.
188. Smith, L., S.L. Fantella, and S. Pellis, *Playful defensive responses in adult male rats depend on the status of the unfamiliar opponent.* Aggressive Behavior: Official Journal of the International Society for Research on Aggression, 1999. **25**(2): p. 141-152.
189. Aldis, O., *Play Fighting*. 1975: Academic Press.
190. Bateson, G., *The message "This is play.".* Group processes, 1956. **2**: p. 145-241.
191. Bekoff, M., *The communication of play intention: Are play signals functional?* 1975.
192. Pellis, S.M. and V.C. Pellis, *On knowing it's only play: The role of play signals in play fighting.* Aggression and violent behavior, 1996. **1**(3): p. 249-268.
193. Bekoff, M., *Cognitive ethology, vigilance, information gathering, and representation: Who might know what and why?* Behav Processes, 1995. **35**(1-3): p. 225-37.
194. Van Hooff, J., *The facial displays of the catarrhine monkeys and apes*, in *Primate ethology*. 2017, Routledge. p. 7-68.
195. Siviy, S.M., *A Brain Motivated to Play: Insights into the Neurobiology of Playfulness.* Behaviour, 2016. **153**(6-7): p. 819-844.
196. Himmler, S.M., et al., *Play, variation in play and the development of socially competent rats.* Behaviour, 2016. **153**(9-11): p. 1103-1137.
197. Grant, E., *An analysis of the social behaviour of the male laboratory rat.* Behaviour, 1963. **21**(3-4): p. 260-281.
198. Mackintosh, J. and E. Grant, *A comparison of the social postures of some common laboratory rodents.* Behaviour, 1963. **21**(3-4): p. 246-259.
199. Poole, T.B. and J. Fish, *An investigation of playful behaviour in Rattus norvegicus and Mus musculus (Mammalia).* Journal of Zoology, 1975. **175**(1): p. 61-71.
200. Finlayson, K., et al., *Facial Indicators of Positive Emotions in Rats.* PLoS One, 2016. **11**(11): p. e0166446.
201. Sales, G.D., *Ultrasound and aggressive behaviour in rats and other small mammals.* Anim Behav, 1972. **20**(1): p. 88-100.
202. Kisko, T.M., et al., *From Play to Aggression: High-Frequency 50-kHz Ultrasonic Vocalizations as Play and Appeasement Signals in Rats.* Curr Top Behav Neurosci, 2016.
203. Bradbury, J. and S. Vehrencamp, *Principles of animal communication, 2nd edn Sunderland.* MA: Sinauer Associates.[Google Scholar], 2011.
204. Wöhr, M. and R.K. Schwarting, *Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat.* Behav Neurosci, 2008. **122**(2): p. 310-30.
205. Fendt, M., et al., *Predator odour but not TMT induces 22-kHz ultrasonic vocalizations in rats that lead to defensive behaviours in conspecifics upon replay.* Sci Rep, 2018. **8**(1): p. 11041.
206. Schleidt, W.M., *Tonic communication: continual effects of discrete signs in animal communication systems.* J Theor Biol, 1973. **42**(2): p. 359-86.
207. Pellis, S.M. and V.C. Pellis, *Are agonistic behavior patterns signals or combat tactics - or does it matter? Targets as organizing principles of fighting.* Physiol Behav, 2015.

208. Yanagi, A. and C.M. Berman, *Body signals during social play in free-ranging rhesus macaques (Macaca mulatta): A systematic analysis.* Am J Primatol, 2014. **76**(2): p. 168-79.
209. Diamond, J. and A.B. Bond, *Kea, bird of paradox: the evolution and behavior of a New Zealand parrot*. 1999: Univ of California Press.
210. Schwing, R., S. Parsons, and X.J. Nelson, *Vocal repertoire of the New Zealand kea parrot Nestor notabilis.* Current Zoology, 2012. **58**(5): p. 727-740.
211. Neunuebel, J.P., et al., *Female mice ultrasonically interact with males during courtship displays.* Elife, 2015. **4**.
212. Heckman, J.J., et al., *High-precision spatial localization of mouse vocalizations during social interaction.* Sci Rep, 2017. **7**(1): p. 3017.
