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Vocal Communication During Early Mother-Infant Interaction: Studies Using the Wistar-Kyoto Rat Model of Depression

Idil Tuncali

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A Thesis Presented

By

IDIL TUNCALI

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Approved as to style and content by:

_____
Mariana Pereira, Chair

_____
Luke Remage-Healey, Member

_____
Joseph Bergan, Member

_____
John Lopes, Interim Director
Interdepartmental Graduate Program

ABSTRACT

VOCAL COMMUNICATION DURING EARLY MOTHER-INFANT INTERACTION:

STUDIES USING THE WISTAR-KYOTO RAT MODEL OF DEPRESSION

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Postpartum depression is a serious psychiatric condition that has deleterious effects on the mother and poses a risk for the mother-infant relationship and ultimately the infant's development. Maternal anhedonia and social communication deficits are two major clinical features central to postpartum depression that likely contribute to deficits in parenting. The present study used Wistar-Kyoto (WKY) mother rats, an animal model of depression which we have developed to examine the postpartum disorder, to investigate the relationship between maternal anhedonia, social communication deficits and parenting disturbances. Rats produce ultrasonic vocalizations (USVs) in different social contexts, and USVs are becoming an increasingly valuable assay for behavioral phenotyping. Alterations of the ultrasound patterns have been reported in several models of neuropsychiatric disorders, including those associated with communicative/social deficits, and can also provide reliable insight into the affective state of the mother rat during social interactions with her litter. In the first study, WKY and control Sprague-Dawley (SD) postpartum females were examined for their affective responses to social cues from pups, as measured by their ultrasonic vocalizations (USVs) during a 30-minute maternal behavior test following 20 minutes of mother-litter separation. Total number of calls, acoustic frequency and duration of calls, and individual USV profiles were analyzed in conjunction with maternal behavior. Both WKY and SD mothers predominantly produced ~50 kHz USVs when interacting with the pups in the maternal behavior test. WKY mothers emitted more trill-type USVs as

compared with SD mothers. Similarly, WKY mothers exhibited substantial disturbances in their maternal behavior. A second experiment evaluated the therapeutic efficacy of adenosine A_{2A} receptor antagonism as a novel treatment strategy for postpartum depression. Emerging evidence indicates that the neuromodulator adenosine, particularly through actions on adenosine A_{2A} receptors, modulates behavioral functions associated with the mesocorticolimbic DA system, including cognitive and motivational processes. Results indicate that acute MSX-3 administration did not attenuate the parenting disturbances of WKY or affect the USV emissions of either strain. Taken together, these results provide evidence for the presence of maternal USVs during mother-litter interactions, and further suggest that variations in USVs produced by mothers during social interaction with their pups may function as an index of their affect. Rat USVs may be used to study the neurobiological mechanisms underlying maternal affect in animal models of postpartum disorders.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 Postpartum Depression: Consequences of Maternal Anhedonia and Social Communication Deficits on Early Mother-Infant Interactions

Postpartum depression is a highly prevalent psychiatric condition worldwide that affects up to 20% of new mothers, with serious deleterious effects on both the mother and her infant (O'Hara & McCabe, 2013). Postpartum depression usually begins within 1 - 12 months after delivery, having the potential to disrupt the mother-infant relationship during a critical developmental period for the infant (Stewart et al., 2003). Early social interactions between the mother and her infant have direct consequences on the emotional and cognitive developmental course and outcome of the infant and are optimal when the maternal behavior is attuned to the infant's affect and changing needs (Bowlby, 1969; Trevarthen, 2001).

Anhedonia, a decreased ability to experience pleasure, and social communication deficits are two major clinical features central to postpartum depression which can compromise a mother's ability to sensitively interact with her infant. The consequences of social anhedonia can include decreased feelings of attachment towards one's infant and disrupted breastfeeding, while further emotional dysregulation can cause high levels of hostility during mother-infant interactions (Field, 2010; Eydie L. Moses-Kolko et al., 2010). Analysis of early interactions indicated that depressed mothers engage less in face-to-face interaction and play behavior, smiling and imitation, exhibit less emotion and warmth, touch their infants less frequently, and exhibit deficits in vocal behavior and synchrony to their infants' cues (Field et al., 1985; Herrera, Reissland, & Shepherd, 2004; Lovejoy, Graczyk, O'Hare, & Neuman, 2000; Zlochower & Cohn, 1996; Lyons-Ruth, Zoll, Connell, & Grunebaum, 1986). Such compromised mother-infant interactions can result in long-term behavioral, emotional and health problems in children (Beardslee, Gladstone, & O'Connor, 2011).

The neural circuitry that supports maternal behavior undergoes considerable plasticity in preparation for birth and further develops in response to ongoing interactions with the developing young (Kim, Strathearn, & Swain, 2016; Stolzenberg & Champagne, 2016). Converging evidence from both animal and humans studies has revealed that parenting is regulated by a distributed network of brain structures, including the prefrontal cortex (PFC), the amygdala, the hippocampus, the medial preoptic area (mPOA), the nucleus accumbens (NA), and its dopaminergic inputs from the ventral tegmental area (VTA) (Lonstein, Lévy, & Fleming, 2015; Numan & Insel, 2003). These structures play a role in cognitive, motivational and affective processes that are key for parenting, such as social recognition and understanding, attention, memory, effort-related functions, and other processes (E. L. Moses-Kolko, Horner, Phillips, Hipwell, & Swain, 2014; Numan & Insel, 2003). Depressed mothers show dysregulation of brain circuits involved in parenting (Laurent & Ablow, 2012; E. L. Moses-Kolko et al., 2014). For instance, depressed mothers showed decreased activity in the dorsomedial prefrontal cortex, an area associated with social processing, along with a dampened amygdala response to negative emotional face that was associated with greater depression severity and more impaired maternal attachment processes (Barrett et al., 2012; Eydie L. Moses-Kolko et al., 2010). Moreover, deficits in maternal behavior observed in depressed mothers has been associated with attenuated maternal responses in the striatum, including the nucleus accumbens, and disrupted connectivity between the amygdala and the medial prefrontal cortex (Atzil et al., 2017).

1.2 Modeling Postpartum Depression in Animals

Because of the clinical significance of parenting disturbances in depression, and the growing emphasis on identifying neural circuits related to specific psychiatric symptoms (i.e., NIH Research Domain Criteria {RDoC}), it is critical to develop animal models that specifically assess social anhedonia processes. The present study used the Wistar-Kyoto (WKY) rat model of depression. The WKY strain was initially developed as the normotensive control strain for the

spontaneously hypertensive rat strain (OKAMOTO & AOKI, 1963). Compared to many other strains, including Sprague-Dawley (SD), WKY demonstrates a syndrome of hormonal, behavioral, and physiological dysfunctions that recapitulate aspects of human depression. WKY male rats show heightened plasma levels of corticosterone and ACTH (Solberg, Olson, Turek, & Redei, 2001), less activity in the open field and the forced swimming tests (Will, Aird, & Redei, 2003), and effort-related motivational deficits in a progressive ratio operant tasks (De La Garza, 2005) compared with control Wistar or SD rats. In addition, WKYs exhibit decreased sucrose intake when compared with control strains, indicative of anhedonia (Malkesman et al., 2005) and show greater social avoidance when presented with a novel conspecific (Nam, Clinton, Jackson, & Kerman, 2014). WKY also exhibit neurochemical abnormalities consistent with those observed in depressed patients, including altered serotonergic, noradrenergic and dopaminergic systems (Jiao, Beck, Pang, & Servatius, 2011).

Prior research in the Pereira Lab demonstrated that the WKY strain recapitulates, with considerable face validity, the major clinical features of postpartum depression in mothers, including the cognitive, motivational and parenting disturbances. For instance, WKY mothers exhibit performance deficits on the attentional set-shifting task that is a rodent analogue of the Wisconsin Card Sorting Task used in humans, indicative of impaired cognitive flexibility. Furthermore, WKY mothers exhibit reduced work output in tasks that assess effort-related symptom domains. In addition to cognitive and motivational deficits, WKY females exhibit severe disturbances in maternal behavior. Yet, to date limited studies have examined the relationship between maternal anhedonia and parenting disturbances.

1.3 The Role of Dopamine in Depression and Parenting

The mesocorticolimbic dopamine (DA) system regulates behavioral and neural processes which are essential to parenting, including social cognition, motivation and affect. The mesocorticolimbic DA system consists of DAergic midbrain projections from the VTA (A10) and

the neighboring substantia nigra (SN, A9) to limbic and cortical structures, including the nucleus accumbens, the basolateral amygdala (BLA), and the prefrontal cortex (Björklund & Dunnett, 2007; Haber, 2003).

Mesocorticolimbic DA, particularly in the nucleus accumbens, plays an important role in regulating aspects of maternal behavior. fMRI studies on parenting showed increased activity in components of the mesocorticolimbic DA system of human mothers responding to own versus unknown (or familiar but unrelated) infant stimuli (Atzil et al., 2017; Swain, Kim, & Ho, 2011). In rats, lesions of the VTA and injections of DA receptor antagonists into the accumbens severely disrupts activational aspects of maternal behavior, while leaving directional aspects relatively intact (Numan et al., 2005; Pereira et al., 2011). Furthermore, DA is released in the NA of postpartum rats during active maternal interaction with the pups (Afonso, King, Chatterjee, & Fleming, 2009; Champagne, 2004; Robinson, Zitzman, & Williams, 2011).

1.4 Dopamine-Adenosine Interactions

Emerging evidence indicates that the neuromodulator adenosine, particularly through actions on adenosine A_{2A} receptors, modulates behavioral functions associated with the mesocorticolimbic DA system, including cognitive, motivational and affective processes (Worden et al., 2009). Adenosine A_{2A} receptors are primarily localized in striatal areas, including both neostriatum and accumbens (Ferré et al., 2004; Schiffmann, Libert, Vassart, & Vanderhaeghen, 1991). Furthermore, there is a functional interaction between DA D2 and adenosine A_{2A} receptors (Ferré, 1997; Fuxe et al., 2003; Hillion et al., 2002). The adenosine A_{2A} receptor antagonist MSX-3 has been shown to reverse the maternal behavioral impairments caused by the DA D2 receptor antagonist haloperidol (Pereira et al., 2011).

1.5 Rodent Ultrasonic Vocalizations, Social Communication and Affect

Rats emit ultrasonic vocalizations (USVs) in a variety of social and behavioral contexts, that are thought to serve social communicative functions and reflect the animal's affective state (S. M. Brudzynski, 2009). USV frequencies can range from as low as 15-kHz to as high as 120-kHz, developing throughout evolution along with the rodent auditory cortex to allow for within-species communication without risk of detection by predators (Stefan M. Brudzynski, 2005).

Adult rats produce two overarching types of USVs, classified primarily on the basis of their sound frequency: 22-kHz (range: 18-28kHz) and 50-kHz (range: 30-120kHz) (Portfors, 2007; Wöhr & Schwarting, 2013). USVs as a reflection of rodent affect has been confirmed through numerous behavioral and pharmacological studies. By exposing the rodent to inherently positive or negative situations, researchers have been able to associate distinct USV emissions with positive or negative affective states of the animal. Low-frequency calls, termed 22-kHz USVs, are typically emitted in aversive situations, such as during inter-male aggression, painful stimuli, exposure to predators, stress, and drug withdrawal (Barker et al., 2014; Blanchard, Blanchard, Agullana, & Weiss, 1991; Borta, Wöhr, & Schwarting, 2006; Han, Bird, Li, Jones, & Neugebauer, 2005; Thomas, Takahashi, & Barfield, 1983; Vivian & Miczek, 1993). These calls are considered to signal alarm and reflect negative affect. High-frequency USVs, termed 50-kHz USVs, are emitted in association with appetitive or reinforcing stimuli, such as in anticipation of or during play and mating, or in response to sucrose and drugs of abuse (Brenes & Schwarting, 2014; Browning et al., 2011; Jeffrey Burgdorf et al., 2008; Heyse, Brenes, & Schwarting, 2015; Mateus-Pinheiro et al., 2014; Schwarting, Jegan, & Wöhr, 2007; Simola, Frau, Plumitallo, & Morelli, 2014; Wright, Deng, & Clarke, 2012). Human simulation of rough-and-tumble play in juvenile rats elicits 50-kHz calls (Panksepp & Burgdorf, 2000; Schwarting et al., 2007). Based on the evidence of context-specific emission of 22 and 50kHz USVs, it is widely accepted that USVs serve as a reliable index of positive or negative affect in the rat.

Emission of USVs also has direct effects on the behavior and brain activity of conspecifics. Playback of 22-kHz USVs causes freezing behavior in rats and increased neuronal

activity in brain regions regulating fear and anxiety, such as the amygdala and periaqueductal gray in rats (Stefan M. Brudzynski & Chiu, 1995; Wöhr & Schwarting, 2007, 2013). Playback of 50-kHz vocalizations elicits social approach behavior accompanied by reduced activity levels in the amygdala but enhanced activity in the nucleus accumbens in conspecifics, indicating that these calls could signal "friendliness" and are used for social communication (Willadsen, Seffer, Schwarting, & Wöhr, 2014; Wöhr & Schwarting, 2007). Taken together, it is thought that USVs function as a reflection of rodent affective state in order to facilitate within-species social interaction.

As more researchers are investigating USVs, the complexity of social signaling in rodents is becoming more apparent. For example, while 22kHz calls are almost exclusively emitted in aversive contexts, 50-kHz calls have been seen during aversive events as well, such as during aggressive interactions and morphine withdrawal (Burman, Ilyat, Jones, & Mendl, 2007; Van Zyl, Dimatelis, & Russell, 2014). In fact, the sonographic structures of 50-kHz USVs vary greatly and researchers have begun to link specific 50-kHz USVs with social and emotional context as well. Two major and distinct categories of 50-kHz USVs exist: Flat calls, which are of constant sound frequency, and Frequency Modulated (FM) calls, which can include up to 14 documented sub-categories of calls with varying degrees of frequency modulation (Himmler, Kisko, Euston, Kolb, & Pellis, 2014; Simola et al., 2012; Wright, Gourdon, & Clarke, 2010). Recent studies have shown that the prevalence of certain 50-kHz USV subtypes is context-dependent. For instance, flat calls increase after social isolation when the rodent is returned to the cage where social contact had once occurred (Garcia, McCowan, & Cain, 2015) and following reunion of a mother rat with her pup (Stevenson et al., 2009). On the other hand, the majority of calls emitted in playful contexts are FM subtypes (Wright et al., 2010). It has been suggested that flat USVs serve a social coordination function (Wöhr, Houx, Schwarting, & Spruijt, 2008), while FM calls more reliably reflect the affective state of the rat (Jeffrey Burgdorf, Panksepp, & Moskal, 2011; Van Zyl et al., 2014).

