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# Behavioral Alterations in Prairie Voles (*Microtus ochrogaster*) after Parent-Pup Separation

Mihoko Yamamoto

*University of Massachusetts Amherst*, [myamamot@nsm.umass.edu](mailto:myamamot@nsm.umass.edu)

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**BEHAVIORAL ALTERATIONS IN PRAIRIE VOLES (*MICROTUS  
OCHROGASTER*) AFTER PARENT-PUP SEPARATION**

A Thesis Presented

by

MIHOKO YAMAMOTO

Submitted to the Graduate School of the  
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Approved as to style and content by:

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UnJa L. Hayes, Chair

---

Cynthia J. Gill, Member

---

Jeffrey D. Blaustein, Member

---

Eric L. Bittman, Member

---

Jerrold S. Meyer, Director  
Neuroscience and Behavior Program

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## ABSTRACT

### BEHAVIORAL ALTERATIONS IN PRAIRIE VOLES (*MICROTUS OCHROGASTER*) AFTER PARENT-PUP SEPARATION

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MIHOKO YAMAMOTO, B.S., STONY BROOK UNIVERSITY

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor UnJa L. Hayes

The prairie vole (*Microtus ochrogaster*), a highly social species, offers a unique opportunity to examine the effects of parent-pup separation in a biparental family system similar to humans. We hypothesized that 1) repeated separation from pups affects parental behavior and emotionality in parents, and 2) neonatal parental separation affects emotional and physiological development in pups, and thus induces altered adult parental, emotional, and social behaviors. During postnatal day (PND) 1-10, pups were removed from their parents for 0, 15, or 360 min and housed either individually or with siblings. Unhandled controls experienced only daily lid opening. Tests for parental responsiveness and emotionality were conducted on PND11 for parents and PND90-92 for their offspring. Emotionality tests included the elevated plus maze, open field, and forced swim tests. Starting at PND150, half of each litter was paired with an opposite-sex vole for 24 hours and tested for partner preference. Additionally, behavioral response to stress was measured in all animals 0, 30, or 60 min after

exposure to a forced swim. Generally, the behavior of the parents and adult offspring was influenced by daily handling, the length of the separation, and presence of siblings. Parental behaviors in parents did not differ among groups, while their anxiety- and depression-like behaviors were influenced by pup separation. For the adult offspring, separation treatment altered parental behavior, emotionality, partner preference, and stress response. Our results demonstrated that parent-pup separation affects emotional and social behaviors in prairie vole parents and adult offspring.

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# CHAPTER 1

## INTRODUCTION

Behaviors in individuals are regulated by environmental and genetic factors. There is increasing evidence that neonatal parental care alters various behaviors in adulthood, including parental responsiveness, emotionality, pair-bond formation, and stress response. However, contradictions have accumulated, as the number of reports increase. This may be in part because each study focuses on one or two specific behaviors, and that each laboratory uses modified procedures for separation. Aiming to add clarity to our understanding, the present study was specifically designed to examine consequences of neonatal separation using multiple behavioral tests within one single experimental setting. Our findings provide an integrated view on how early-life environment influences individuals. In addition, how parents react to separation from their offspring is an important question to address, since it affects the quality of their parental care in various species. Thus, the effect of pup separation on parents was also examined in this study.

Prairie voles (*Microtus ochrogaster*) are highly social animals, exhibiting biparental care and pair bonding similar to humans. This study is particularly important, because the long-term effect of separation has never been explored using prairie voles. Studying behavioral changes induced by exposure to parent-pup separation in this species provides significant insights into the development

of psychological patterns and possible deficits that are organized during early life in humans.

## **1.1. Parental Responsiveness**

### **1.1.1. General Overview**

Parental behaviors facilitate survival of offspring into adulthood by providing nutrition, warmth, tactile stimulation, and protection. Parental behaviors include retrieving, licking and crouching over pups, and nest building. In mammals, the majority of parental behaviors are displayed by females, reflecting the fact that newborn mammals receive milk from mothers. In addition to such sex differences, variable parental responsiveness is observed within the same sex. Notable variation is found especially in nulliparous animals. Some individuals display parental behaviors, while others avoid and even fatally attack pups.

#### **1.1.1.1. Parental Responsiveness in Rodents**

The behaviors and underlying neurobiology of parental care have been studied extensively using rodents, specifically rats and prairie voles.

##### **1.1.1.1.1. Rats**

Virgin female rats actively avoid pups (Weisner and Sheard, 1933; Rosenblatt, 1967). Avoidance continues in pregnancy until 24-48 hours prior to parturition, when females display maternal behavior upon exposure to pups (Bridges, 1984; Moltz et al., 1970; Novakov and Fleming, 2005; Terkel and

Rosenblatt, 1968). This change in behavior is driven by hormonal fluctuations during pregnancy, specifically the decrease in circulating levels of progesterone and the concomitant increase in estradiol (Bridges, 1984). Avoidant female rats also can become maternal through a process called sensitization (discussed further on page 7).

Paternal behavior in rats has not been explored as much, since males typically do not contribute to the care of sired offspring. Male rats are infanticidal and usually not responsive to sensitization (Jakubowski and Terek, 1985). However, virgin, castrated male rats can be sensitized to pups, indicating non-hormonal regulation of paternal behavior in males (Rosenblatt, 1967).

#### **1.1.1.1.2. Prairie Voles**

The prairie vole is a biparental species, with males contributing to the care of offspring along with females. Unlike rats, sexually-naïve adult females are usually infanticidal (*i.e.*, severely or fatally harm pups; Lonstein and De Vries, 1999b) rather than avoidant. The shift from infanticidal to maternal behaviors is triggered by parturition, specifically pelvic distention of the cervix, rather than hormonal fluctuation (Hayes and De Vries, 2007). Interestingly, most females around the age of postnatal day (PND) 20-30 are maternal (Lonstein and De Vries, 2001), as found in rats (Stern, 1987; Zaias et al., 1996), but this likelihood decreases with age. Underlying mechanisms that explain the reduction of maternal responsiveness in virgin females have not yet been fully understood.

Although both males and females are highly parental postpartum, sex differences in parental care are observed (Lonstein and De Vries, 1999a). Dams exhibit longer time in contact with pups and time being quiescent in crouching position. On the other hand, sires lick and carry pups longer.

Incidences of infanticide occur less frequently in virgin males than in females (Lonstein and De Vries, 1999). Although prairie vole males are generally parental across age, infanticidal males are found occasionally (12.58%; Hayes, unpublished data). Similar to infanticidal females, infanticidal males display paternal behavior toward their sired offspring (Hayes, unpublished data). The induction of paternal behavior may be linked to mating-induced inhibition of infanticide, as has been reported for infanticidal male mice (vom Saal, 1985).

### **1.1.2. Underlying Mechanisms**

Induction of parental behaviors in avoidant and infanticidal animals is regulated in hormone-dependent and independent manners. The hormone-dependent mechanisms involve gonadal and peptide hormones that are involved in some aspects of reproduction. The hormone-independent mechanism, often called sensitization, results from continuous exposure to pups.

#### **1.1.2.1. Gonadal Hormones**

Depending on species, the importance of gonadal hormones in the regulation of parental behavior varies. In rats, hormonal fluctuations during pregnancy influence maternal responsiveness. Specifically, the decrease in

circulating levels of progesterone and increase in estradiol simultaneously occurs with induction of maternal behavior in female rats. Mimicking such hormonal changes after ovariectomy facilitates the display of maternal behaviors in nulliparous female rats (Bridges, 1984; Bridges and Ronsheim, 1989; Molts et al., 1970).

In prairie voles, however, parental behavior is not dependent on gonadal hormones. Although virgin females show distinct behaviors towards pups, they are hormonally quiescent until activated by male stimuli (Carter et al., 1995; Sawrey and Dewsbury, 1985). As mentioned earlier, pelvic distention, rather than hormonal fluctuations associated with pregnancy and parturition, is important for induction of maternal behavior in prairie voles (Hayes and De Vries, 2007).