213. Coffey, K.R., R.G. Marx, and J.F. Neumaier, *DeepSqueak: a deep learning-based system for detection and analysis of ultrasonic vocalizations.* Neuropsychopharmacology, 2019. **44**(5): p. 859-868.
214. Noldus, L.P., A.J. Spink, and R.A. Tegelenbosch, *EthoVision: a versatile video tracking system for automation of behavioral experiments.* Behav Res Methods Instrum Comput, 2001. **33**(3): p. 398-414.
215. Kisko, T.M., et al., *haploinsufficiency leads to pro-social 50-kHz ultrasonic communication deficits in rats.* Dis Model Mech, 2018. **11**(6).
216. Seffer, D., et al., *Pro-social 50-kHz ultrasonic communication in rats: post-weaning but not post-adolescent social isolation leads to social impairments-phenotypic rescue by re-socialization.* Front Behav Neurosci, 2015. **9**: p. 102.
217. Burleson, C.A., et al., *Social play in juvenile hamsters alters dendritic morphology in the medial prefrontal cortex and attenuates effects of social stress in adulthood.* Behav Neurosci, 2016. **130**(4): p. 437-47.
218. Schneider, P., et al., *Adverse social experiences in adolescent rats result in enduring effects on social competence, pain sensitivity and endocannabinoid signaling.* Frontiers in behavioral neuroscience, 2016. **10**: p. 203.
219. Rogers-Carter, M.M. and J.P. Christianson, *An insular view of the social decision-making network.* Neurosci Biobehav Rev, 2019. **103**: p. 119-132.
220. Brudzynski, S.M. and F. Barnabi, *Contribution of the ascending cholinergic pathways in the production of ultrasonic vocalization in the rat.* Behavioural brain research, 1996. **80**(1): p. 145-152.
221. Brudzynski, S.M., *Ultrasonic calls of rats as indicator variables of negative or positive states: Acetylcholine–dopamine interaction and acoustic coding.* Behavioural brain research, 2007. **182**(2): p. 261-273.
222. Thompson, B., K.C. Leonard, and S.M. Brudzynski, *Amphetamine-induced 50 kHz calls from rat nucleus accumbens: A quantitative mapping study and acoustic analysis.* Behavioural brain research, 2006. **168**(1): p. 64-73.
223. Bell, H.C., et al., *The role of the medial prefrontal cortex in the play fighting of rats.* Behav Neurosci, 2009. **123**(6): p. 1158-68.
224. Bell, H.C., S.M. Pellis, and B. Kolb, *Juvenile peer play experience and the development of the orbitofrontal and medial prefrontal cortices.* Behav Brain Res, 2010. **207**(1): p. 7-13.
225. Pellis, S.M., et al., *The role of the striatum in organizing sequences of play fighting in neonatally dopamine-depleted rats.* Neuroscience letters, 1993. **158**(1): p. 13-15.

226. Phillips, A.G., S. Ahn, and J.G. Howland, *Amygdalar control of the mesocorticolimbic dopamine system: parallel pathways to motivated behavior.* Neurosci Biobehav Rev, 2003. **27**(6): p. 543-54.
227. Warren, M.R., D.T. Sangiamo, and J.P. Neunuebel, *High channel count microphone array accurately and precisely localizes ultrasonic signals from freely-moving mice.* Journal of neuroscience methods, 2018. **297**: p. 44-60.
228. Snoeren, E.M.S. and A. Ågmo, *The Incentive Value of Males' 50-kHz Ultrasonic Vocalizations for Female Rats (Rattus norvegicus).* Journal of comparative psychology (1983), 2014. **128**(1): p. 40-55.
229. Emmerson, M.G., K.A. Spencer, and G.R. Brown, *Social experience during adolescence in female rats increases 50 kHz ultrasonic vocalizations in adulthood, without affecting anxiety-like behavior.* Developmental psychobiology, 2020. **62**(2): p. 212-223.
230. Redecker, T.M., et al., *Effects of Cacna1c haploinsufficiency on social interaction behavior and 50-kHz ultrasonic vocalizations in adult female rats.* Behavioural brain research, 2019. **367**: p. 35-52.