USVs emitted by WKY adults have been characterized in previous literature. WKY adults display behavioral inhibition and lower levels of vocalization during social interactions (Rao & Sadananda, 2015). However, another studied showed that WKY increased USV emission more than the control after removal of the cage-mate in an attempt to re-establish contact (Braw et al., 2008; Van Zyl et al., 2014). This has been suggested as a demonstration of anxiety-like behavior in the WKY and was ameliorated by treatment with antidepressant.

1.6 The Role of Dopamine in USV Production

The experience of positive and negative affective states are mediated by the mesolimbic DA (positive) and mesolimbic cholinergic (negative) systems, which consequently are also associated with the production of positive and negative USVs (S. Brudzynski, 2015). Both pharmacological and electrical stimulation of these systems preferentially elicits 50 or 22 kHz USVs in rodents, respectively (Jeffrey Burgdorf, Knutson, & Panksepp, 2000). For instance, stimulation of accumbens DA with amphetamine elicits 50kHz USVs while stimulation of the ascending cholinergic system with cholinomimetics induces 22kHz USVs (S. M. Brudzynski, 2009). Manipulation of dopamine signaling has direct consequences on vocalization rate and acoustic properties of rodent USVs, therefore suggesting that rat vocalizations can provide insight into the neurobiological mechanisms affecting mood that occur in the awake, behaving animal.

1.7 Vocal Communication between Mother and Infant Rat

Auditory signaling between the litter and mother is essential to the survival of the pups. Rat pups typically emit USVs in the 40-kHz range when separated from their mother and littermates (Hofer & Shair, 1978, 1980). These calls can be distinguished from adult USVs by duration, frequency range, and repetition rate, suggesting that pup and adult USVs likely belong to different perceptual categories and consequently have different functional effects on conspecifics (Portfors, 2007). Pup isolation calls elicit pup-seeking and retrieval behavior from

the mother and facilitate mother-offspring reunions (Smotherman, Bell, Starzec, Elias, & Zachman, 1974; Wöhr & Schwarting, 2008). Alterations in pups USVs are associated with reduced maternal behavior and quality of maternal caregiving in the rat.

Signaling in the auditory cortex is enhanced in maternal rodents, allowing the mother to optimize her retrieval response to pup isolation vocalizations (Elyada & Mizrahi, 2015). Studies show that olfactory cues of pups alone do not provide sufficient information for the mother to locate the pup, and that auditory cues are essential for directional locating of the pups by the mother (Farrell & Alberts, 2002; Smotherman et al., 1974). Playback of pup USVs elicits the same approach behavior as do calls from a live pup (Farrell & Alberts, 2002; Sewell, 1970).

The relationship between pup vocalizations and maternal behavior is bi-directional. Quality of maternal care has been positively correlated with the amount of USV emission by pups. This could be because greater pup emission rates incite the mother to perform maternal behavior, but some studies have shown that the quality of maternal care affects the emission rate of pups as well (D'Amato, Scalera, Sarli, & Moles, 2005). Consequently, it has been suggested that the absence of maternal behavior from the dam attenuates pup emissions (D'Amato et al., 2005). Additionally, parental affiliation can be quantified by the increase in rat pup vocalization rate following a brief reunion with the mother (Myers et al., 2004). Some studies have suggested that certain pup vocalizations serve to inhibit aggressive behavior from the mother or conspecifics (Boulanger-Bertolus, Rincón-Cortés, Sullivan, & Mouly, 2017). One study found that prolonged vocalization from pups stressed by cold exposure agitated the mothers, causing them to perform fewer maternal behaviors (Bell, 1974). Pup USVs have even been shown to induce prolactin secretion in maternal female rats (Hashimoto, Saito, Furudate, & Takahashi, 2001). These facts suggest a nuanced effect of pup vocalizations on maternal behavior.

WKY pups show lower levels of USV emissions when repeatedly separated from the mother when compared to a control strain (Braw et al., 2008). This is consistent with previous literature that states Wistar-kyoto as a high-anxiety strain of rat and others that state a connection

between experience of low levels of maternal care, anxiety levels and rate of isolation USV emission (Rao & Sadananda, 2015; Wöhr & Schwarting, 2008). Additionally, Hodgson et al. have shown that pup isolation USVs decrease in response to a variety of anxiolytic and antidepressant drugs (2007). Pup USVs can therefore provide information about the affective state of the pups, maternal affiliation, quality of maternal care and communication deficits of the pups.

1.8 Current Studies: Research Questions and Working Hypothesis

The above evidence suggests that USVs may serve as a sensitive measure to evaluate the affective state of new mothers and affiliation in rodent models of postpartum depression (J. Burgdorf, Knutson, Panksepp, & Shippenberg, 2001). To date, however, there have been no studies combining maternal behavior and acoustic vocalization during mother-litter social interactions. The purpose of this thesis was to evaluate USVs of mothers and pups during mother-litter social interactions in the WKY model of depression and control SD strains. An additional goal was to evaluate the effect of the adenosine A_{2A} receptor antagonist MSX-3 on maternal behavior and USV emissions in both SD and WKY strains. The first study evaluated maternal behavior and ultrasonic vocalization profiles emitted by WKY and control SD dyads during dynamic social interaction. It was hypothesized that WKY mothers would exhibit altered vocalizations in parallel with parenting disturbances. The second study evaluated the ability of the adenosine A_{2A} receptor antagonist MSX-3 to ameliorate the behavioral impairments of WKY mothers. It was hypothesized that administration of MSX-3 would ameliorate social and acoustic deficits in WKY mothers.

CHAPTER 2

ANALYSIS OF AFFECTIVE ULTRASONIC VOCALIZATIONS OF MOTHER RATS DURING SOCIAL INTERACTIONS WITH THEIR LITTER – A COMPARISON BETWEEN THE CONTROL SPRAGUE-DAWLEY AND THE DEPRESSIVE-LIKE WISTAR-KYOTO

2.1 Introduction

Postpartum depression is a serious psychiatric condition affecting up to 20% of new mothers that has deleterious effects on the mother and poses a risk for the mother-infant relationship (O'Hara & McCabe, 2013). Depressed mothers are not attuned to their child's needs and signals, vocalize less often, and engage in less positive social interactions with their infants (Braarud et al., 2017; Field et al., 1985; Field, Diego, & Hernandez-Reif, 2006; Herrera et al., 2004; Zlochower & Cohn, 1996). Such compromised parenting results in long-term negative socio-emotional and cognitive developmental outcomes in infants (Beardslee et al., 2011). Although the clinical literature consistently relates postpartum depression to compromised parenting, little is known about how parenting is compromised by postpartum depression. Anhedonia, a decreased ability to experience pleasure, is a major clinical feature central to postpartum depression that likely compromises the mother's ability to sensitively interact with her infant (i.e., the ability to correctly interpret infant social signals and provide the appropriate behavioral responses). Animal models of relevance to depression represent a crucial starting point to examine impaired affiliation and attachment (social anhedonia) in postpartum depression.

Rats perceive and emit ultrasonic vocalizations (USVs) in a variety of social and behavioral contexts that are critically dependent on situational factors and experience (S. M. Brudzynski, 2009). While it is widely accepted that USVs serve an important communicative function during social interaction, there is growing evidence highlighting USVs as an emerging source of insight into the rat's affective state (S. Brudzynski, 2015; Wöhr & Schwarting, 2013).

In adult rats, two main types of USVs have been described: 22-kHz (range: 18-28kHz) and 50-kHz (range: 30-120kHz) (Best, Zhao, Scardochio, & Clarke, 2017; Wöhr & Schwarting, 2013). Low-frequency calls, termed 22-kHz USVs, are typically emitted in aversive situations (Barker et al., 2014; Blanchard et al., 1991; Han et al., 2005; Thomas et al., 1983) while high-frequency USVs, termed 50-kHz USVs, are emitted in association with appetitive or reinforcing stimuli (Jeffrey Burgdorf et al., 2008; Panksepp & Burgdorf, 2000; Willadsen et al., 2014; Wöhr & Schwarting, 2007). Pup isolation-induced vocalizations, typically emitted at 40kHz, have also been extensively characterized and are considered a third, separate category of rat USVs (Portfors, 2007). Pups emit USVs when singly-isolated from the mother and their emission rates relate to anxiety, maternal affiliation and quality of maternal care (Bell, Nitschke, Bell, & Zachman, 1974; Boulanger-Bertolus et al., 2017; Brouette-Lahlou, Vernet-Maury, & Vigouroux, 1992; D'Amato et al., 2005; Hashimoto et al., 2001; Myers et al., 2004; Noirot, 1972; Shair, 2014). To date, however, little is known about vocalization of mother rats during social interactions with her litter, and whether maternal USVs could be used to indicate disturbances in postpartum social affect.

The present study assesses USVs in the Wistar-Kyoto (WKY) rat model of depression, which displays an endogenous depressive phenotype, for identification of altered affiliative mechanisms as characterized by their species-specific USV profile during social interaction with the litter.

2.2 Materials and Methods

2.2.1 Animals

Primiparous postpartum Sprague-Dawley (SD) and Wistar-Kyoto (WKY) female rats (Charles River Laboratories, Kingston, NY) bred in our laboratory were used. Before giving birth, pregnant females were housed in individual clear Plexiglass cages (38.5 cm W x 48.5 cm L x 20.5 cm H) lined with fresh Sani-Chips® bedding (P.J. Murphy Forest Products Corp,

Montville, NJ) and containing Eco-Bedding as nest-building material (Fibercore LLC, Cleveland, Ohio). The day of birth was designated as postpartum day 0 (PPD0) and litters were culled to 8 pups (3–5 males, 3–5 females) on PPD1. All females were kept on a 12-h light/dark cycle (light on at 0700 AM) at 22±1◦C, with *ad libitum* access to water, rat chow (5058 PicoLab® Diet, LabDiet, Brentwood, MI) and sunflowers seeds (5LP8 PicoLab® Sunflower, LabDiet, Brentwood, MI). All behavioral procedures were conducted during the light phase of the light/dark cycle. One day before testing, a 5-cm high Plexiglas divider was inserted into each female's cage to divide the floor into four equal compartments. Females were tested in their home cages, which were placed into the testing room to habituate 15 minutes prior to the start of testing procedures. Ambient temperature in the testing room was maintained at 22 ± 1°C. All procedures used in this study were reviewed and approved by the University of Massachusetts Amherst's Institutional Animal Care and Use Committee.

2.2.2 Experimental Design

WKY (n=7) and SD (n=8) postpartum females were examined for their affective responses to social cues from pups, as measured by their ultrasonic vocalizations (USVs) during a maternal behavior test. In fast-paced, dynamic social interactions, no particular call can be readily attributed to a specific member of the mother-infant dyad. In order to determine which member of the dyad was vocalizing, additional separate recordings of the mother and the pups were included. Postpartum females underwent two phases of behavioral testing occurring on PPD7 and PPD8 (Figure 2.1).

Day 1 Testing began with the litter removed from the home cage and housed in a small cage (18 cm W x 30 cm L x 13 cm H) outside of the testing room (lined with Sani-Chips® bedding and containing nest-building material from the maternal cage). Ten minutes after removal of the litter, a 5-minute recording was taken while the mother was alone in her cage (I: *mother alone).* Immediately after, the cage containing the litter was returned to the testing room

and placed adjacent to the home cage (so that the mother was able to see, smell and hear the pups but not physically interact with them) and a second 5-minute recording was taken (II: *mother with litter separated).* Thereafter, the litter was scattered throughout the home cage opposite to the nest, and a 30-minute recording was taken in conjunction with a maternal behavior test (III: *mother-litter social interaction*). After the maternal behavior test, the mother and litter were removed from the home cage. One pup from the litter was randomly selected and returned to the home cage, and a 5-minute recording was taken from this isolated pup (*IVa: pup isolation*). Following recording, the pup was reunited with his/her mother and littermates. Immediately after, a littermate of the opposite sex was returned to the home cage, and a 5-minute recording was taken (*IVb: pup isolation*). Isolated pups were placed outside of the nest site during isolation recordings due to an observed lack of vocalizations when placed within the nest. The order of the sex of the pups recorded was counterbalanced within groups. The pups' temperatures were measured before and after each isolation recording and their weights taken after testing.

Day 2 Testing began with the mother injected intraperitoneally (IP) with 1.0 mL/kg of a solution that contained ketamine HCl (75.0 mg/mL), xylazine (7.5 mg/mL) and acepromazine maleate (1.5 mg/mL) before being placed into the testing room. Once the mother exhibited loss of palpebral/corneal and pedal withdrawal reflexes (about 5 minutes), the litter was removed from the home cage and housed in a small cage (18 cm W x 30 cm L x 13cm H), lined with Sani-Chips® bedding and containing nest-building material from the maternal cage, outside of the testing room. Fifteen minutes later, a 5-minute recording was taken of the anesthetized mother in her home cage to confirm the absence of maternal vocalizations (V: *mother anesthetized*). The litter was then scattered throughout the home cage opposite to the nest, and a 15-minute recording was taken (VI: *litter with anesthetized mother*). During this recording, after 10 minutes elapsed the researcher manually grouped the litter around the mother in the nest, thus making it possible to compare litter vocalization levels before and after grouping.