The underlying mechanisms of paternal behavior have not been explored as much as for maternal behavior, because paternal care is uncommon in mammals (Kleiman and Malcolm, 1981). Although males do not experience hormonal fluctuations associated with pregnancy and parturition, infanticidal males display parental behaviors towards their own pups (Hayes, unpublished data). Castration in adulthood fails to alter parental responsiveness in male prairie voles (Lonstein and De Vries, 1999). Perinatal manipulation of gonadal hormones (*i.e.* inhibition of androgenic and estrogenic activity) also does not change paternal responsiveness (Lonstein and De Vries, 2000). However, neonatal castration increases the incidence of infanticide in male prairie voles (Lonstein et al., 2002), suggesting organizational involvement of testicular hormones in parental responsiveness in males. Similarly, neonatal castration in male rats

reduces aggressive reactions and increases parental responsiveness towards unrelated conspecific pups (Quadagno and Rockwell, 1972; McCullough et al., 1974; Rosenberg and Herrenkohl, 1976; Rosenberg et al., 1971). As in females, further understanding of the mechanisms involved in the behavioral change from infanticidal to parental is useful to gain insights into the regulation of paternal behavior.

#### **1.1.2.2. Peptide Hormones**

Several peptide hormones that have been implicated in pregnancy and parturition have also been shown to be important factors in parental behaviors.

##### **1.1.2.2.1. Prolactin**

In rats, prolactin (PRL) is released into the circulatory system from the anterior pituitary twice a day during the first half and on last day of pregnancy, with suppressed circulation in the second half of pregnancy (Grattan, 2001). Injection of PRL into the medial preoptic area facilitates maternal responsiveness in nulliparous female rats (Bridges et al., 1990). These studies indicate an involvement of PRL in the induction of parental behaviors. PRL receptors in the brain region implicated in maternal behaviors are upregulated in lactating females compared to diestrous females (Pi and Grattan 1999). Central infusion of prolactin receptor antagonist reduces maternal responsiveness in postpartum female rats (Torner et al., 2002).



Unlike the case for females, prolactin seems to have no major influence on paternal behaviors in rodents. Lowering prolactin by dopamine agonist does not change parental responsiveness in male prairie voles (Lonstein and DeVries, 2000b), and virgin male prairie voles do not alter PRL or its receptor after daily exposure to pup, although upregulation is observed in females (Khatib et al., 2001).

#### **1.1.2.2.2. Oxytocin**

The nonapeptide oxytocin (OT) facilitates uterine contraction during parturition and milk ejection during lactation. OT is synthesized in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. OT is then transported to the posterior pituitary and released into circulation.

In addition to peripheral secretion, OT also functions in the brain. In rats, centrally infused oxytocin facilitates the onset of maternal behaviors (Pedersen and Prange, 1979), while OT antagonist prevented the display of maternal behaviors (Pedersen et al., 1985; Van Leengoed et al., 1987). The expression and distribution of oxytocin receptors (OTR) differ depending on parental responsiveness. Highly maternal rats exhibit a greater OTR density in the PVN, medial preoptic area (MPOA), lateral septum (LS), central amygdala (CeA) and bed nucleus of stria terminalis (BNST) than less maternal rats (Champagne et al. 2001; Francis et al., 2002). OTR expression increases in the PVN, supraoptic

nucleus (SON), BNST, and MPOA throughout gestation in rats (Bealer et al, 2006).

These studies suggest that changes in the oxytocinergic system as a result of parturition may be a key factor in initiating maternal behavior in female voles. In juvenile and adult virgin female prairie vole, a positive correlation of parental responsiveness and OTR density is found in specific brain regions implicated in regulation of maternal behaviors (Hayes, unpublished data; Olazabal and Young 2006a, Olazabal and Young 2006b). Changes in OTR expression pre- and postpartum is observed in the less social montane vole (*Microtus montanus*) when compared with prairie voles. Within 24 hours after parturition, OTR expression at the lateral amygdale and parental responsiveness (time spent with pups) increases to the level of prairie vole females (Insel and Shapiro, 1992). Central infusion of OTR antagonist prevents the display of maternal behaviors (Pederson et al., 1985; Van Leengoed et al., 1987). These findings suggest that OT has an important role in the regulation of maternal behaviors, in addition to parturition and lactation.

#### **1.1.2.2.3. Vasopressin**

Vasopressin (AVP) is a nonapeptide closely related to OT. It is also synthesized in the PVN and SON and released into blood via the posterior pituitary. AVP has an important role in regulation of paternal behavior. Density of AVP-immunoreactive fibers increases after mating, and this change coincides with increased paternal responsiveness (Bamshad et al., 1994). Central infusion of AVP increases paternal care in prairie voles, while reduced parental care is

observed after infusing V1aR antagonist (Wang, et al., 1994). Interestingly, differences in V1aR binding were observed between paternal and non-paternal meadow voles (Parker, et al., 2001). Although the vasopressinergic system seems to regulate paternal behavior, the mechanism for induction of paternal behavior remains to be revealed. Early experience of biparental care increases the display of parental behavior in meadow voles as adults, despite that the meadow voles are uniparental (McGuire, 1988). These findings suggest experience-based regulation of paternal behavior in vole species.

### **1.1.2.3. Sensitization (Non-Hormonal Induction)**

In addition to hormonal fluctuations associated with parturition, sensitization can induce parental behavior. The sensitization procedure entails continuously exposing females to unfamiliar pups, thereby allowing animals to habituate to pup stimuli (*e.g.*, olfactory cues; Mayer and Rosenblatt, 1975; Rosenblatt, 1967). After 5-7 days of continuous exposure, the reactions of virgin females do not differ from dams (Bridges, 1972; Rosenblatt, 1967). A recent study confirmed that only continuous exposure to pups successfully induces maternal behavior in virgin rats. Daily, 1h pup exposures and pup exposures without physical contact with pups for 7 days fails to induce maternal behavior (Seip and Morrell, 2008). Interestingly, anosmia reduces the number of days needed to induce maternal behaviors in virgin females (Mayer and Rosenblatt, 1975), indicating the importance of olfactory processing in pup-induced maternal behavior.

In general, male rats do not take care of pups regardless of their reproductive status, and they are usually not responsive to sensitization (Jakubowski and Terek, 1985). However, virgin, castrated male rats can be sensitized to pups (Rosenblatt, 1967), indicating hormonal suppression and non-hormonal modulation of paternal behavior in male rats.

In prairie voles, however, repeated daily but not continuous exposure fails to sensitize many non-parental animals (Hayes, unpublished data). Interestingly, some infanticidal virgins become maternal after 24h continuous exposure to neonatal pups, while others remain infanticidal even after 4 weeks (BuAbbud, Sigal and Hayes, unpublished data). Further studies that examine individual differences in sensitivity to pup stimuli and parental responsiveness are required.

### **1.1.3. Rearing Condition and Parental Responsiveness**

The way in which an adult rodent reacts to conspecific pups can be influenced by various factors, such as quality of maternal care she received as a neonate. The direct effects of neonatal maternal care on later maternal responsiveness have been observed in studies examining the “handling effect” in rats. The handling effect is a persistent enhancement of maternal care, specifically licking, that results from short periods (~ 15min) of separation from the pups. Female pups raised by briefly separated dams, who exhibit facilitated licking, also lick their pups longer as adults (Francis and Meaney, 1999; Francis et al., 2002; Levine, 1967; Stanton et al., 1988). Conversely, prolonged neonatal separation (3-6 hours) results in less maternal care (Boccia and Pedersen, 2001; Caldji et al.,

1998; Liu et al., 1997; Pryce et al., 2001) and increased stress responses in adulthood (Lovic et al., 2001; Levine, 2002; Ogawa et al., 1994; Plotsky and Meaney, 1993). Artificial tactile stimulation, as well as stimulation from siblings, can compensate for the effects of early isolation (Melo et al., 2006), supporting that licking, a major component of parental care, is an important factor in the degree of maternal responsiveness in adulthood.

A number of studies have shown a correlation between maternal responsiveness and emotionality, as measured by degrees of anxiety- and depression-like behaviors. In rodents, anxiety-like behavior is often measured using the elevated maze and open field tests, because these tests stimulate fear and avoidance responses to being exposed (Montgomery, 1955; Pellow, 1985). These tests have been validated by administration of anxiogenic and anxiolytic drugs. Forced swim test is widely used to assess depression-like behavior in animals.

In an early study by Fleming and Luebke (1981), nulliparous female rats exhibited avoidance of pups and greater timidity as measured by the open field (*i.e.*, test for anxiety expressed by amount of exploratory behavior; Cunha and Masur, 1978) and emergence test (*i.e.*, measurement on neophobic behavior expressed by emergence from home cage; Pare et al., 2001). These females also took longer to display maternal behaviors compared to parturient females.

Likewise, the relationship between emotionality and maternal behaviors is evident within parturient females. Dams repeatedly separated from their pups show more depressive- and anxious-like behaviors: greater immobility in a forced

swim test, and less open arm entry in an elevated plus maze (Boccia and Pedersen, 2001; Boccia et al., 2007). Although the relationship between maternal responsiveness and emotionality has not been fully explored, numbers of studies reported involvement of the HPA axis. Details will be discussed in the section of stress response (page 14).

Maternal deprivation affects responses to those behavioral tests. In rats, pups experienced brief isolation as neonate later exhibited depression-like behaviors including hypoactivity, which is measured by reduced exploratory behavior and inactiveness in the open field test, and anhedonia, which is measured by reduced amount of sucrose intake and food intake (Grippe et al., 2007). Likewise, neonatal separation can also induce anxiety-like behaviors (Huot et al., 2001; Wigger and Neumann, 1999), although another study found no difference (Huot et al., 2004; Rees et al., 2007).

## **1.2. Pair bond**

### **1.2.1. General Overview**

Despite its rarity in mammalian taxa, heterosexual pair bonding is found across various species (Kleiman, 1977). Formation of a pair bond is observed by monogamous animals. Among these, prairie voles (*Microtus ochrogaster*) are widely used as an animal model to study underlying mechanisms of pair bonding. Once established, a pair bond persists for a lifetime, and both male and female prairie voles raise offspring together (Carter et al., 1995). However, mating outside the social bond has been observed in a field study (Wolff and Dunlap,

2002). Thus, prairie voles are used to study social monogamy, rather than sexual monogamy. Formation of a pair bond can be triggered by mating or by mating-independent cohabitation (Williams et al., 1992; Winslow et al., 1993).

The partner preference test is a widely used choice test to assess pair bond formation in rodents. After a certain duration of cohabitation (varies depending on experimental design) with an opposite-sex partner, a subject animal is given simultaneous access to a partner and a stranger, who are confined in separate cages. The amount of time the experimental animal spends with each stimulus animal is recorded. Animals that spend more time with their partners are considered pair-bonded (Insel and Hulihan, 1995; Williams et al., 1992; Winslow et al., 1993).

Minimum duration of cohabitation required for induction of partner preference in prairie voles has been controversial. Typically, 6h cohabitation with mating or 24h cohabitation without mating is sufficient to induce significant partner preference in females. On the other hand, females in the study by DeVries et al. (1996) significantly preferred partners after 6h mating-independent cohabitation. Minimum duration of cohabitation required for partner preference in males is unknown. Six hours of cohabitation without mating fails to induce partner preference (DeVries, et al. 2002), while 24h cohabitation with an ovariectomized female induces partner preference in males (DeVries et al., 1996). Regardless, multiple studies indicate that 24 hours cohabitation with the opportunity to mate (as used in my study), is sufficient for both male and female prairie voles to develop a preference for their partner.

### **1.2.2. Underlying Mechanisms**

A series of studies with prairie voles has revealed several key neurotransmitters that regulate formation and maintenance of pair bond. When compared with non-monogamous, closely related vole species, prairie voles exhibit different patterns of OT (Insel and Shapiro, 1992), AVP (Insel et al., 1994), and corticotropin releasing factor (CRF; Lim et al., 2005) receptor expression. In addition to these three regulatory molecules, dopamine (DA) also has an important role in induction and maintenance of a pair bond. After discussion of key molecules, neural circuits involved in pair bonding will be introduced.

#### **1.2.2.1. Oxytocin and Vasopressin**

Centrally administered OT facilitates formation of a pair bond in females even in the absence of mating, and blocking OTR prevents mating-induced pair bond formation (Insel and Hulihan, 1995; Williams et al., 1994). In addition, OT administration induces partner preference in both males and females even after 1h cohabitation, which is insufficient to form a pair bond in control group (Cho et al., 1999). In the same study, pretreatment with OTR antagonist prevents the effect of OT in those who cohabitated with their partner for 1h. Similarly, 1h cohabitation was sufficient to exhibit partner preference when AVP was centrally administered in both males and females (Cho et al., 1999). When the cohabitation was extended to 24h, partner preference in males with AVP infusion was greater than males received vehicle control (Winslow et al., 1993).



### **1.2.2.2. Corticotropin-Releasing Factor (CRF)**

CRF, a protein synthesized in the PVN, has an important role in the hypothalamic-pituitary-adrenal axis, which regulates the stress response (detailed mechanism can be found in the section on Stress Response, page 14). CRF also modulates pair bond formation. Central infusion of CRF facilitates partner preference in males after 3h cohabitation (DeVries et al., 2002). CRF has an important role in the hypothalamic-pituitary-adrenal axis. Exposure to stress and peripheral injection of corticosterone enhances partner preference in males, while females show reduced partner preference (DeVries et al., 1995, 1996). Adrenalectomized males prefer their partner when a corticosterone pellet is provided (DeVries, 1996). These findings indicate sexually dichotomous effects of stress on the pair-bond formation in prairie voles.

### **1.2.2.3. Dopamine (DA)**

DA is a catecholamine neurotransmitter synthesized in DA neurons in the ventral tegmental area, substantial nigra, and arcuate nucleus of the hypothalamus. DA is involved in the reward system. DA induces partner preference in females that have cohabitated with males even without the opportunity for mating (Wang et al., 1999). In prairie vole males, the activation of the D1 receptor prevents, whereas D2 receptor facilitates pair-bond formation (Aragona et al., 2006). Subsequent studies suggest complementary effects of DA receptors in the formation and maintenance of a pair bond.

Examining distributions of key neurotransmitters and their receptors revealed neural pathways important in pair-bond formation. Young and Wang (2004) proposed a model that shows two key neural pathways involved in vole partner preference (Figure 1.1). Formation of a pair bond is initiated by tactile and olfactory signals from

a mate. Tactile signals activate the ventral tegmental area that release DA into the nucleus accumbens (NAcc) and prefrontal

cortex. Olfactory signals activate the olfactory bulb, from

which the signal is transmitted to the medial nucleus of the amygdala (MeA). Olfactory learning is facilitated at the MeA and lateral septum by oxytocinergic and vasopressinergic systems. Maintenance of a pair bond is regulated by dopaminergic, oxytocinergic, and vasopressinergic systems at the NAcc and ventral pallidum. Aforementioned brain regions and neurotransmitters are also implicated in infant-mother bonding, emotional regulation, and stress response. Underlying mechanisms of affiliative social behaviors are likely to overlap in the reward system.

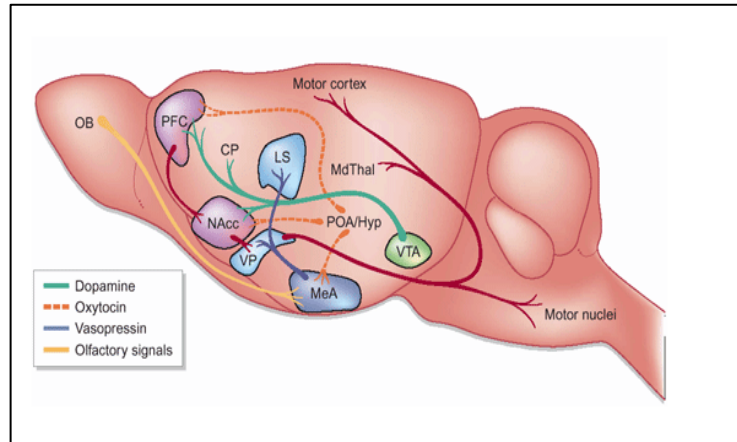


Figure 1.1. Sagittal view of a prairie vole brain illustrating a proposed neural circuit model for pair bonding (Young & Wang, 2004. *Nature Neuroscience* 7:1048-1054).