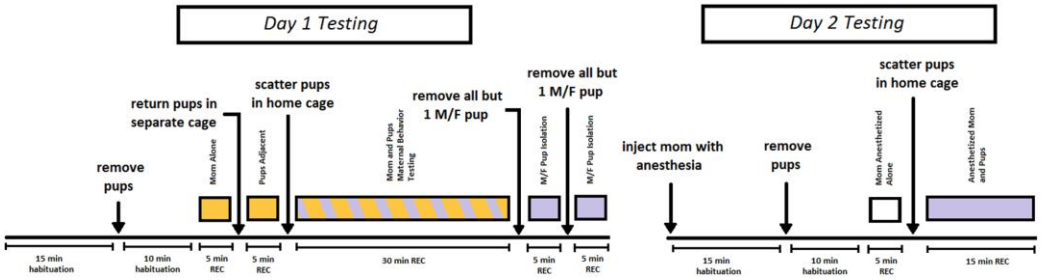

Figure 1. Schematic representation of experimental procedure. Yellow boxes indicate recordings that contain exclusively maternal USVs while purple indicates those recordings that contain exclusively pup USVs. Yellow and purple stripes indicate both maternal and pup USVs are present.

2.2.3 Maternal Behavior Testing

Following 20 minutes of maternal separation, the litter was scattered in the home cage opposite to the nest, and the number of the following maternal behaviors were continuously scored for 30 min: retrievals of the pups into the nest, mouthings (oral repositioning of the pups into the nest), full body and anogenital lickings, and nest building. In addition, the total duration of huddling behaviors, including lying in contact with pups and hovering over the pups in the nest while actively performing other behaviors (i.e. licking of pups or self-grooming), and the nursing posture kyphosis (a quiescent upright crouching over pups) were recorded. Total time in contact with pups was considered as the summed durations of huddling plus nursing behaviors. Also, the latency to begin retrieving pups (i.e. time from the introduction of the pups to the first retrieval by the female), to reunion of the entire litter into the nest as well as to begin hovering over and nursing was registered. Only those postpartum females that retrieved each of their pups into the nest were considered to have grouped the litter. The latency to begin hovering over or nursing the pups is the first occurrence of a bout of each behavior ≥2 min in duration. A latency of 1800 s was given for any category of behavior that was not initiated (or completed, i.e. reunion of the litter) within the 30-minute observation period. Other behaviors recorded included general exploration (line crosses and rearings), self-grooming and eating/drinking.

2.2.4 Ultrasonic Vocalization Recording and Analysis

USVs were recorded using the Avisoft Ultrasound Gate 116H acquisition device (Avisoft Bioacoustics, Berlin, Germany) connected to a condenser ultrasound microphone (CM16/CMPA Avisoft Bioacoustics, Berlin, Germany) and stored for offline analysis. The microphone was placed at a fixed height of 12-15 cm above the cage. Spectrograms were displayed in real time by Avisoft-RECORDER USGH (version 2.7; Avisoft Bioacoustics, Berlin, Germany) on a personal computer.

Acoustic analysis of the recordings was performed using Avisoft SASLab Pro (Version 5.2.10, Avisoft Bioacoustics, Berlin, Germany). The amplitude of each sound file was increased by 200% to strengthen USV signals and allow for comparability between spectrogram-derived parameter measurements. Spectrograms were generated with a fast Fourier transformation (FFT) length of 512 points and a time window overlap of 75% (FlatTop window, 100% frame size). Correspondingly, spectrograms had a frequency resolution of 488 Hz and a temporal resolution of 0.512 ms. Due to the substantial background noise picked up by the microphone, a spectrogram bandwidth of 30-90 kHz was set to reduce automatic measurement errors in Avisoft. Preliminary analysis of the data indicated that less than 1.5% of the vocalizations emitted during maternal interaction were below 30 kHz for both SD and WKY (n = 6).

Following manual adjustment of duration measurements and classification of the USVs, a Linear Prediction Coding (LPC) procedure in Avisoft was applied to each spectrogram, and the Avisoft automatic parameter measurement tool was used to calculate duration, start time, and peak frequency (frequency at the relative point of maximum amplitude) at 50 evenly-spaced time points within each USV. To minimize erroneous peak frequency measurements taken at points of low USV amplitude, the program was set to exclude frequency measurements taken from spectrogram points with amplitudes of less than -69 dB. This reduced but did not eliminate erroneous measurements at times when the background noise had a higher amplitude than the

USV signal, so a custom R script was created to remove remaining outliers and inaccurate measurements, accounting for the limitations of the Automatic Detection feature of Avisoft and manual classification. Because there was considerable variability in the amplitude of each call, almost all of the discrete USVs had missing data points. which were then interpolated as explained below.

2.2.5 Post-Processing and Acoustic Parameter Analysis

Labels that contained more than one USV (overlapping calls) were excluded from follow up processing procedures and all parameter analyses (0.15%). Discrete calls of duration between 2 – 400 ms that had at least 23 out of 50 evenly-spaced peak frequency measurements were considered for interpolation procedures. Prior to interpolation, erroneous peak frequency measurements at the extremes of the spectrogram were identified and deleted from the data. Missing peak frequency data points were reconstructed using a spline interpolation function in R. Spline over other methods was selected based on visual determination of fit of a random sample of 1000 USVs balanced for call type. However, spline tended to interpolate endpoint values to the extreme. The resulting outlier frequency measurements were removed and the missing data points reconstructed using a step-wise regression, which was determined to be the simplest and most effective final interpolation method for USV endpoints. The peak frequency measurements were then averaged for each USV signal to yield the average peak frequency.

2.2.6 Manual Classification of Ultrasonic Vocalizations

Each USV was manually labelled on the spectrogram by a single coder. A second trained coder independently analyzed a random sample of USV recordings to ensure objectivity and reliability in the manual analysis. Inter-rater reliability measures were confirmed to be high (ICC = 0.98, $p<0.01$). Labelled USVs had to meet three spectrographic inclusion criteria (Wright et al.,

2010): (1) temporal continuity (i.e., interruption < 20 ms), (2) sound frequency between 30 and 90 kHz, and (3) signal composition visually distinct from background noise.

2.2.7 Statistical Analysis

Behavioral data were analyzed using non-parametric Mann-Whitney U tests and acoustic data were analyzed with linear mixed models (SPSS v25), using the best-fitting covariance structure. For each analysis, only those USV categories that made up more than 2% of the emissions in at least one of the groups were included. All acoustic data are expressed as mean ± standard error of the mean (SEM). Statistical significance was set at $p<0.05$.

2.3 Results

2.3.1 Overview of USV Results

A total of 24,776 USV emissions were manually detected and analyzed in this experiment. Based on visual inspection of the spectrograms, 20 USV categories were recognized (Figure 2), including 3 novel categories (Table 2). Less than 1% of the total USVs did not fit into one of these 20 categories and were classified as unclear. Of all the USVs analyzed, flat USVs made up the greatest proportion at 58%, followed by short calls at 14%. 98% of the data was made up by flat, short, flat-z, flat-mz, trill, short-c, complex, short-ur, step down, inverted-u, step up and trill-c USVs, ordered by most to least percentage. The remaining 7 categories made up less than 2% of the observed USVs.

No USV categories were emitted in every recording by every individual. 3/8 SD dyads emitted flat or short USVs in all 6 recordings and 1/7 WKY dyads emitted short USVs in all 6 recordings. In general, flat and short USVs were the most ubiquitous across recordings, with their proportions of occurrence (number of recordings containing that USV category / the total number of recordings, 6) significantly larger than all other categories (all ps < 0.05).

USV acoustic signals overlapping in time could not be measured accurately for duration or peak frequency. Of the 24,776 USVs included in statistical analysis of counts, 21,117 consisted of a single sound wave unique in time and were included in duration analysis. Of these non-overlapping USVs, 15,060 had adequate signal-to-noise ratios to allow for accurate detection of peak sound frequency by the analysis software and were included in the acoustic frequency analysis. The proportion of non-overlapping USVs included in acoustic frequency analysis was similar between strains and across most USV categories (Table 1).

Of note, 22kHz USVs (mean peak frequency less than 30kHz) accounted for only 1% of the measurable USVs. This USV category was only emitted by mothers when separated from their pups (see "Mother USV Profile" below for details).

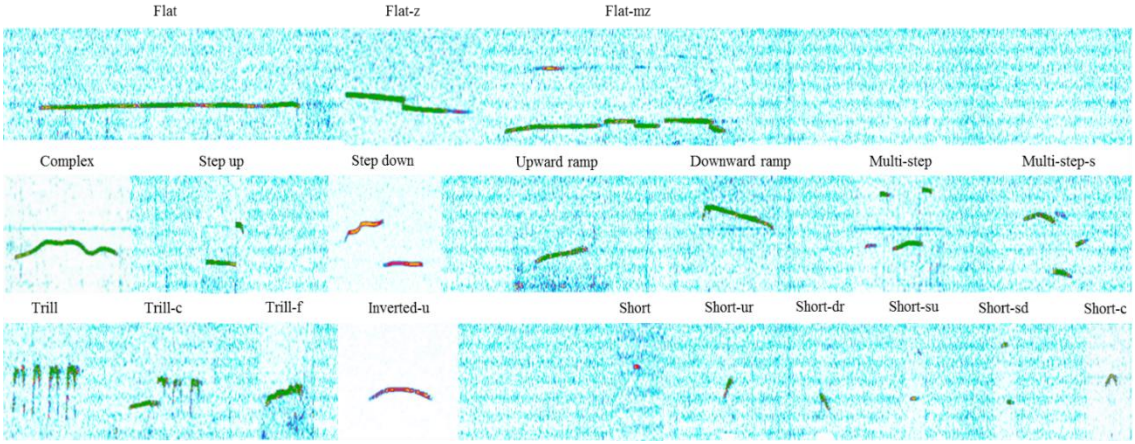

Figure 2. Representative spectrogram images of the 20 USV categories recognized in this study.

Table 1. Percentage of non-overlapping calls in each category that were included in acoustic frequency analysis. Asterisk indicates significant difference of proportions between strains (One-way ANOVA with strain as the between-subjects factor).

	SD (%)	**WKY (%)**
Complex	78.7	75.86
Flat	75.74	75.87
Flat-mz	94.86	92.44
Flat-z	83.06	89.17
Inverted-u	79.53	65.81
Short	66.21	49.78
Short-c	66.47	32.08*

Short-ur	56.65	16.26*
Step down	74.81	54.55
Step up	60.36	61.4
Trill	64.52	17.92*
Trill-c	85.71	60.56
Upward ramp	82.14	61.54

Table 2. Descriptions of USV categories and existing literature which identifies them.

	Description	**Existing literature**
Flat	Strain line with mean slope < 0.2 kHz/ms	Clark et al, 2010, Burgdorf et al, 2008; Brudzynski 2015; Burke et al, 2017; Takahashi et al, 2010; Rao et al, 2015; Portfors, 2007; Geyer, 2009
Flat-z	Flat with one instantaneous frequency jump < 10 kHz to another flat	Zeskind et al, 2011; Geyer, 2009; Hashimoto et al, 2004
Flat-mz	Flat with more than one instantaneous frequency jump < 10 kHz to another straight line	Zeskind et al, 2011; Geyer, 2009; Hashimoto et al, 2004
Short	Duration < 12 ms with no change in frequency	Clark et al, 2010; Brudzynski, 1999 (dot-type); Burke et al, 2017; Takahashi et al, 2010; Portfors, 2007
Short-ur	Steady increase in frequency with mean slope > 0.2 kHz/ms and duration < 12 ms	Brudzynski, 1999 (rising sweep)
Short-dr	Steady decrease in frequency with mean slope < -0.2 kHz/ms and duration < 12 ms	Brudzynski, 1999 (falling sweep); Hashimoto et al, 2004
Short-su	Instantaneous frequency jump to a higher frequency and duration < 12 ms	NA
Short-sd	Instantaneous frequency jump to a lower frequency and duration < 12 ms	NA
Short-c	Inverted-u shape < 12 ms	NA
Complex	Mean slope of frequency changes sign (+/-) twice or more with frequency change > 5 kHz each	Clarke et al, 2010; brudzynski, 1999; Burke et al, 2017
Step up	Instantaneous frequency jump > 10 kHz to a higher frequency	Clarke et al, 2010; Zeskind et al, 2011; Brudzynski, 2015; Burke et al, 2017
Step down	Instantaneous frequency jump > 10 kHz to a lower frequency	Clarke et al, 2010; Zeskind et al, 2011; Brudzynski, 2015; Burke et al, 2017
Upward ramp	Steady increase in frequency with mean slope > 0.2 kHz/ms	Clarke et al, 2010; Burke et al, 2017
Downward ramp	Steady decrease in frequency with mean slope < -0.2 kHz/ms	Clark et al, 2010
Multi-step	More than one instantaneous frequency jump >10 kHz	Clark et al, 2010; Zeskind et al, 2011; Brudzynski, 2015; Burke et al, 2017

Multi-step-s	More than one instantaneous frequency jump >10 kHz and part of the call contains a harmonic	Clark et al, 2010; Burke et al, 2017
Trill	Rapid oscillations in frequency	Clark et al, 2010; Burke et al, 2017; Takahashi et al, 2010; Rao et al, 2015
Trill-c	Flat or complex with instantaneous jump to a trill	Clark et al, 2010; Burgdorf et al, 2008; Brudzynski, 2015; Burke et al, 2017; Rao et al, 2015
Trill-f	Flat with micro-oscillations in frequency	Rao et al, 2015
Inverted-u	Steady increase followed by a decrease in frequency > 5kHz each	Clark et al, 2010; Brudzynski 1999; Burke et al, 2017; Takahashi et al, 2010

2.3.2 Mother USV Profile

Two 5-min recordings were taken from the mother alone in the home cage first in the absence and then in the presence of her pups in the testing room. The pups were removed and housed outside of the testing room for the first recording. For the second recording, the litter was returned to the room, and positioned next to the home cage so that the mother could see, smell and hear her pups but not physically interact with them.

There was no main effect of strain ($F_{(1,13)}$=0.600, p=ns), or a strain x USV category interaction ($F_{(7, 195)}$=0.172, p=ns) for the overall number of maternal USVs emitted, with both SD and WKY mothers emitting low levels of calls, mostly flat and short categories (all ps<0.05).