### 1.2.3. Rearing Condition and Pair Bonding

Although early life experience is crucial for adult affiliative behaviors, whether or not maternal deprivation influences pair-bond formation has not been examined in prairie voles. Manipulations of neural systems implicated in the formation of prairie vole pair bonds suggest possible outcomes of neonatal separation. Neonatal OT injections increase AVP immunoreactivity in males and OT immunoreactivity in females in the PVN on PND21 (Yamamoto et al., 2004; Yamamoto et al., 2006). More recent studies show that neonatal OT treatment results in different degrees of alloparental behaviors and partner preference in females, and that the effect is dose-dependent (Bales et al., 2007b). Furthermore, OT injection within 24h of birth in monogamous mandarin voles (*Microtus mandarinus*) facilitated formation of pair bond in females, while no effect was observed in males (Jia et al., 2008). Interestingly, the same treatment suppressed the maintenance of pair bond in female mandarin voles, suggesting differential involvement of oxytocinergic system in formation and maintenance of pair bond. No previous study examined the degree of OT exposure in neonatal pups, even though oxytocinergic system is upregulated in dams around parturition. Studies that examine the effects of physiological and behavioral changes in mothers on her offspring would be necessary to understand exactly how neonatal OT manipulation influence pair bonding.

## **1.3. Stress Response**

### **1.3.1. General Overview**

Ability to cope with stressful situations is extremely important for successful survival and reproduction, because failure can lead to serious damage or even death. Since Selye's discovery of a physiological response to handling (1937), studies have revealed behavioral and neurobiological systems that regulate responses to stress. Upon exposure to a stressor, acute physiological changes are triggered via sympathoadrenal system, known as a 'fight or flight' response. Outcomes include increased cardiac output, respiration and catabolism, redirecting blood flow to the brain, heart and muscles, and heightened attention. Another mechanism, the hypothalamic-pituitary-adrenal (HPA) axis, is also activated by stress. Unlike the first system, HPA axis produces sustained physiological responses (discussed in detail in the Underlying Mechanisms). Such reactions are adaptive; however, prolonged exposure to stressor can lead to disrupted reactivity of HPA axis and results in physiological and physiological deficits.

Although both behavioral and physiological measurements are commonly used in the field of stress response studies, circulating levels of stress-related hormones, particularly corticosterone in rodents, is widely accepted as an indication of stress response in an animal. Detailed mechanism of corticosterone regulation is discussed in the following section. In behavior, increased fear and anxiety are typically observed after exposure to a stressor.

### **1.3.2. Underlying Mechanisms**

Especially after the sequencing of corticotropin releasing factor (CRF; Spiess et al., 1981), regulation of stress response via the hypothalamic-pituitary-adrenal (HPA) axis has been studied extensively. The HPA axis consists of three major molecules: CRF synthesized in the parvocellular cells in the PVN, adrenocorticotrophic hormone (ACTH) synthesized in the anterior pituitary, and corticosterone (CORT) synthesized in the adrenal cortex, in which other glucocorticoids are also produced. CRF-containing neurons in the PVN project their axon to the median eminence via vascular portal system and deliver CRF to the anterior pituitary. At the anterior pituitary CRF activates CRF1 receptor, leading to release of ACTH. ACTH is then delivered to adrenal gland through the vascular system and stimulates release of CORT (Axelrod and Reisine, 1984; Dallman et al., 1987). CORT can be transported to the central nervous system and reduce CRF and ACTH production, forming a negative feedback loop in this system.

### **1.3.3. Rearing Condition and Stress Response**

Neonatal isolation evokes increased vocalization and plasma corticosterone level in prairie vole pups when compared with less-social meadow vole pups (Shapiro and Insel, 1992), suggesting that separation is more stressful in neonatal prairie voles. However, effects of separation persisting into adulthood have not been studied in prairie voles.

In rats, altered HPA axis activity after exposure to maternal deprivation has been observed. Prolonged, repeated maternal separation induces sustained elevation of the basal plasma CORT level, as well as the central CRF and CRF mRNA level (Biagini et al., 1998; Plotsky and Meaney, 1993). Furthermore, animals with history of neonatal isolation show higher ACTH reactivity in response to acute stressors (Ladd et al., 1996; Liu et al., 2000; Plotsky and Meaney, 1993). Increased CORT is also found in rats reared in the artificial rearing paradigm, in which pups are isolated from dams for 24 hours while nutrition and minimal warmth and tactile stimulation are provided (Workel et al., 1997). Compensatory effect of tactile stimulation also suggests that deprivation of licking (tactile stimulation from dams) induces dysfunction in HPA axis (Melo et al., 2006). In fact, a direct correlation of the amount of licking received as a neonate with behaviors in adulthood has been observed (reviewed in Champagne and Meaney, 2001).

Subsequent studies revealed that altered methylation in the promotor regions of the glucocorticoid receptor affect expression of the receptor in the hippocampus (Weaver, et al., 2005 and 2006). Such epigenetic modification increases sensitivity of HPA axis to glucocorticoids and eventually to a stressor in adulthood (Weaver et al., 2005 and 2006). A similar effect of high licking on gene methylation in pups is found at the promoter gene sequence for estrogen receptor (ER)-alpha (Champagne et al., 2003 and 2006). ER-alpha promotes expression of OTR, which also modulates reactivity of HPA-axis (Champagne et al., 2001).

Although a direct correlation between neonatal care and adult behaviors has not been examined in prairie voles, the above findings suggest that adult prairie voles exposed to parental separation would exhibit altered HPA-axis activities in adulthood.

#### **1.4. Current Study**

Many studies have repeatedly shown that adult physiology and behavior reflect early life experience. Reduced amount of neonatal care results in a decrease in parental behavior, heightened anxiety- and depression-like behaviors, and altered HPA-axis activity in adulthood. Importance of neonatal care has been well addressed; however, the influence of early experience on a wide range of adult behaviors in prairie voles remains to be examined. The purpose of the present study is to provide an integrative perspective on what aspects of adult behaviors are influenced by parent-pup separation.

The prairie vole was chosen because 1) previous prairie vole studies provide significant amount of background information, especially neurobiological mechanisms that might explain the effects of early life experience, and 2) biparental social system in prairie voles is similar to the family structure in humans, providing insight into psychological deficits primed during early life.

In this study the effects of parent-pup separation were examined in both parents and adult offspring. We hypothesized that 1) repeated separation from pups affects parental behavior and emotionality in parents, and 2) neonatal

parental separation affects emotional and physiological development in pups, and thus induces altered adult parental, emotional, and social behaviors.

In order to test the first hypothesis, parents were subjected to short (15min), long (360min), or no separation during the postpartum day 1 to 10, and tested for their parental behaviors and emotionality (anxiety- and depression-like behaviors). Based on the previous rat studies, parents subjected to long pup separation were predicted to exhibit reduced parental behaviors and increased anxiety- and depression-like behaviors, while parents subjected to short pup separation were expected to show increased parental behaviors and reduced anxiety- and depression-like behaviors.

In order to test the second hypothesis, pups were subjected to short (15min), long (360min), or no separations. In rats, artificial tactile stimulation partially compensates the effects of maternal deprivation (Melo et al., 2006). Thus, pups that were subjected to short and long separations were either kept with siblings or isolated during the separation. Once pups became adults, they were tested for parental behaviors, emotionality, partner preference, and behavioral response to a stressor. Based on the previous rat studies, a working model that predicts behavioral changes in adult offspring was created (Figure 1.2). When duration of separation is considered, animals subjected to long separation were expected to exhibit less parental behaviors and partner preference, and greater anxiety- and depression-like behaviors than those subjected to short separation. When housing condition is considered, isolated animals were expected to show less parental behaviors and partner preference, and greater anxiety- and



depression-like behaviors than those kept with siblings. Combining two variables, the model predicts that behaviors in animals that experience both isolation and long-separation would be altered the most by early-life separation.

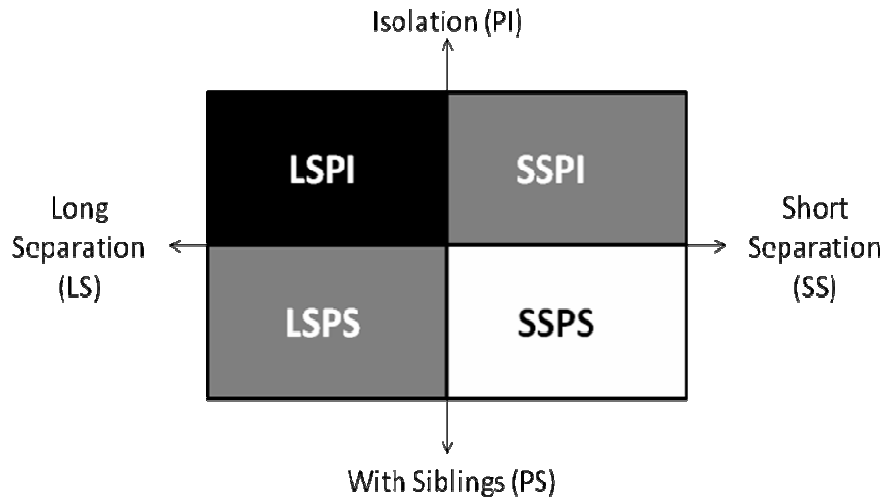


Figure 1.2. Suggested Model Predicting Behavioral Changes by Early Life Separation in Adult Offspring. X-axis represents duration of separation (short vs. long) and Y-axis represents housing condition (siblings vs. isolation).

## CHAPTER 2

### METHODS

#### 2.1. General Methodology

##### 2.1.1. Subjects

Subjects were male and female prairie voles (*Microtus ochrogaster*) bred in our colony. The colony was established in 1996 at the University of Massachusetts, Amherst, from voles captured in 1994 from Urbana, IL, by McGuire (Smith College, Northampton, MA, USA) and Wang (Florida State University, Tallahassee, FL, USA), and outbred in 2000 with animals provided by Carter (University of Illinois, Chicago, IL, USA). The vivarium in which the voles were housed is temperature- (21°C) and light-controlled (14hr light: 10hr dark). The animals are housed in plastic cages (48 x 28 x 16 cm) containing wood chips, shavings, and Carefresh (wood pulps) with food (Purina rabbit chow, sunflower seeds, cracked corn, and whole oats) and water provided *ad libitum*. In general, litters are weaned at postnatal day (PND) 20 and sorted according to sex approximately 20 days later. Typically, at PND90-120 they are prescreened for their spontaneous levels of parental responsiveness using a standard parental behavior test.

##### 2.1.2. Behavior Tests

###### 2.1.2.1. Parental Behavior Test (PBT)

To test parental behavior, an animal is placed in a test cage (plastic, 48 x 28 x 16 cm), allowed to habituate for 30 minutes, and then presented with two

unrelated pups placed in corners opposite to the subject's location. Behaviors displayed by the subject are recorded for 10 minutes (SONY Handycam, DCR-SR80). If a subject does not make contact with either pup during the 10-minute period, testing is continued for an additional 10 minutes, during which pups are moved towards the subject, reducing the distance by half every 3 minutes. In the event of an attack, pups are quickly removed from the cage and euthanized. The video is scored for parental and non-parental behavior by an observer blind to subject group using a behavioral scoring apparatus (Teklogix Workabout, PSION TEKLOGIX Inc. Ontario, Canada) and software (The Observer, Noldus Information Technology, Wageningen, Netherlands). Parental behaviors include crouching (hover over pups with arched back posture), licking pups, nesting (carrying bedding to a nest site, chewing bedding, building a nest), and retrieving (carrying one pup to the other pup). Non-parental behaviors are exploration (moving around and rearing), feeding, self-grooming, and sniffing pups. Latency to initial contact and duration of each behavior are also measured. Due to ambiguity of interpretation, moving (carrying pups from one site to another except to another pup) is not included in either category and is analyzed on its own. Animals that attack pups during the test are categorized as infanticidal, and those who display more than 100 second of parental behaviors and 15 second of licking as parental. If an animal does not fall in these two categories, it is labeled as non-responsive.

#### **2.1.2.2. Elevated Plus Maze Test (EPM)**

The apparatus (Plexiglas, opaque gray) consists of two opposing open arms (10 x 50cm, 110cm entire length) and two opposing closed arms (10 x 50 x 50cm) placed approximately 50 cm above the floor. To test anxiety-like behavior, voles are placed on the center of the apparatus facing the closed arms, and their movements are recorded for 10 minutes (SONY Handycam, DCR-SR80). The number of entries into and the time spent in each type of arm is measured by a blind scorer.

#### **2.1.2.3. Open Field Test (OF)**

To test anxiety-like behavior, voles are placed at the central region of a wide open-top box (76 x 92 x 30cm) with a grid floor, creating sixteen 19 x 23 cm grids and 1748cm<sup>2</sup> of central area. Behaviors of subjects are video-taped for 10 minutes (SONY Handycam, DCR-SR80) and scored by a blind scorer. Measured variables include the number of grid line crossings (within center, within peripheral area, and from periphery to center) and the time spent in the peripheral and central regions.

#### **2.1.2.4. Forced Swim Test (FST)**

To test depression-like behavior, adult voles are placed in a glass cylinder (diameter/height-12.5/19cm) filled with enough warm water (30-32°C) to prevent subjects from touching the bottom. The temperature is chosen to avoid reduced activity due to hypothermia (Drugan *et al.*, 2005; Taltavull *et al.*, 2003). The test

lasts 10 minutes and is recorded using a SONY Handycam, DCR-SR80. After 10 minutes, the subject is towel-dried and returned to its home cage with a heat lamp attached at one corner of the cage. The duration of immobility and struggling (vertical swimming and diving) are measured by a blind scorer.

#### **2.1.2.5. Partner Preference Test (PPT)**

The apparatus consisted of three plastic cages (36 x 24 x 31cm) connected by Plexiglas tubing (7.5 x 15cm), with vole food mix, wood chips, and Carefresh in each cage. Following 24h-cohabitation with a randomly assigned, age- and weight-matched opposite sex animal from general colony in a pairing cage (plastic, 48 x 28 x 16 cm), a subject is placed in the middle, neutral cage. Its mate (partner vole) and stranger vole are tethered in opposite end-cages using a plastic collar and flexible, plastic-coated steel wire. The test lasts 3 hours, and time-lapse images are recorded at every 15sec (Live! Cam VF0050, Creative Technology Ltd., Singapore). Location of the subject animal in each frame is scored by an observer blind to experimental animal sex or treatment and to partner and stranger position.

## **2.2. Experiment 1: Effects of Pup Separation in Parents**

### **2.2.1. Subjects**

Fifty-six animals (28 males and 28 females) served as subjects for Experiment 1. Based on the results of prescreening, only highly parental males and females were selected and paired, meaning animals showed at least 8 minutes

of parental behaviors, including crouching, licking, nesting and retrieving within a ten minute testing period. Randomly chosen males and females were paired and allowed to reproduce. Before pairing, males were isolated for 48 hours, and females for 24 hours with urine-soiled bedding from male cages.

### **2.2.2. Design**

On postpartum day (PPD) 0, each pair was randomly assigned to one of four groups. Daily pup separation (described in more detail in next section) occurs for 10 days (PPD1-10). On PPD11, dams and sires were tested for parental behavior (PBT) and given one of the following tests: elevated plus maze (EPM), open field (OF), or forced swim test (FST). All animal experienced parental behavior test first, then either OF, EPM or FST 4-6 hours later. Between tests, animals were returned to their home cage.