There were, however, significant main effects of context ($F_{(1, 195)}$=15.848, p<0.001) and USV category ($F_{(7,195)}$=4.458, p<0.0001), as well as a significant context x USV category ($F_{(7, 195)}$=2.328, p<0.05) interaction effect for the number of USV emissions. Both strains emitted increased numbers of maternal flat and short USVs when the litter was returned and placed adjacent to the home cage (Figure 2.3, all ps<0.001). WKY mothers also showed an increase in trill-c USVs, however this difference did not reach significance (Figure 3). ANOVA also showed a significant strain x context interaction effect ($F_{(1,195)}$=3.973, p<0.05), with only WKY mothers significantly increasing their USV emissions upon the return of their pups to the testing room (p<0.001). WKY mothers also emitted 22kHz USVs when the pups were placed adjacent to the

home cage, with relative numbers (number of 22kHz USVs / total number of measurable USVs) ranging from 2/2 (100%), through 14/40 (33%) to 5/71 (7%).

During the first recording, 3 of 8 SD moms and 5 of 7 WKY moms did not vocalize while alone in the home cage. Because of the low USV emission levels, mean peak frequency and duration of relevant call categories were calculated using pooled data from both recordings. No significant strain differences were found on the peak frequencies of USVs ($F_{(1,9.378)}$=1.659, p=ns), however, there was a significant main effect of USV category ($F_{(6,9.378)}$=5.435, p<0.05), with both strains emitting trill, trill-c and upward ramp USVs at higher frequencies than the other categories (all ps<0.05), and an interaction of strain x USV category ($F_{(6,9.378)}$=5.971, p<0.01), with WKY mothers emitting step up USVs at a higher peak frequency than SD mothers (p<0.01). In addition, there was no significant difference between strains on the duration of calls ($F_{(1,4.311)}$=0.000, p=ns), but there was a significant main effect of USV category ($F_{(6,7.386)}$=23.169, p<0.001), with both strains emitting short USVs with significantly shorter durations than all other USVs (all ps<0.05), excluding upward ramp (p=ns).

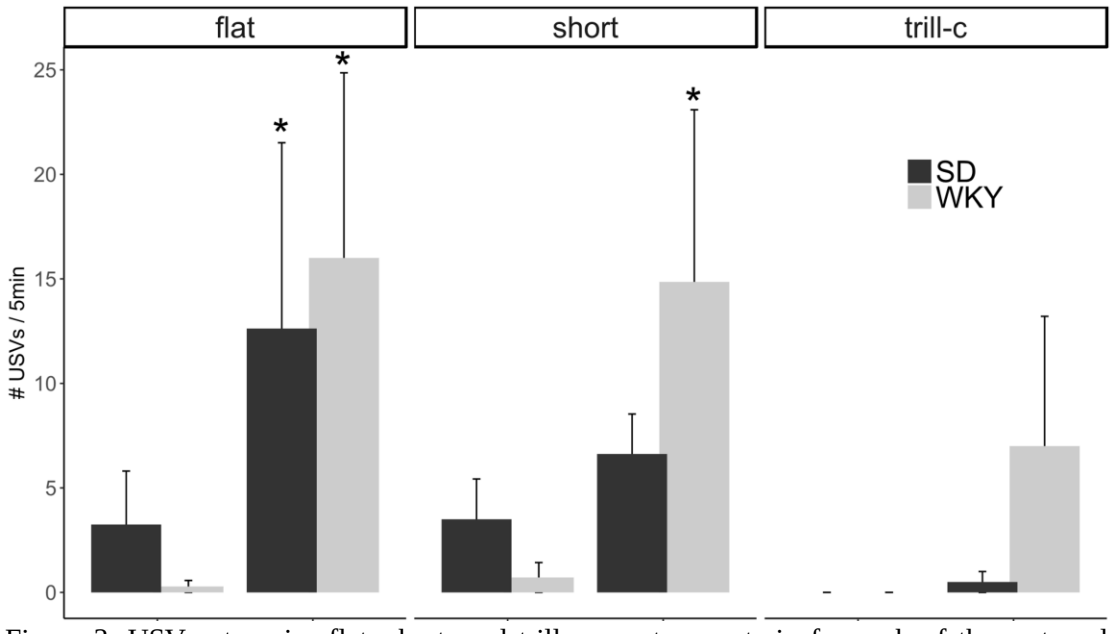

Figure 3. USV categories flat, short, and trill-c counts per strain for each of the maternal recordings. * indicates within-strain significance of recordings.

2.3.3 Isolation-induced USVs Emitted in the Home Cage

Isolation-induced USVs were collected for 5min on PND7-8 in the home cage. Pups were selected randomly from the litter and placed in their home cage outside of the nest quadrant for the duration of the recording. As shown in Figure 4A, the total number of isolation-induced USVs was not different between WKY and SD pups. A three-way ANOVA with strain and sex as the between-subjects factors and USV category as the within-subjects factor showed no main effect of strain ($F_{(1,31.634)}$=0.981, p=ns) or sex ($F_{(1,31.634)}$=0.077, p=ns) on the number of isolation-induced USVs during the 5-minute recording. There was a significant main effect of USV category ($F_{(4,46.546)}$=4.905, p<0.01), with both WKY and SD pups emitting almost exclusively flat, flat-z, flat-mz, and short calls (Figure 4B). In addition to the total USV count and call count by USV categories, the latency to start calling ($F_{(1,25)}$=1.178, p=ns), total calling time ($F_{(1,25)}$=0.290,p=ns), and emission rate (number of calls/min; $F_{(1, 25)}$=1.580, p=ns) were not different between singly-isolated WKY and SD pups (Figure 4C).

Further analysis of acoustic properties of these USVs revealed significant differences between strains (Figure 4D). WKY pups emitted isolation-induced USVs at a higher frequency than SD pups (main effect Strain: $F_{(1, 17.866)}$=4.417, p=0.050; main effect USV category: $F_{(3,30.131)}$=12.269, p<0.0001; interaction Strain x USV category: $F_{(3,30.131)}$=0.510, p=ns). Both strains emitted short USVs at a higher peak frequency than the other USVs (all ps<0.001). Most isolation-induced flat USVs emitted by WKY pups had peak frequencies between 40 and 50kHz, whereas those emitted by SD pups had peak frequencies between 38 and 48kHz (first and third quartiles). Also, the duration of isolation-induced flat calls differed between strains (main effect Strain: $F_{(1,20.303)}$=16.918, p < 0.01; main effect USV category: $F_{(3,18.081)}$=135.765, p<0.0001; interaction Strain x USV category: $F_{(3,18.081)}$=6.565, p<0.01), with WKY pups emitting flat, flat-z and flat-mz USVs of shorter duration than SD pups (all ps<0.05). There was no main effect of sex

for mean peak frequency ($F_{(1,17.866)}$=0.491, p=ns) or mean duration ($F_{(1,20.303)}$=2.210, p=ns) of isolation-induced USVs.

Body weight and core temperature were also collected, as these variables are known to alter pup USV emission (Brunelli, Vinocur, Soo-Hoo, & Hofer, 1997; Shair, Masmela, Brunelli, & Hofer, 1997). Litter weights were taken on the day of recording, then divided by the total number of pups in the litter to yield average pup weights. Pup weight differed between strains (main effect of Strain: ($F_{(1,13)}$=5.073, p<0.05), with WKY pups weighing significantly less than SD pups. Peak frequencies were negatively correlated with pup weight (Pearson Coefficient r=-

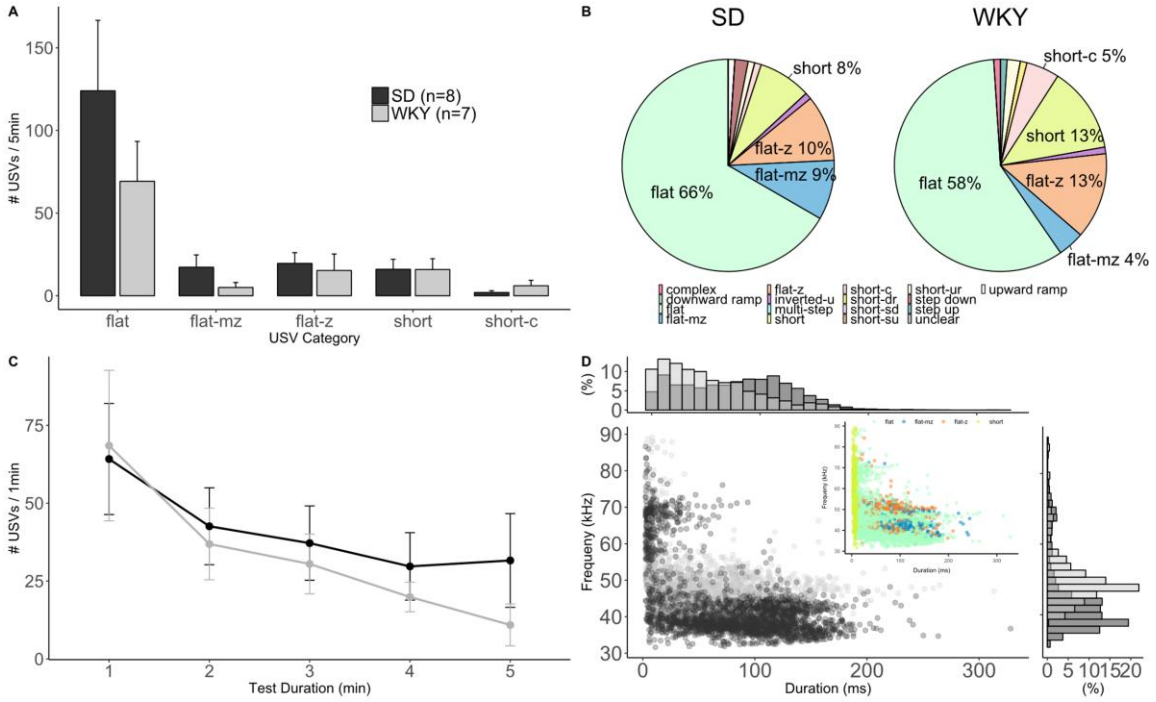

Figure 4. Isolation-induced USVS in WKY and control SD pups tested in their home cage. (A) Total number (n) of isolated-induced USV emitted during 5-min isolation from mother and littermates. (B) Pie graph shows the percentage of the different call categories emitted by WKY and SD pups. No sex differences were detected on any USV metric scored and these data were pooled across sex within each strain. (C) call rate (number of isolation-induced USV per min). (D) Distribution of duration and peak frequency of individual isolation-induced USVs in WKY and SD pups (inset: distribution colored by USV category).

0.216, p<0.05), while duration of USVs and total USV count were not correlated with pup weight. Body temperatures were registered at the beginning and end of the 5 min recording. No differences were detected on body temperatures between SD and WKY pups (p=ns).

2.3.4 Pup Call Repertoire in the Presence of Littermates and Anesthetized Mother

The litter was first moved to another cage, after which the mother was anesthetized and returned to the nest quadrant of the home cage. A 5 min recording of the anesthetized mother verified the absence of maternal calls. Following 20 minutes of mother-litter separation, pups were then scattered in three quadrants of the home cage (nest located in fourth quadrant, separated by 5cm high plexiglass dividers), and their USVs recorded for 15 minutes. The USV emissions during the first 5 minutes of this test were compared with those emitted during the 5-minute pup isolation tests. When the number of pups vocalizing was controlled for, three-way ANOVA showed a significant interaction effect of USV category x recording ($F_{(5,16.387)}$=5.100, $p<0.01$), with both strains emitting significantly less flat, flat-mz, flat-z and short USVs in the presence of the littermates and anesthetized mother (all $ps<0.05$). There was also a change in USV profile (interaction strain x USV category x recording: $F_{(5,112.836)}$=4.133, $p<0.01$), with WKY pups decreasing the proportion of flat USV emissions and increasing the proportion of short USV emissions in the presence of the litter and anesthetized mother (all $ps<0.01$). With 2-3 pups in each of the three quadrants, it was noted that the pups tended to group themselves with their littermates within each quadrant before 5 minutes had elapsed (early postpartum pups were unable to climb over the 5cm high plexiglass divider). Once 10 minutes had elapsed, the experimenter grouped the pups with the mother manually, causing an increased rate of pup USV emission for 30 seconds during manual grouping. This increase in call rate, which persisted for only 30 seconds after the onset of grouping, was analyzed separately and was ignored for comparisons between strains.

As shown in Figure 6A, the number of USVs emitted was different between WKY and SD litters. A two-way ANOVA with strain as the between-subjects factor and USV type as the within-subjects factor showed significant main effects of strain ($F_{(1,23.728)}$=10.857, $p<0.01$) and USV type ($F_{(7,19.498)}$=8.104, $p<0.0001$), as well as a strain x USV type interaction ($F_{(7,19.498)}$=5.554,

p<0.01) on the number of USV emissions during the 15-minute recording. WKY pups emitted significantly less flat, flat-z and flat-mz USVs compared to control SD pups (all ps<0.05) while the counts of all other USV categories did not differ between strains (all ps=ns).

The litter was manually grouped around the anesthetized mother by the experimenter 10 minutes into the recording. The number of USV emissions during manual grouping (30 seconds following the onset of manual grouping) were compared to the USV emissions during the 30 seconds preceding manual grouping in both strains. There was a significant increase in USVs in both strains following onset of manual grouping (main effect of grouping: $F_{(1,153.942)}$=57.074, p<0.001; interaction grouping x strain: $F_{(1,153.942)}$=0.602,p=ns). There was a three-way interaction effect of strain x grouping x USV category ($F_{(10,225.001)}$=2.850, p<0.01). Both SD and WKY pups increased flat USVs (p<0.0001 and p<0.01, respectively) and short USVs (p<0.001 and p<0.001, respectively) in response to manual grouping, with SD pups showing a greater increase in flats compared to WKY pups (Figure 5). The USV profiles also differed between strains (interaction strain x USV category: $F_{(10,44.150)}$=3.136, p<0.01) with WKY pups emitting a smaller proportion of flat USVs (p<0.01) before and during grouping.