### **2.2.3. Pup Separation**

Litters were culled to 4 pups (2 males and 2 females) per litter on the day of birth. Starting on postnatal day (PND) 1, the litters were removed from their parents for either 15 minutes (short separation; SS) or 360 minutes (long separation; LS). During the separation period, pups were kept warm on a heating pad. There were two control groups. First control group (C) had the pups removed from the dams and immediately returned to the home cage. The other control group (control-undisturbed; CU) only had the lid of the cage opened and closed. Sires also were picked up and returned immediately for SS, LS and C groups.

This procedure continued for 10 consecutive days. When returned to the home cage,

<b>Group</b>	<b>Separation Duration [min]</b>
Undisturbed Control (CU)	0
Control (C)	0
Short Separation (SS)	15
Long Separation (LS)	360

separated pups were

Table 2.1. Group Assignment of Parents

placed at the end opposite to the nest. The location of dam and sire, number of pup retrieval, and latencies of retrieval to the nest, licking, crouching, and other non-parental behaviors were recorded for the first 10 min once pups were returned.

## **2.3. Experiment 2: Effects of Parent Separation in Adult Offspring**

### **2.3.1. Subjects**

The offspring from Experiment 1 were used in Experiment 2. Pups were weaned at postnatal day (PND) 20. On PND40, animals were sexed and housed with another same-sex sibling. If there were only three pups in a litter, all pups were kept together. Weight was measured on PND0, 20, and 90.

### **2.3.2. Design**

Pups were separated on PND1-10. On PND90, the parental behavior test was conducted on all animals. Two days later (PND92), each animal experienced one of the following tests: EPM, OF or FST. During PND 150-200, one male and one female from each litter were exposed to forced swimming for 5min. The other

half of the litter (one male and one female) was paired with untreated, opposite-sex voles from our general colony for 24 hrs and tested for partner preference (PPT). Twenty-four hours after the PPT, animals were exposed to 5min forced swimming.

### 2.3.3. Parental Separation

Starting on PND1, the litters were removed from parents for either 15 (Short Separation; SS) or 360 (Long Separation; LS) minutes and kept warm on a heating pad. During the separation period, pups were isolated (Pup Isolation; PI) or with siblings (Parental Separation; PS). The control group (C) only had the pups removed from the dams and immediately returned to the home cage. The other control group (Control-Undisturbed; CU) only had the lid of the cage open and closed. This procedure continued for 10 consecutive days. When returned, separated pups were placed at the end of the cage opposite to the nest.

<b>Group</b>	<b>Separation duration (min)</b>	<b>Housing conditions during separation</b>
<b>CU</b>	None (0)	N/A
<b>C</b>		
<b>SSPI</b>	Short (15)	Isolated
<b>SSPS</b>		With Siblings
<b>LSPI</b>	Long (360)	Isolated
<b>LSPS</b>		With Siblings

Table 2.2. Group Assignment of Pups



## **2.4. Statistical Analyses**

### **2.4.1. Parental Responsiveness (PBT)**

Durations of parental and non-parental behaviors were analyzed by factorial ANOVA with sex and separation treatment as independent variables. Separation treatments for parents were C, CU, SS and LS. Separation treatments for adult offspring were C, CU, SSPI, SSPI, LSPI, and LSPS.

### **2.4.2. Emotionality (EPM, OF, and FST)**

Dependent variables were analyzed by factorial ANOVA with sex and separation treatment as independent variables.

### **2.4.3. Pair Bond (PPT)**

To examine the effects of previous experiences on partner preference, the number of observations in the partner, neutral, and stranger cages were analyzed by one-way ANOVA with two factors: whether or not a subject attacked an unrelated pup during the parental behavior test and type of emotionality test. Previous studies have shown a sex difference in the minimum time necessary to form a pair bond in prairie voles (DeVries *et al.*, 1995). Thus, the effect of sex on partner preference was also analyzed by repeated measures ANOVA.

### **2.4.4. Weight**

Weight of each pup was measured on PND0, 20 and 90. Individual weights for each date were categorized based on separation treatment for analysis.

Repeated measures ANOVA was used to compare weight gain among groups. On PND90, weight and sex of each individual was recorded. Factorial ANOVA was used with sex and separation treatment as independent variables.

## CHAPTER 3

### RESULTS

#### 3.1. Experiment 1: Effects of Pup Separation in Parents

##### 3.1.1. Parental Responsiveness

There were no significant differences in duration of total parental behaviors and each parental behavior among treatment groups (Table 3.1). Total duration of non-parental behaviors was generally longer in dams ( $66.75 \pm 8.90$ ) than sires ( $43.42 \pm 8.35$ ;  $F_{(1, 105)} = 5.71$ ,  $p < 0.019$ ). An interaction of treatment and sex was also found ( $F_{(3, 105)} = 3.25$ ,  $p < 0.025$ ). Post-hoc analyses (Fisher's LSD) revealed that dams in LS and CU groups exhibited longer non-parental behavior than sires, while SS and C groups had no sex difference. Among four non-parental behaviors (exploration, feeding, grooming, and sniffing), the same pattern was observed for grooming. Dams self-groomed longer than sires ( $37.75 \pm 5.54$  vs.  $13.16 \pm 1.64$ ;  $F_{(1, 105)} = 19.76$ ,  $p < 0.001$ ), and an interaction of sex and separation treatment in the duration was found ( $F_{(3, 105)} = 4.09$ ,  $p = 0.009$ ). Similar to the total non-parental behaviors, only LS and CU groups showed a sex difference in post-hoc analyses (Fisher's LSD). Duration of exploration differed depending on the treatment, regardless of sex ( $F_{(3, 105)} = 3.01$ ,  $p = 0.033$ ). Post-hoc analyses (Fisher's LSD) revealed that exploration was longer in CU groups ( $48.34 \pm 11.57$ ) than C and LS groups ( $16.00 \pm 3.91$  and  $14.62 \pm 3.61$ , respectively).

### 3.1.2. Emotionality

#### 3.1.2.1. Elevated Plus Maze Test

No differences were found in the time spent in the open arms and closed arms, the number of total entries, or the % entries into each type of arm (Table 3.2).

#### 3.1.2.2. Open Field Test

C and CU were combined since they did not differ (Control, CO). An interaction of separation and sex was found in the percent entry to the central area ( $F_{(2, 30)}=4.20, p=0.025$ ). Post-hoc tests (Fisher's LSD) revealed that SS sires entered to the central area more frequently than SS dams, while LS and CO sires entered the central area more frequently than dams (Figure 3.1). Total number of crossings was higher in dams ( $258.28\pm 20.84$ ) than sires ( $141.11\pm 18.41$ ), regardless of treatment ( $F_{(1, 30)}=18.02, p<0.001$ ).

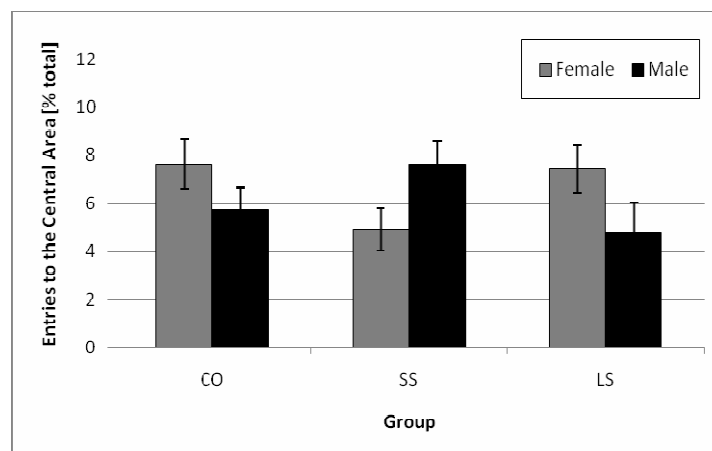


Figure 3.1. Anxiety-like Behaviors in Parents as Measured by Entries to the Central Area in Open Field Test (Mean $\pm$ SEM)

Group	C		CU		LS		SS	
	Female	Male	Female	Male	Female	Male	Female	Male
N	11	11	9	9	18	18	19	18
Contact	587.85±5.06	569.75±11.81	526.84±19.77	569.02±14.79	585.25±4.94	581.77±9.67	573.43±13.17	543.69±21.47
Crouching	118.15±29.13	139.85±31.20	61.17±22.12	126.22±24.54	100.87±19.85	124.24±18.31	141.39±25.65	83.79±16.84
Licking	290.86±32.14	238.05±34.36	257.78±28.18	265.46±35.15	282.58±25.09	295.39±26.79	239.68±20.73	278.75±21.04
Nesting	6.38±3.00	6.64±4.52	5.89±3.77	7.92±3.32	10.34±3.02	20.06±10.22	6.24±2.61	26.87±12.97
Retrieving	1.46±0.46	1.23±0.36	1.29±0.33	1.27±0.41	10.86±8.96	1.49±0.31	1.65±0.37	1.32±0.33
Total Parental Behavior	416.86±21.59	385.76±25.76	326.12±37.96	400.87±31.41	404.66±22.11	441.18±25.90	388.97±21.06	390.74±25.67
Exploration	12.72±4.71	19.29±6.30	66.22±17.02	30.47±14.12	14.28±4.60	14.95±5.69	21.97±9.37	46.23±20.09
Feeding	1.25±1.25	0.00±0.00	0.00±0.00	0.09±0.09	2.88±2.71	0.22±0.22	3.06±2.52	0.00±0.00
Grooming	17.71±5.39	14.31±3.29	58.69±14.01	7.54±1.86	49.13±12.85	7.71±1.64	28.65±7.20	20.73±3.70
Sniffing	5.60±4.71	1.46±1.14	1.30±0.52	2.81±1.99	1.34±0.22	0.37±0.13	1.14±0.46	2.99±1.26
Total Non-Parental Behavior	37.28±10.51	35.06±7.80	126.21±30.53	40.91±16.76	67.64±14.99	23.25±6.00	54.82±13.33	69.95±22.56
Moving	2.94±1.07	1.23±0.50	9.94±4.77	2.39±1.02	2.09±0.91	2.11±0.65	16.72±11.56	1.09±0.33

Table 3.1. Parental and Non-Parental Behaviors in Parents (Mean±SEM [sec])

Group	C		CU		SS		LS	
	Female	Male	Female	Male	Female	Male	Female	Male
<b>Elevated Plus Maze Test</b>								
N	4	4	4	4	8	8	5	6
Open arms [sec]	186.25±62.43	92.25±54.19	150.50±60.32	99.50±53.82	156.50±40.38	170.00±41.21	114.60±55.58	196.83±38.58
Closed arms [sec]	320.50±89.55	374.50±52.77	315.50±74.96	359.75±63.04	324.75±43.59	310.38±46.91	379.80±62.99	279.83±30.53
Entries to Open Arms	11.00±3.81	5.50±2.63	6.50±2.33	8.25±3.86	8.25±1.62	9.13±2.11	7.60±2.36	10.00±2.68
Entries to Closed Arms	15.00±5.15	12.50±2.53	13.75±3.45	16.75±2.17	13.38±1.03	13.38±1.10	14.60±2.73	14.50±3.03
Total Entries	26.00±8.71	18.00±4.18	20.25±4.59	25.00±6.03	21.63±2.50	22.50±2.86	22.20±4.68	24.50±5.40
<b>Open Field Test</b>								
N	3	3	3	3	6	6	6	6
Duration in Central Area [sec]	60.00±25.36	22.33±6.23	58.33±12.78	64.33±14.62	57.17±7.78	46.33±11.17	62.67±13.92	43.00±11.00
Entries to Central Area	24.33±9.26	8.33±1.33	21.00±8.08	9.33±4.10	9.57±2.60	10.67±2.43	21.50±2.42	8.33±4.20
Crossings in Central Area	25.33±9.02	8.00±1.00	30.00±10.12	11.33±3.48	13.86±2.81	11.17±3.53	28.17±7.32	11.50±4.25
Crossings within Periphery	186.00±28.11	122.33±22.81	219.33±58.38	129.33±23.25	159.29±21.35	100.50±15.01	224.00±20.40	106.17±39.08
Total Crossings	260.67±55.35	148.00±24.58	292.33±84.50	160.33±25.98	193.29±27.26	133.83±22.65	296.00±20.83	135.33±51.29
<b>Forced Swim Test</b>								
N	3	3	3	3	6	6	6	6
Immobility [sec]	141.67±42.48	157.00±49.37	56.33±22.60	51.33±25.46	125.50±34.20	121.00±45.36	178.67±21.84	95.17±27.82
Struggling [sec]	243.67±81.11	188.67±20.42	235.33±26.62	249.33±96.07	276.50±47.32	326.00±47.86	251.00±38.78	209.83±56.16

Table 3.2. Emotionality Tests in Parents (Mean±SEM)

### 3.1.2.3. Forced Swim Test

Groups did not differ in the durations of immobility and struggling (Table 3.2). However, animals that were handled daily (SS, LS and C) were immobile longer than unhandled animals (CU;  $F_{(1, 32)}=5.60$ ,  $p=0.024$ ; Figure 3.2). No differences were found in duration of struggling between type of handling or sex.

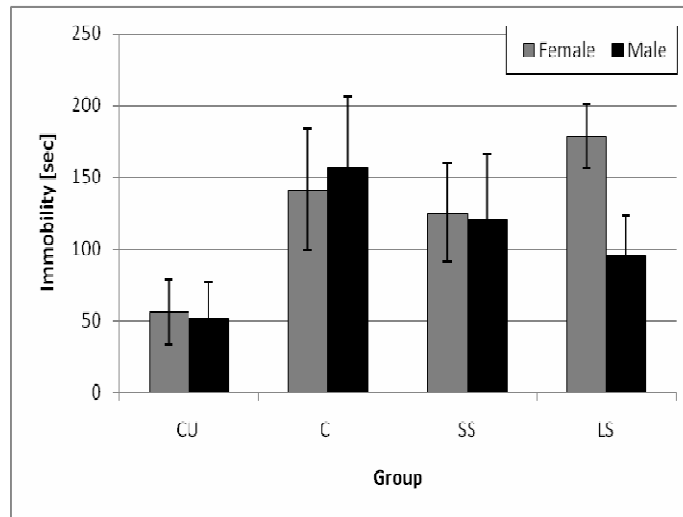


Figure 3.2. Depression-like Behaviors in Parents as Measured by Immobility Time in Forced Swim Test (Mean±SEM)

## 3.2. Adult Offspring

### 3.2.1. Development

Weight of each pup was measured on PND 0, 20 and 90. Because individual pups were not identified before PND40, we were unable to record weight gain for each animal. C and CU were combined as a control group (CO) because these two groups did not differ. Difference in weight gain was found among groups (Figure 3.3;  $F_{(8, 292)}=5.52$ ,  $p<0.0001$ ). Post-hoc tests (Fisher's LSD)

revealed that weights did not differ among groups on PND0. On PND20, LSPI animals were significantly lighter than CO animals. On PND90, LSPI animals weighed significantly less than the other groups, and CO animals weighed significantly more than other groups. When analyzed based on individual weight on PND90, males ( $46.68\text{g}\pm 0.96$ ) were generally heavier than females ( $38.33\text{g}\pm 1.21$ ;  $F_{(1, 144)}=23.41$ ,  $p<0.001$ ). There was no interaction of sex and treatment. The ratio of males did not differ among groups ( $X^2_{(5)}=4.73$ ,  $p=0.450$ ).