Number of USVs were also compared during minutes 1-3 (beginning), 7-9 (middle) and 13-15 (end) of the test to discern the effects of different contexts on pup USV emission (Figure 6C). During minutes 1-3 pups were scattered throughout the cage without direct skin contact with litter-mates, and by minutes 7-9 pups had grouped themselves with the littermates in their quadrant of the cage. Finally, minutes 12-15 were after manual grouping of the litter with their anesthetized mother in the nest quadrant. Three-way ANOVA (between = strain, within = time in test and USV category) showed a significant main effect of time in test ($F_{(2,494)}$=16.690, p<0.001) and no interaction effect of strain x time in test ($F_{(2,494)}$=1.921, p=ns) on the number of USV emissions (Figure 6C). Additionally, there was an interaction effect of strain x time in test x USV category ($F_{(24,494)}$=4.379, p<0.001). Both SD and WKY pups emitted more short USVs during the beginning of the test than during the middle or the end (all ps<0.05), but only SD pups emitted

more flat USVs during the beginning of the test than during the middle or the end (both ps<0.0001). WKY pups emitted more short-ur USVs during the beginning of the test compared to the middle and the end (both ps<0.01) and more short-c USVs during the beginning of the test compared to the middle and the end (p<0.05; p=0.058, respectively). The USV profile was also different between strains (interaction strain x USV category: $F_{(12,233.443)}$=9.986, p<0.0001) with SD pups emitting a greater proportion of flat USVs (p<0.0001) and WKY pups emitting a greater proportion of short (p<0.001) and short-c (p<0.001) USVs. This difference was consistent across test times (interaction strain x USV category x time in test: $F_{(24,382.176)}$=0.805, p=ns).

Further analysis of USV parameters revealed a difference in the peak frequencies of USVs emitted by WKY and SD litters (Figure 6D). A two-way ANOVA with strain as a between-subjects factor and USV type as a within-subjects factor showed significant main effects of strain ($F_{(1,14.963)}$=35.513, p<0.001) and USV type ($F_{(5,16.536)}$=19.345, p<0.001) on mean peak frequency of USVs with no interaction effect ($F_{(5,16.536)}$=1.210, p=ns). WKY pups emitted each USV type at a higher frequency than SD pups (all ps<0.05), except for step down USVs, which

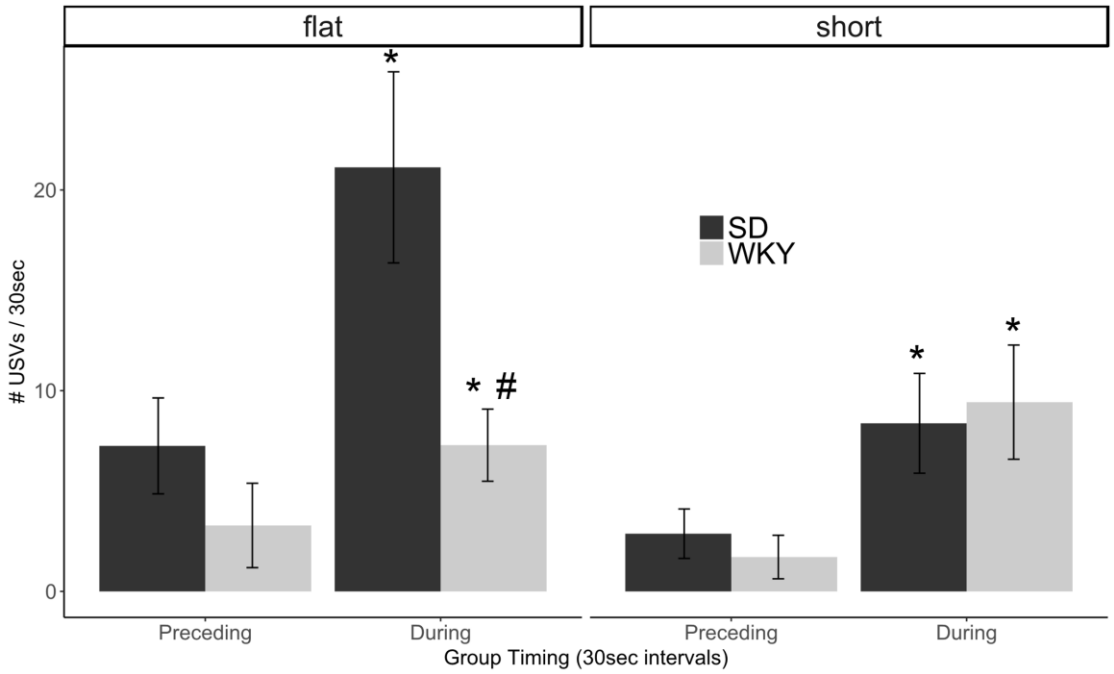

Figure 5. Pup USV counts of flat and short USVs before and during grouping. * indicates within-strain differences before and during grouping, # indicates between-strain differences.

were emitted at a marginally higher peak frequency by WKY pups (p=0.066). Short USVs were emitted at a higher peak frequency than other USV categories in both strains (all ps<0.05), excluding step down (p=ns). The duration of the USVs emitted differed between strains ($F_{(1,14.051)}$=7.384, p<0.05), and a two-way ANOVA showed a significant effect of USV category ($F_{(7,57.861)}$=21.928, p<0.0001) and no interaction of strain x USV Category ($F_{(5,56.197)}$=1.273, p=ns). Flat-mz USVs had the longest durations in both strains (all ps<0.05) while short USVs had the shortest duration (all ps<0.05). Litter weights were taken and divided by the number of pups in the litter to yield average pup weight, as pup weight is known to affect USV emission rate. Average pup weight was different between strains (F(1,13)=5.073, p<0.05), and was positively correlated with number of USV emissions (Pearson's Cor r=0.523, p < 0.05) and duration of flat-

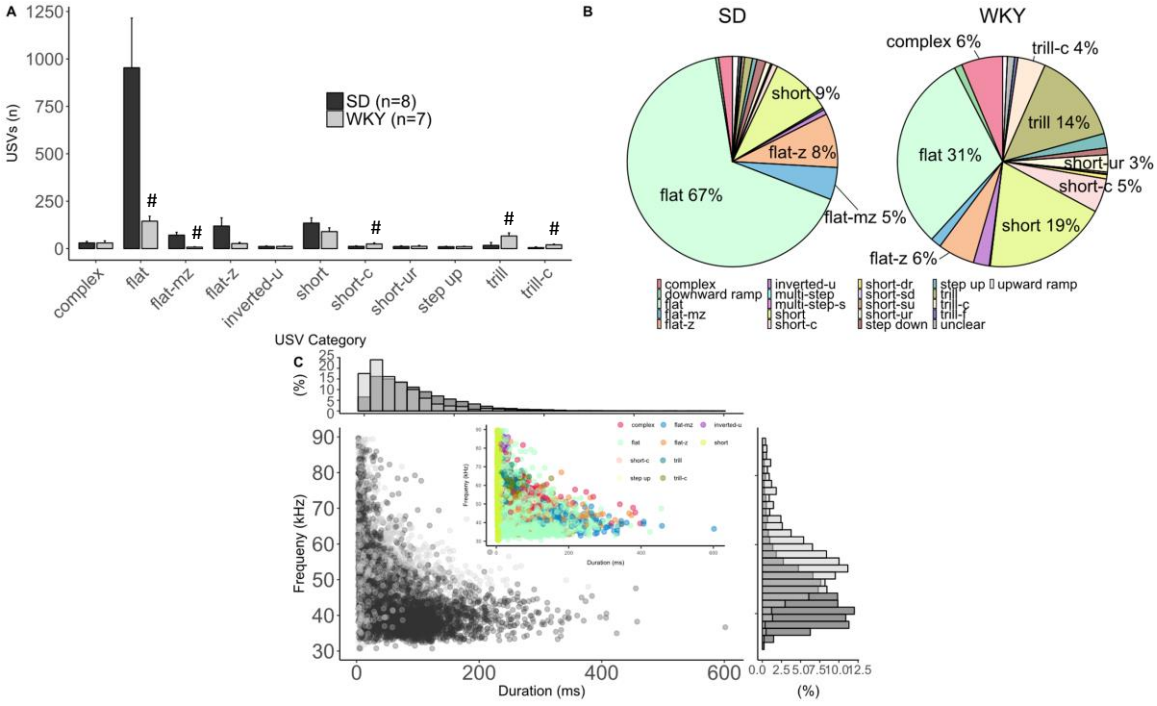

Figure 6. USVs in WKY and SD pups tested in the presence of littermates and anesthetized mother in their home cage. (A) Mean ± SEM counts of USV categories emitted during 15-min recording. # indicates significance between strains. (B) Pie graph shows the percentages of all call categories emitted by WKY and control SD litters. (C) Number of USVs when pups were scattered, after grouping with littermates in each quadrant, and after grouping with mother in WKY and SD pups. * indicates significant difference from scattered across strains. # indicates significance between strains. (D) Distribution of duration and peak frequency of individual USVs emitted by WKY (grey) and SD (black) pups (inset: distribution colored by USV category).

z USVs (r=0.619, p<0.05).

2.3.5 USVs During Mother-Litter Social Interaction

WKY dyads emitted significantly fewer USVs than SD dyads (main effect of strain: $F_{(1,19.208)}$=10.020, p<0.01). Five call categories were particularly prevalent across both strains (significant main effect of USV category: $F_{(10,23.162)}$=7.008, p<0.0001): flat (58%), short (11%), flat-z (8%), trill (5%) and flat-mz USVs (5%) (Figure 7B). In addition, there was a significant strain x USV category interaction effect ($F_{(10,23.162)}$=3.932, p<0.01): SD dyads emitted significantly more flat and flat-mz USVs than WKY dyads (all ps<0.01) and marginally more flat-z USVs (p=0.076), while WKY dyads emitted more short-c (p=0.059), trill (p=0.051) and trill-c (p<0.05) USVs (Figure 7A). The USV profiles also differed between strains (interaction strain x USV category: $F_{(10,30.530)}$=12.353, p<0.0001), with WKY dyads emitting a smaller

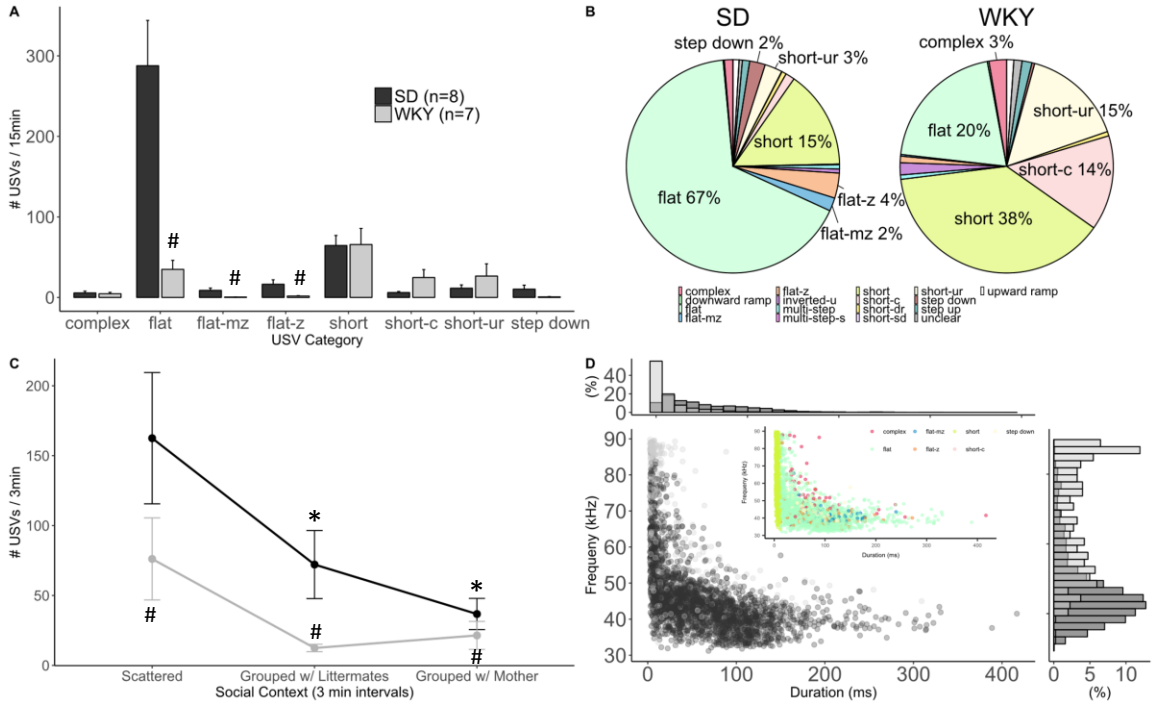

Figure 7. USVs during mother-litter interactions. (A) Mean ± SEM counts of USV categories emitted during 30-min recording. (B) Pie graph shows the percentages of all call categories emitted by WKY and control SD dyads. (C) Distribution of duration and peak frequency of individual USVs emitted by WKY (grey) and SD (black) pups (inset: distribution colored by USV category).

proportion of flat and flat-mz USVs (all ps<0.05) calls, and a greater proportion of short, short-c, step up, trill and trill-c USVs than the control SD strain (all ps<0.05).

In addition, the mean peak frequencies of USVs were significantly different between strains (Figure 7C, $F_{(1,19.959)}$=62.472, p<0.0001) and USV category ($F_{(8,24.315)}$=39.289, p<0.0001), with WKY dyads emitting USVs at a higher peak frequency overall (strain x USV category interaction effect: $F_{(4,48)}$=1.630, p=ns) (Figure 7C). Short, inverted-u, trill and trill-c USVs were emitted at higher peak frequencies relative to the other USV categories in both strains (all ps<0.05). Durations of these calls were also significantly different between strain (Figure 7C, $F_{(1,32.282)}$=6.052, p<0.05) and USV category ($F_{(8,27.064)}$=168.304, p<0.0001), and showed a significant strain x USV category interaction effect ($F_{(8,27.064)}$=6.714, p<0.001), with WKY dyads emitting flat, flat-mz and short USVs of shorter duration than SD dyads (all ps<0.01) and trill and trill-c USVs of longer duration than SD dyads (p<0.01; p=0.059, respectively).

2.3.5.1 Relationship between USVs and Maternal Behaviors

WKY mothers exhibited deficits in their maternal behavior (Figure 8 and Table 3). As shown in Figure 8A, WKY mothers exhibited significantly fewer retrievals (Mann-Whitney U tests: U=49, p<0.05) corporal (U=55.5, p<0.01) and anogenital lickings (U=51, p<0.01) than the SD mothers. Not all WKY mothers completed retrievals and grouped all pups into the nest, however the latency to group was not different between strains (U=19, p=ns; Table 3). WKY mothers spent less time in contact with pups compare to SD mothers, although this difference did not reach statistical significance (U=39, p=ns; Table 3).

The number of USV emissions changed according to the behavior of the mother. The number of USV emissions while mothers were grouping the litter in the nest, hovering over and nursing their pups was significantly different between strains (strain x behavior interaction effect: $F_{(2,20.746)}$=8.381, p<0.01). WKY dyads emitted less USVs during retrieval than did SD dyads

(p<0.01), and USVs were not emitted during nursing by either strain. There was also a significant strain x behavior x USV category interaction effect ($F_{(26,34.469)}$=3.323, p<0.001), with SD dyads emitting more flat, flat-mz, flat-z, short, step down, step up and upward ramp USVs during retrieval and hover over than during nursing (all ps<0.05), whereas this difference was not seen in WKYs (all ps=ns). WKY dyads emitted more short-c USVs during retrieval and hover over than during nursing (all ps<0.05) and more trill and trill-c USVs during retrieval than during hover over and nursing (all ps<0.05). These differences in emissions were not seen in SD dyads (all ps=ns).

The call profile (relative proportions of the USV categories emitted during maternal behaviors) also differed between strains and behaviors (strain x behavior x USV category interaction effect: $F_{(26,44.139)}$=1.718, p=0.056). WKY dyads emitted a smaller proportion of flat USVs during retrieval compared to during hover over and a larger proportion of short-c, trill and trill-c USVs during retrieval compared to during hover over and nursing (all ps<0.05). USV profiles of SD dyads also differed between behaviors, with a greater proportion of flat-mz USVs emitted during retrieval than during hover over and nursing (all ps<0.05). SD and WKY dyads

Table 3. Latencies and durations of maternal behaviors.

Behavior	**Strain**	**Latency (m)**				**Duration (m)**			
		Min	Median	25th and 75th SIQR	Max	Min	Median	25th and 75th SIQR	Max
Retrieve	SD	0.07	0.58	0.08, 1.08	6.4	1.15	5.01	2.2, 7.82	12.87
	WK	0.35	0.52	0.42, 0.61	1.08	0.7	7.67	3.73, 11.61	24.38
Group	SD	2.15	5.08	-0.59, 10.76	14.17	-	-	-	-
	WK	1.15	18.93	9.78, 28.09	30	-	-	-	-
Hover Over	SD	3.1	6.6	3.64, 9.56	15.68	5.8	12.5	10.11, 14.88	22.63
	WK	1.58	12.83	8.51, 17.16	18	7.42	8.62	7.97, 9.26	14.4
Nursing	SD	7.97	21.39	13.61, 29.18	30	0	8.07	3.57, 12.57	18.33
	WK	6.18	21.17	19.05, 23.28	30	0	8.78	4.87, 12.7	11.23

both emitted greater proportions of short USVs during hover over compared to during retrieval (all ps<0.05). No differences were found in duration (interaction strain x behavior x USV category: $F_{(18,258.298)}$=1.364, p=ns) or peak frequency (interaction strain x behavior x USV category: $F_{(18,225.598)}$=0.584, p=ns) of USVs emitted by either strain during maternal behaviors.

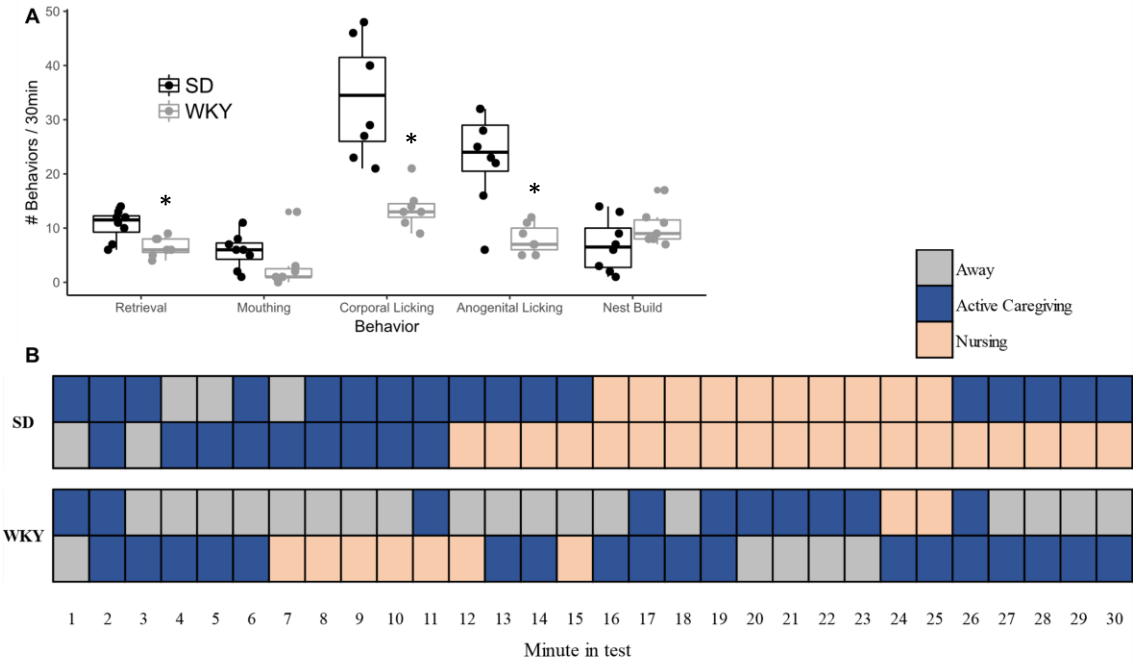

Figure 8. Maternal Behavior of WKY and SD rats. (A) Number of active maternal behaviors over a 30-minute behavioral test. * indicate significant differences between strains. (B) Representative examples (2 SD, 2 WKY) of maternal behaviors during each minute of the 30 min behavioral test: considered away if the mother was not in direct contact with pups, active caregiving includes retrieval and corporal licking, anogenital licking and nest building.

2.3.5.2 Who's Talking?

USVs emitted by pups in the presence of littermates and anesthetized mothers were compared to USV emissions during the first 15 minutes of the mother-litter interactions to account for changes in USV emissions in the presence of an awake, behaving mother. The number of USVs emitted and the USV profiles differed markedly between the two mother-litter recordings. Specifically, in the first 15 minutes of recordings with an awake behaving mother, dyads emitted approximately three times the average number of calls as dyads with the mother anesthetized (main effect of recording: $F_{(1,6.135)}$=14.872, p<0.01; strain x recording x USV

category interaction effect: $F_{(11,15.408)}=3.159$, $p<0.05$). Post-hoc analysis revealed that only SD dyads increased the emission of complex and flat USVs in the presence of an awake behaving mother ($p<0.05$) while only WKY dyads increased emissions of step up, trill and trill-c USVs (all $ps<0.05$). WKY dyads also decreased emissions of short-c USVs in the presence of an awake behaving mother ($p<0.05$).

The call profiles of each strain also differentially changed with recording (strain x USV category x recording interaction effect: $F_{(11,203.480)}=2.316$, $p<0.05$), with WKY dyads emitting a higher proportion of trill USVs and a lower proportion of short-c and short-ur USVs in the presence of an awake behaving mother (all $ps<0.05$). In fact, trill and trill-c USV categories were emitted neither by isolated pups nor pups in the presence of littermates and anesthetized mother but were emitted by mothers separated from pups by a barrier, indicating that trill and trill-c USVs during the mother-litter interaction were emitted exclusively by mothers.

The recordings taken of isolated pups and pups in the presence of littermates and anesthetized mother yielded a robust USV repertoire for pups, and acoustic parameters of these USVs were consistent across social testing conditions (USV category x recording interaction for peak frequency: $F_{(6,25.929)}=1.492$, p=ns; and duration: $F_{(6,17.187)}=1.027$, p=ns) allowing for reliable spectrographic characterization of pup USVs which were then used to distinguish between mother and pup USVs during the 30-minute mother-litter interaction. Lower and upper bounds of peak frequency and duration were used for flat, flat-z and short USVs of pups within each dyad to determine maternal USVs during the 30-minute mother-litter interaction.

Using this information, 1240 flat (13.5%), flat-z (13%), trill (48.7%), trill-c (14.8%) and short (10%) maternal USVs were determined during the 30-minute mother-litter interaction, accounting for approximately 8% of the total USVs emitted. There was a significant effect of strain ($F_{(1,13)}=5.120$, $p<0.05$), USV category ($F_{(4,52)}=6.195$, $p<0.001$) and a significant strain x USV category interaction effect ($F_{(4,52)}=4.302$, $p<0.01$), on maternal USV counts, with WKY mothers emitting significantly more trills than SD mothers ($p<0.001$). Of note, all WKY mothers

emitted trills, whereas only one SD mother emitted trills to levels characteristics of WKY mothers (Figure 9). Also, WKY mothers emitted most trills during the first 3 minutes of the mother-litter interaction recording (Figure 9). The peak frequencies of maternal USVs did not differ between strains ($F_{(1,13)}$=0.807, p=ns). Durations of maternal USVs were different between

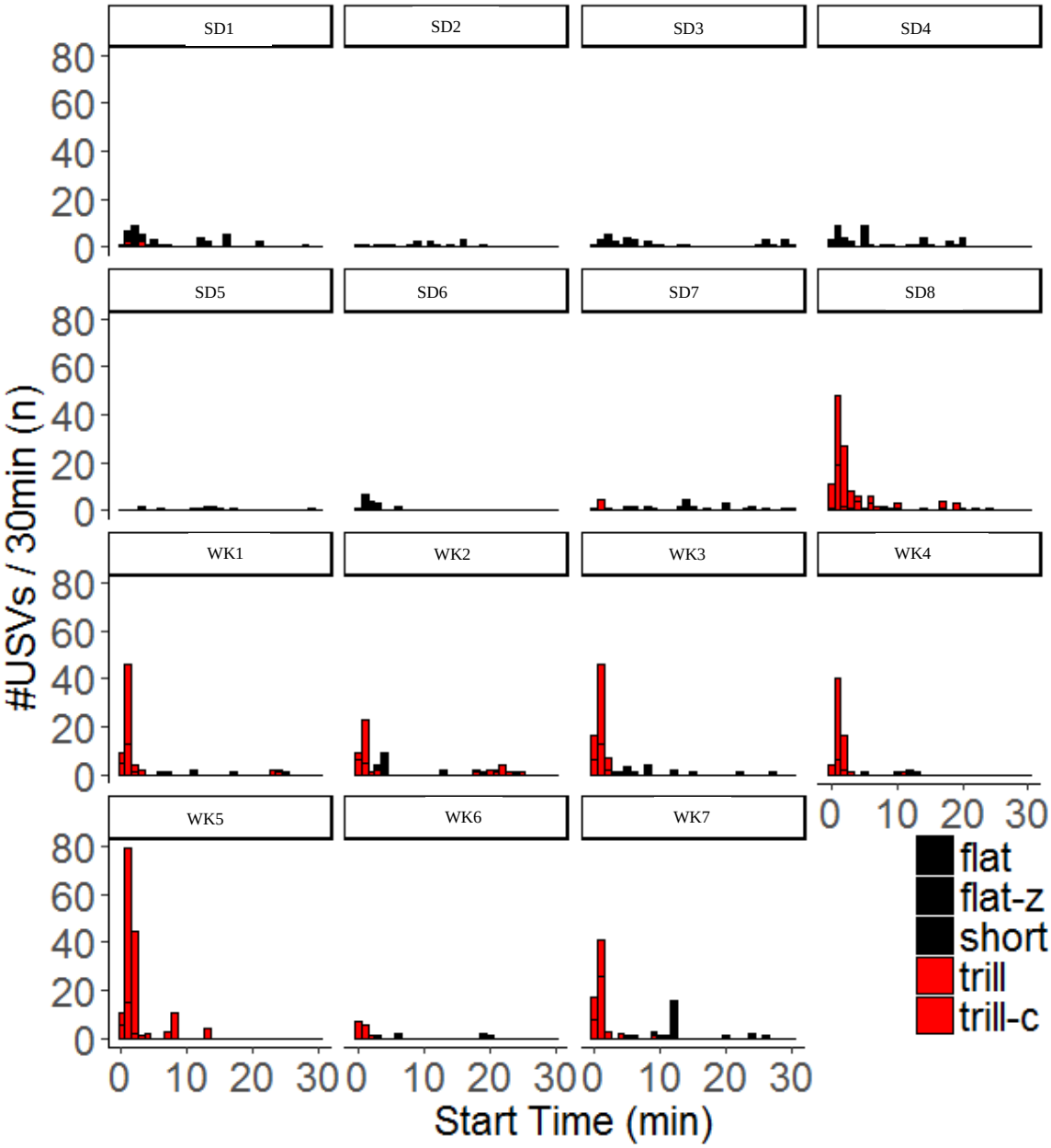

Figure 9. Histograms of start times (s) of maternal USVs by individual colored red to highlight trill and trill-c USV emission levels. Black arrows indicate time of grouping of the litter. Absence of a black arrow indicates the mother did not group all pups within the duration of the test (30 minutes).

strains ($F_{(1,13)}$=4.800, $p<0.05$) and had a significant interaction effect of strain x USV category ($F_{(4,41)}$=2.900, $p<0.05$), with WKY mothers emitting flat and flat-z USVs of shorter duration than SD mothers (both $ps<0.05$).

2.4 Discussion

The present study provides a detailed analysis of mother and litter USVs emitted during social interaction. Separate analysis of the vocal repertoire of the mother and pups confirmed maternal USVs during social interaction with the pups. Significant alterations were found in the vocal repertoire of WKY mothers, which were paralleled by deficits in their maternal behavior. Vocalizations and acoustic parameters emitted by the pups were different between strains, likely mediating, at least partially, behavioral and vocal differences between SD and WKY mothers.

2.4.1 Vocal Repertoire of Pups

Isolation-induced vocalization are thought to serve a communicative role by eliciting pup-seeking and retrieval behavior in the mother (Branchi, Santucci, & Alleva, 2001). Consistent with previous studies, WKY and SD pups emitted USVs, mostly flats and shorts, when isolated from the mother and littermates (Boulanger-Bertolus et al., 2017; Harmon et al., 2008; Noirot, 1972; Okon, 2009). A trend was seen for WKY pups to emit reduced isolation-induced vocalization rates compared to SD pups. This result is in agreement with one previous report showing that WKY pups emitted lower rates of USVs during separation from their mothers when compared with control strains (Braw et al., 2008). It is important to note that our recordings were taken in the pups' familiar home cage (but away from the nest), which has been shown to elicit lower levels of USVs versus a novel environment (Barfield et al., 1979; Hofer & Shair, 1978; Oswalt & Meier, 1975), which could account for the lack of strain differences in our study.

The presence of littermates and (anesthetized) mother in the home cage elicited fewer calls in SD and WKY pups than when alone in the home cage. In this context, WKY pups also

emitted less USVs than SD pups. Physical contact with a littermate or the mother reduced call production even more in both WKY and SD pups (i.e., contact quieting; Hofer & Shair, 1978). Contact quieting is considered to be a comfort response to termination of isolation by socially relevant stimuli (Shair et al., 2003), and was most pronounced after reunion with the mother in the nest. Pup USVs were not necessarily extinguished after grouping in the nest, which could have been due to tactile stimulation from the mother as she entered or departed the nest, as this has been known to modulate pup vocalizations (Geyer, 2009). During manual grouping, both SD and WKY pups increased their emissions of flat and short USVs, however WKY pups continued to emit significantly fewer flats compared to SD pups. This result is consistent with previous studies showing that pups increase the number of USVs during retrievals, regardless of whether retrievals were performed by the mother or by the experimenter (Blumberg & Sokoloff, 2001; Okon, 2009; Shair et al., 2003), and demonstrates differing effects of tactile stimulation on WKY and SD pup vocalizations.

The acoustic parameters of call types emitted by pups were constant within strains across recordings, suggesting inherent differences between strains. WKY pups emitted USVs at higher peak frequencies and of shorter duration than SD pups. Pup calls have been suggested serve similar functions as the crying of human babies, including the ability to elicit caregiving behaviors (Brouette-Lahlou et al., 1992; Zeskind et al., 2011). It is unclear if higher frequencies have an effect on the response of the mother. However, frequency in human infant crying is an important variable in facilitating mother's recognition of infant's needs and is recognized to be higher in infants later diagnosed with autisms (Zeskind & MTR, 1988; Young et al., 2010). Call duration is socially relevant as mothers preferentially respond to calls with longer durations (Smith et al., 1976; Ehret et al., 1992).

In addition, the call profile was different between strain, with a 7:1 ratio of flat to short calls in SD versus 2:1 in WKY pups. It has been suggested that different types of pup USVs have differing effects on maternal behavior. Specifically, calls of long duration such as flats serve to

elicit maternal retrieval, while short USVs serve to inhibit maternal behavior in order to prevent rough handling or aggression (Noirot, 1972), although this interpretation has been questioned (Bell, 1974).

It is tempting to assume that the reduced USVs seen in WKY pups reflects a communication deficit. Isolation-induced ultrasonic vocalizations emitted by rat pups serve important communicative functions and are crucial for pup survival (Bell et al., 1974; Boulanger-Bertolus et al., 2017; Brouette-Lahlou et al., 1992; D'Amato et al., 2005; Hashimoto et al., 2001; Myers et al., 2004; Noirot, 1972; Shair, 2014). As shown in playback experiments, pup ultrasonic vocalizations elicit maternal search and retrieval behavior (Farrell & Alberts, 2002; Wöhr & Schwarting, 2008). Reduced levels of pup ultrasonic vocalizations or unusual calling patterns decrease the ability to signal for maternal caregiving (communication potential), likely mediating maternal behavior deficits in WKY mothers. In support of this, daily social isolation of pups has been shown to reduce the number of calls emitted by pups in the presence of their mother (Zimmerberg et al. 2002). Moreover, a previous study showed that WKY pups do not increase isolation USV production after brief reunion with the mother (Braw et al., 2008), indicative of reduced maternal affiliation (Shair, 2014).

Another possible explanation underlying reduced USVs in WKY pups might be related to alterations in their development. In this sense, and consistent with previous studies, a negative correlation was found between pup body weight and peak frequency of USVs (Blumberg & Sokoloff, 2001). WKY strain is overall smaller than SD strain. However, work from our lab evaluating pup developmental physical landmarks and neurological reflexes revealed similar weight gain and age of reaching developmental milestones in WKY and SD pups, arguing against developmental delays in WKY pups underlying their vocalization profile.

2.4.2 Vocal Repertoire of Mother

The call rate was three times as high in dyads with an active mother, suggesting the presence of maternal USVs during mother-litter social interactions. In this context, WKY dyads also exhibited reduced call rate compared with SD dyads, highlighting once more social and communication deficits between mother and pups.

Trill call category was only emitted by mothers in a strain-dependent manner, such that all WKY mothers emitted trills and none but one SD did. WKY mothers emitted more trill USVs during mother-litter separation and the mother-litter interaction than did SD mothers. Significantly, these trills were exclusively emitted during mother-litter separation and during the beginning of the test while pups were scattered in the home cage. WKY mothers also emitted 22-kHz calls during these times. This higher emission of calls may reflect a hypersensitivity of WKY mothers to social isolation. This would be consistent with a study by Van Zyl et al. (2014) showing that adult WKY rats emitted more frequency-modulated USVs in response to removal of the cage-mate (social isolation), and that this increase in vocalization rates was ameliorated by antidepressants (Van Zyl et al., 2014).

Mothers of both strains also emitted flat, flat-z and short USVs during the mother-litter interaction. SD mothers emitted more flat USVs during active caregiving than WKY mothers despite the fact that durations of these behaviors were similar between strains. Adult 50 kHz flat USVs serve a social coordination function in interactions between adult conspecifics (Willadsen et al., 2014; Wöhr & Schwarting, 2007), and are also emitted in association with rewarding stimuli (Jeffrey Burgdorf et al., 2000). Lower levels of 50 kHz flat USVs emitted by WKY mothers could be a reflection of lower social valence of the pups.

In conclusion, significant differences in social communication between mother and litter were found in the WKY rat model of depression and the control SD strain. This indicates maladaptive socio-emotional regulation in the WKY model, consistent with the depressive-like symptomatology of the strain. We also found early indications of social communication deficits

in WKY pups, as indicated by their low levels of USV emissions compared to the control SD pups.

WKY mothers exhibited both substantial disturbances in maternal caregiving and altered vocal signaling during social interaction with the litter, suggesting that the experience of interacting with their pups is different from that of the control SD mothers. It is important to note that our method of identifying maternal vocalizations could only distinguish those that fell outside of the acoustic frequency and duration range of pups, meaning there were possibly more maternal USVs emitted during mother-litter interaction that could not be identified in this study. Procedures such as electromyographic monitoring of the larynx during maternal behavior would help to irrevocably distinguish maternal USVs during this dynamic interaction.

Examining USVs emitted by rodents is an important method of assessing neurobiological mechanisms of social anhedonia and the effectiveness of antidepressant treatments in ameliorating these symptoms. In the next chapter, we explore the impact of a drug that interacts with dopaminergic pathways involved in reward on the experience of WKY mothers while they interact with their pups.

CHAPTER 3

EFFECTS OF THE ADENOSINE A2A RECEPTOR ANTAGONIST MSX-3 ON MATERNAL BEHAVIOR AND USV EMISSIONS OF WKY MOTHER RATS

3.1 Introduction

Postpartum depression (PPD) is a serious psychiatric disorder affecting up to 20% of new mothers and is characterized primarily by cognitive, motivational and affective disturbances that impact the ability of the mother to parent (O'Hara & McCabe, 2013). Social anhedonia is a core symptom of PPD that involves a reduced ability to experience pleasure, reduced motivation to socialize and deficits in social functioning leading to social avoidance. Importantly, it is recognized that these symptoms have profound disruptive effects on the quality of maternal caregiving (Field, 2010). Specifically, depressed mothers show a lack of attunement to their child's particular needs and signals, are less proactive, and less likely to engage in social contact with their infants relative to healthy mothers (Carter et al. 2001; Jameson et al. 1997).

Rats emit various types of ultrasonic vocalizations (USVs) that are thought to serve as context-dependent affective signals and accomplish important communicative functions (Stefan M. Brudzynski, 2005; Portfors, 2007; Wöhr & Schwarting, 2013). Three main categories of USVs have been identified: (1) Pups emit 40 kHz USVs ("distress calls") when socially isolated from mother and littermates that induced retrieval by the mother, (Portfors, 2007; Vivian & Miczek, 1993). (2) Adult rats emit low-frequency 22 kHz USVs during or in response to aversive situations including drug withdrawal, inter-male aggression, and foot-shock stimulation (Barker et al., 2014; Blanchard et al., 1991; Han et al., 2005; Thomas et al., 1983) (3) High-frequency 50 kHz USVs are emitted mainly during appetitive social interactions such as rough-and-tumble play and mating (Jeffrey Burgdorf & Panksepp, 2001; Harmon et al., 2008; Willadsen et al., 2014; Wöhr & Schwarting, 2007), although certain 50 kHz USVs, such as trill USVs that contain rapid

oscillations in sound frequency, could reflect a negative affective state akin to anxiety (Van Zyl et al., 2014; Wöhr & Schwarting, 2008).

Expression of 50 kHz USVs depend upon mesolimbic DA neurotransmission. Release of DA in the NA is linked to both the production and perception of 50 kHz (but not 22kHz) USVs (Barker et al., 2014; Jeffrey Burgdorf & Panksepp, 2001; Wright et al., 2012), suggesting that NA DA functions to close a perception-and-action-loop, which is particularly relevant for appetitive social and reciprocal communicatory signals. On the other hand, interference with DA neurotransmission, following either depletion of accumbens DA, as well as administration of DA antagonists selectively reduce 50 kHz USVs, but not 22 kHz USVs (Jeffrey Burgdorf & Panksepp, 2001; Wright et al., 2012).

Recent studies have focused upon the involvement of the purine nucleoside adenosine and adenosine A_{2A} receptors in functions associated with the DAergic system, including cognitive, motivational, and affective processes (Ferré, 1997; Worden et al., 2009). Adenosine A_{2A} receptors are almost exclusively concentrated in striatal areas, including both neostriatum and the NA, where they are predominantly co-localized with DA D2 receptors (DeMet and Chicz-DeMet 2002; Fink et al. 1992; Hettinger et al. 2001). Considerable evidence indicates that there is a functional antagonistic interaction between DA D2 receptors and adenosine A_{2A} receptors at the cellular level (Ferré, 1997; Fuxe et al., 2003). In recent years, there has been increasing interest in the use of adenosine A_{2A} antagonists for their potential antidepressant effects (Salamone 2007). This is supported by studies showing antidepressant-like effects on traditional animal models of depression during tail suspension and forced swim tests (Hodgson et al. 2009; Hanff et al. 2010). Moreover, adenosine A_{2A} antagonism has been shown to reverse the behavioral effects of DA D2 antagonists on effort-related functions (Mott et al., 2009; Worden et al., 2009), and on the maternal behavior of postpartum rats. A recent study showed that activation of A_{2A} receptors attenuated 50-kHz USV emission in male rats during social interactions (Simola et al., 2014).

Our previous results using the WKY animal model of depression demonstrated that WKY mothers exhibit deficits in maternal behavior and USV profile compared to SD mothers, indicative of social anhedonia. Notably, the parenting deficits observed in WKY mother rats resemble those of SD mothers treated with DA antagonists (Pereira et al. 2011; Li et al. 2004). The present study investigated the ability of the adenosine A_{2A} receptor antagonist MSX-3 to ameliorate the impairments on maternal behavior and USV profile observed in WKY mothers during mother-litter social interactions.

3.2 Materials and Methods

3.2.1 Animals

Primiparous postpartum Sprague-Dawley (SD) and Wistar-Kyoto (WKY) female rats (Charles River Laboratories, Kingston, NY) bred in our laboratory were used in this study. Before giving birth, pregnant females were housed in individual clear Plexiglass cages (38.5 cm W x 48.5 cm L x 20.5 cm H) lined with fresh Sani-Chips® bedding (P.J. Murphy Forest Products Corp, Montville, NJ) and containing Eco-Bedding as nest-building material (Fibercore LLC, Cleveland, Ohio). The day of birth was designated as postpartum day 0 (PPD0) and litters were culled to 8 pups (3–5 males, 3–5 females) on PPD1. All females were kept on a 12-h light/dark cycle (light on at 0700 AM) at 22±1◦C, with *ad libitum* access to water, rat chow (5058 PicoLab® Diet, LabDiet, Brentwood, MI) and sunflowers seeds (5LP8 PicoLab® Sunflower, LabDiet, Brentwood, MI). One day before testing, a 5-cm high Plexiglas divider was inserted into each female's cage to divide the cage floor into four equal compartments. Ambient temperature in the testing room was maintained at 22 ± 1°C. All procedures used in this study were reviewed and approved by the University of Massachusetts Amherst's Institutional Animal Care and Use Committee.

3.2.2 Pharmacological agents and selection of doses

The adenosine A_{2A} receptor antagonist MSX-3 (Sigma Chemical, St. Louis, MO, USA) was freshly dissolved in 0.9% saline, which was also used as the vehicle condition. The MSX-3 dose and injection time (1.0 mg/kg IP; 20 min before testing) chosen for the present study were selected on the basis of a previously published report showing that this dose effectively ameliorated haloperidol-induced maternal behavior deficits in SD postpartum females (Pereira et al. 2011).

3.2.3 Experimental Design

All behavioral testing was conducted on PPD 7-8, during the light phase of the light/dark cycle. Separate groups of SD (n=16) and WKY (n=16) postpartum females were randomly assigned to receive either (1) VEH: saline vehicle IP (20 min before testing) or (2) 1.0 mg/kg MSX-3 injected IP (20 min before testing). Twenty minutes before the test, both mother and litter were removed from the home cage, the mother received an injection of either MSX-3 or the same volume of vehicle and was immediately returned to her home cage. The litter was housed in a small cage (18 cm W x 30 cm L x 13cm H, lined with Sani-Chips® bedding and containing nest-building material from the maternal cage) outside of the testing room.

3.2.4 Maternal Behavior Testing

At the beginning of the test, the litter was scattered throughout the home cage opposite to the nest and the number of the following maternal behavioral components were continuously scored for 30 min: retrievals of the pups into the nest, mouthings (oral repositioning of the pups into the nest), full body and anogenital lickings, and nest building. In addition, the total duration of huddling behaviors, including lying in contact with pups and hovering over the pups in the nest while actively performing other behaviors (i.e. licking of pups or self-grooming), and the nursing posture kyphosis (a quiescent upright crouching over pups) were recorded. Total time in contact

with pups was considered as the summed durations of huddling plus nursing behaviors. Also, the latency to begin retrieving pups (i.e. time from the introduction of the pups to the first retrieval by the female), to reunion of the entire litter into the nest as well as to begin hovering over and nursing was registered. Only those postpartum females that retrieved each of their pups into the nest were considered to have grouped the litter. The latency to begin hovering over or nursing the pups is the first occurrence of a bout of each behavior ≥2 min in duration. A latency of 1800 s was given for any category of behavior that was not initiated (or completed, i.e. reunion of the litter) within the 30-minute observation period. Other behaviors recorded include general exploration (line crosses and rearings), self-grooming and eating/drinking.

3.2.5 Ultrasonic Vocalization Recording and Analysis

USVs were recorded using the Avisoft Ultrasound Gate 116H acquisition device (Avisoft Bioacoustics, Berlin, Germany) connected to a condenser ultrasound microphone (CM16/CMPA Avisoft Bioacoustics, Berlin, Germany) and stored for offline analysis. The microphone was placed at a fixed height of 12-15 cm above the cage. Spectrograms were displayed in real time by Avisoft-RECORDER USGH (version 2.7; Avisoft Bioacoustics, Berlin, Germany) on a personal computer.

Acoustic analysis of the recordings was performed using Avisoft SASLab Pro (Version 5.2.10, Avisoft Bioacoustics, Berlin, Germany). The amplitude of each sound file was increased by 200% to strengthen USV signals and allow for comparability between spectrogram-derived parameter measurements. Spectrograms were generated with a fast Fourier transformation (FFT) length of 512 points and a time window overlap of 75% (FlatTop window, 100% frame size). Correspondingly, spectrograms had a frequency resolution of 488 Hz and a temporal resolution of 0.512 ms. Due to the substantial background noise picked up by the microphone, a spectrogram bandwidth of 30-90 kHz was set to reduce automatic measurement errors in Avisoft. Preliminary

analysis of the data indicated that less than 1.5% of the vocalizations emitted during maternal interaction were below 30 kHz for both SD and WKY (n = 6).

Each USV was manually labelled on the spectrogram by a single coder. Labelled USVs had to meet three spectrographic inclusion criteria (Wright et al., 2010): (1) temporal continuity (i.e., interruption < 20 ms), (2) sound frequency between 30 and 90 kHz, and (3) signal composition visually distinct from background noise. Following manual adjustment of duration measurements and classification of the USVs, a Linear Prediction Coding (LPC) procedure in Avisoft was applied to each spectrogram, and the Avisoft automatic parameter measurement tool was used to calculate duration, start time, and peak frequency (frequency at the relative point of maximum amplitude) at 50 evenly-spaced time points within each USV. To minimize erroneous peak frequency measurements taken at points of low USV amplitude, the program was set to exclude frequency measurements taken from spectrogram points with amplitudes of less than -69 dB. This reduced but did not eliminate erroneous measurements at times when the background noise had a higher amplitude than the USV signal, so a custom R script was created to remove remaining outliers and inaccurate measurements, accounting for the limitations of the Automatic Detection feature of Avisoft and manual classification. Because there was considerable variability in the amplitude of each call, almost all of the discrete USVs had missing data points. which were then interpolated as explained below.

3.2.6 Post-Processing and Acoustic Parameter Analysis

Labels that contained more than one USV (overlapping calls) were excluded from follow up processing procedures and all parameter analyses (0.15%). Discrete calls of duration between 2 – 400 ms that had at least 23 out of 50 evenly-spaced peak frequency measurements were considered for interpolation procedures. Prior to interpolation, erroneous peak frequency measurements at the extremes of the spectrogram were identified and deleted from the data. Missing peak frequency data points were reconstructed using a spline interpolation function in R.

Spline over other methods was selected based on visual determination of fit of a random sample of 1000 USVs balanced for call type. However, spline tended to interpolate endpoint values to the extreme. The resulting outlier frequency measurements were removed and the missing data points reconstructed using a step-wise regression, which was determined to be the simplest and most effective final interpolation method for USV endpoints. The peak frequency measurements were then averaged for each USV signal to yield the average peak frequency.

3.2.7 Statistical Analysis

Behavioral data were analyzed using non-parametric Kruskal-Wallis rank sum tests followed by Mann-Whitney U tests and acoustic data were analyzed with linear mixed models (SPSS v25), using the best-fitting covariance structure. Acoustic data are expressed as mean ± standard error of the mean (SEM) and behavioral data are expressed as median ± semi inter-quartile range (SIQR). Statistical significance was set at $p<0.05$.

3.3 Results

3.3.1 Effect of MSX-3 on USV Emissions During Mother-Litter Interaction

Both strains emitting predominantly flat (53%), short (16%), trill (9%), flat-z (5%), trill-c (4%), complex (2%), short-c (2%) and flat-mz (2%) USVs. The number of USV emissions were not different between strains ($F_{(1,50.165)}=0.019$, p=ns) and there was no effect of treatment on the emissions of either strain ($F_{(1,50.165)}=0.447$, p=ns). There was an interaction effect of strain x USV category ($F_{(10,53.852)}=6.886$, $p<0.001$). WKY dyads emitted more complex, short, short-c, step up, trill and trill-c USVs than SD dyads ($p<0.01$), while SD dyads emitted more flat USVs ($p<0.05$) across treatments (Figure 10).

3.3.2 Effect of MSX-3 on Maternal Behaviors During Mother-Litter Interaction

Consistent with experiment 1, WKY females receiving vehicle treatment exhibited severe deficits in their maternal behaviors (Figure 11). Kruskal Wallace rank sums test showed significant difference between treatment groups in corporal licking (chi-squared=13.861, $p<0.01$) and anogenital licking (chi-squared=14.853, $p<0.01$) and a marginally significant difference between treatment groups in retrievals (chi-squared=6.620, $p=0.085$). As shown in Figure 11, vehicle-treated WKY mothers exhibited significantly fewer retrievals ($U=55$, $p<0.05$), corporal ($U=64$, $p<0.001$) and anogenital ($U=63.5$ $p<0.01$) lickings compared to vehicle-treated SD mothers. The latency to group the litter was also different between groups (chi-squared=17.155, $p<0.001$), with WKY mothers treated with vehicle showing a higher latency to group the litter compared with SD mothers treated with vehicle ($U=2$, $p<0.01$).

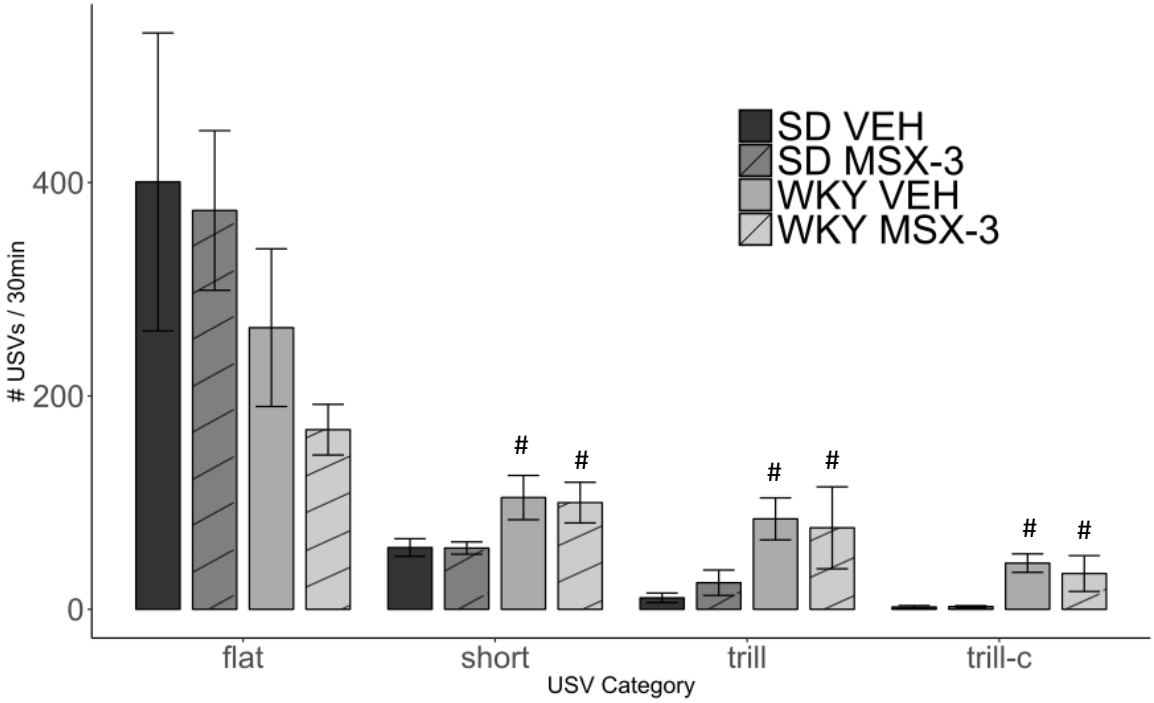

Figure 10. Mean ± SEM counts of USV categories emitted during the 30-min recording. # indicates significant difference between strains within treatment groups.

Administration of MSX-3 to WKY mothers did not produce a statistically significant increase in retrievals ($U=41.5$, p=ns), corporal ($U=48.5$, $p=0.092$) or anogenital licking ($U=42.5$, p=ns), nor did it decrease the latency to retrieve ($U=35.5$, p=ns) relative to vehicle-treated WKY

mothers. As shown in Figure 11, SD mothers receiving MSX-3 exhibited full maternal behavior that was not different from the control group.

Lastly, WKY mothers treated with MSX3 performed similar levels of retrievals (U=34.5, p=ns) and corporal licking (U=45, p=ns) compared to SD mothers treated with MSX3, but still performed lower levels of anogenital licking (U=51, p=0.051). MSX-3 ameliorated behaviors to levels characteristic of SD mothers (vehicle-treated control group). WKY mother treated with MSX-3 performed a similar number of retrievals (U=37.5, p=ns), corporal (U=46, p=ns) and anogenital lickings (U=49.5, p=0.073) compared to SD mothers treated with vehicle, but still showed a significantly higher latency to group (U=4, p<0.01).

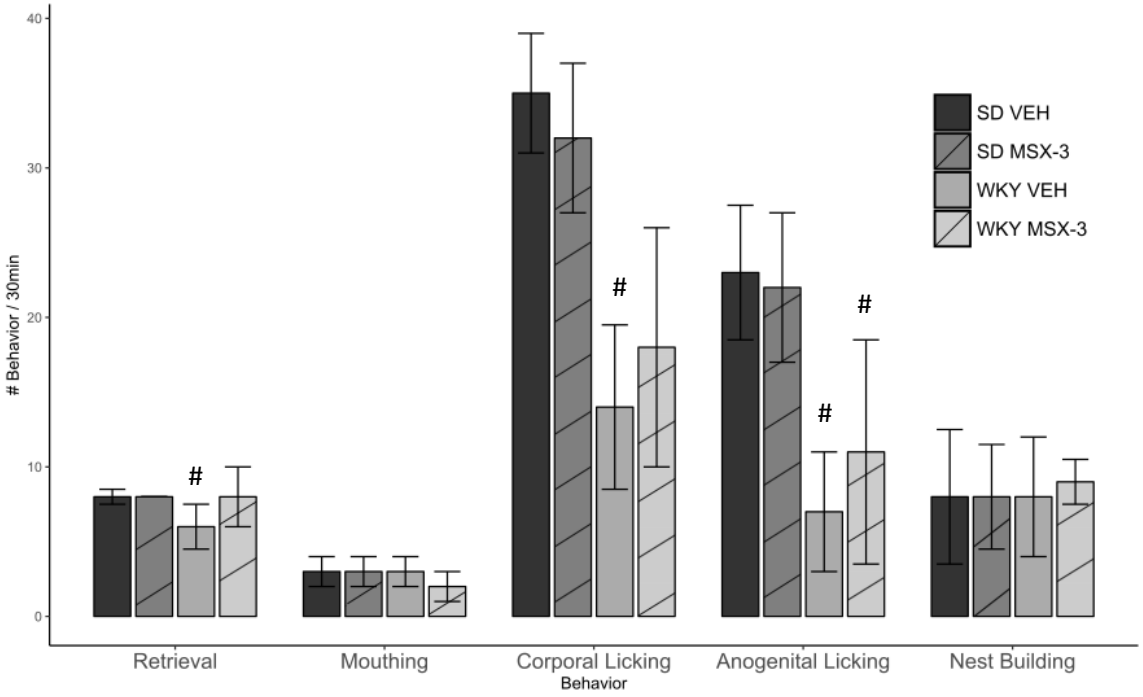

Figure 11. Counts of maternal behaviors performed during the 30-min mother-litter interaction. # indicates significant difference between strains within treatment groups.

3.4 Discussion

The findings of this study support the use of MSX-3 as a potential antidepressant treatment for postpartum depression. MSX-3 attenuated maternal behaviors of WKY mothers to

levels more characteristic of SD mothers, suggesting alterations in DA signaling as the root cause of the maternal behavioral deficits seen in WKY mothers.

Consistent with our previous study, WKY mothers emitted more trill and trill-c USVs during the first 5 minutes of the maternal behavior test compared to SD mothers. MSX-3 did not affect USV emissions during the mother-litter interaction. However, the lack of differences between treatment groups were thought to be driven by three outliers in the data. When these animals' vocalizations were removed from the data, an effect of MSX-3 on trill and trill-c USVs emitted by WKY mothers was revealed. Specifically, MSX-3 seemed to decrease the emissions of trill and trill-c USVs emitted by WKY mothers.

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