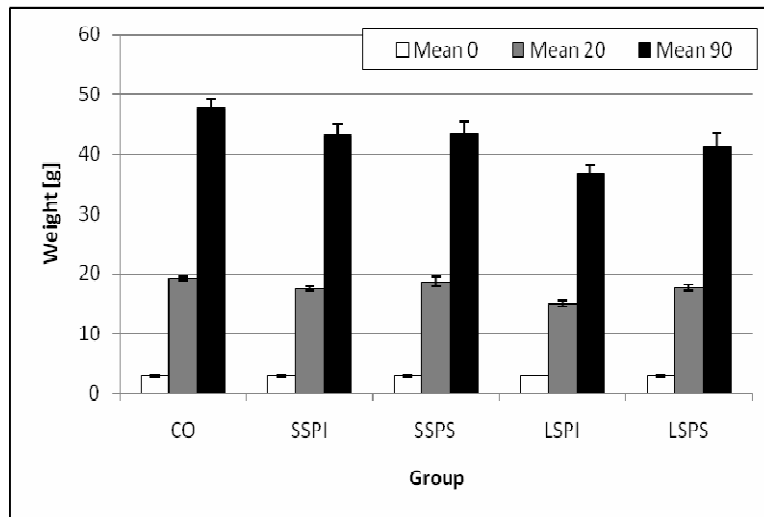


Figure 3.3. Weight Gain in Pups (Mean±SEM)

### 3.2.2. Parental Responsiveness

#### 3.2.2.1. Incidence of Infanticides

There was no difference in the percentage of infanticidal animals among groups (Table 3.3;  $X^2_{(5)}=1.73$ ,  $p=0.885$ ). Generally, females were more likely to be infanticidal than males (32.14% vs. 2.52%, respectively;  $X^2_{(1)}=34.3$ ,  $p<0.0001$ ).



### 3.2.2.2. Frequencies of Parental Animals

Within non-infanticidal animals, there were no group ( $X^2_{(5)}=2.26$ ,  $p=0.812$ ) or sex ( $X^2_{(1)}=0.24$ ,  $p=0.625$ ) differences in the percentage of parental versus non-responsive animals (Table 3.3).

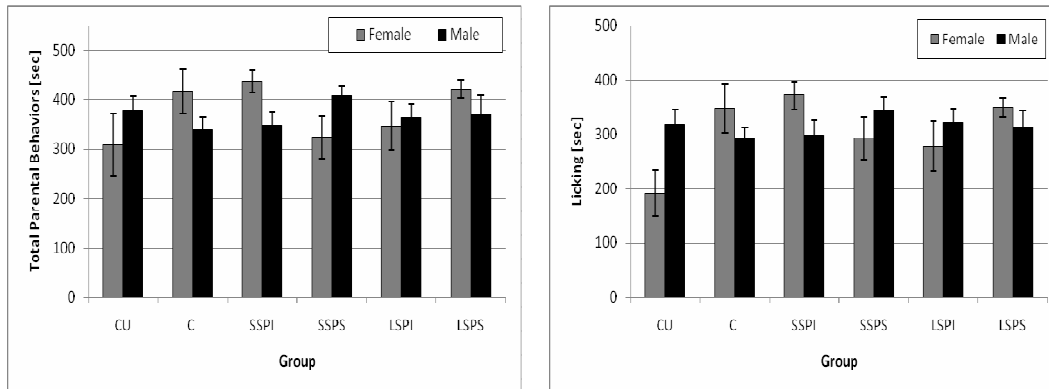
Treatment		Number of animals							
Duration of Separation	Housing Condition	Infanticidal		Non-responsive		Parental		Total	
		Female	Male	Female	Male	Female	Male	Female	Male
C		3	0	0	2	7	19	9	20
CU		4	0	1	1	7	19	11	20
SS	PI	3	1	0	1	12	21	15	23
	PS	3	0	1	0	9	21	11	19
LS	PI	6	0	2	1	10	16	18	16
	PS	6	1	0	1	8	14	13	13

Table 3.3. Frequency of Infanticidal, Non-Responsive, and Parental Adult Offspring

### 3.2.2.3. Parental Behavior Test

Degree of parental responsiveness was analyzed in animals that did not attack pups during the parental behavior test ( $N_{\text{male}}=116$ ,  $N_{\text{female}}=57$ ; Table 3.4). No differences among groups were found in the durations of total non-parental behaviors and each non-parental behavior. In general, females sniffed pups ( $F_{(1, 161)}=5.13$ ,  $p=0.025$ ) and moved pups around ( $F_{(1, 161)}=5.88$ ,  $p=0.016$ ) longer than males. Interactions of treatment and sex were found in the total duration of parental behavior ( $F_{(5, 161)}=2.38$ ,  $p=0.041$ ) and licking ( $F_{(5, 161)}=2.79$ ,  $p=0.019$ ). Post-hoc analyses (Fisher's LSD) revealed that SSPI females exhibited parental care longer than SSPI males, SSPI females, CU females and C males (Figure

3.4a). In the duration of licking, only CU animals showed a sex difference (males licked longer than females; Figure 3.4b). No correlations of parental behaviors between parents and pups were found.



Left: Figure 3.4a. Duration of Total Parental Behaviors in Adult Offspring (Mean±SEM)

Right: Figure 3.4b. Duration of Licking in Adult Offspring (Mean Mean±SEM)

### 3.2.3. Emotionality

For all tests reported below, C and CU were combined as a control group (CO), because these two groups did not differ.

#### 3.2.3.1. Elevated Plus Maze Test

No effect of treatment was found in durations in open arms and closed arms, % entries to each type of arms, or total number of entries (Table 3.5). Males spent longer times than females in the open arms ( $F_{(1, 56)}=5.72, p=0.020$ ).

### 3.2.3.2. Open Field Test

An effect of separation was found in the % crossings within the central area ( $F_{(4, 57)}=2.81$ ,  $p=0.034$ ; Figure 3.5). The % crossings within the central area were higher in SSPI females than SSPI males. However, SSPS males crossed more in the central area than SSPS females. No sex differences were found in the CO, LSPI and LSPS groups.

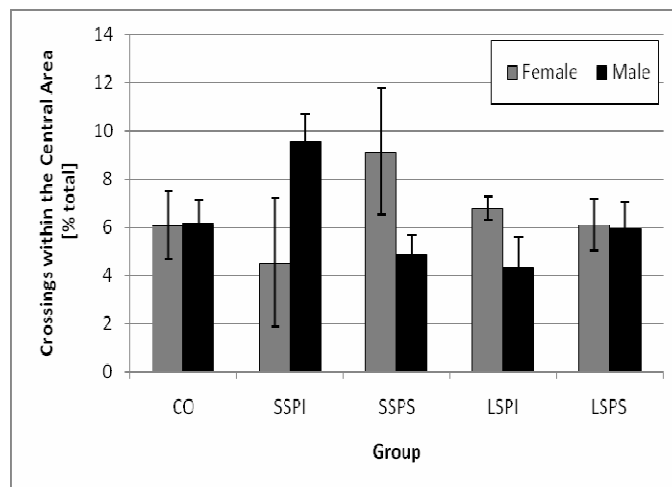


Figure 3.5. Anxiety-like Behaviors in Adult Offspring as Measured by Crossings within the Central Area in Open Field Test (Mean $\pm$ SEM)

### 3.2.3.3. Forced Swim Test

No differences were found in the durations of immobility and struggling according to treatments (Table 3.5).

Group	C		CU		SSPI		SSPS		LSPI		LSPS	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
N	7	21	8	20	12	22	10	21	12	17	8	15
Contact	540.1±36.36	462.0±35.05	472.3±71.12	488.0±31.38	516.2±20.69	466.9±31.51	493.9±48.51	528.1±17.36	491.9±52.51	496.8±31.77	583.7±3.31	495.9±39.44
Crouching	54.21±34.27	40.95±12.01	91.98±31.69	47.34±11.60	54.37±16.90	35.58±10.70	25.64±11.69	54.74±13.19	62.82±23.17	37.35±9.37	59.73±24.06	48.95±16.43
Licking	348.7±45.43	292.8±20.73	191.2±41.97	318.4±28.57	371.9±25.19	298.1±26.81	293.0±38.93	344.8±24.15	277.6±46.48	321.4±26.39	349.8±17.60	313.8±30.07
Nesting	12.37±10.50	5.28±1.87	25.18±22.21	12.97±6.16	8.57±3.36	14.14±4.03	1.96±1.42	7.49±5.46	5.22±3.13	3.57±3.30	12.23±7.17	8.55±7.47
Retrieving	1.27±0.75	0.78±0.31	0.75±0.40	0.51±0.19	2.04±0.78	0.23±0.13	2.78±1.56	1.60±0.46	1.83±0.82	1.35±0.31	0.75±0.30	0.38±0.18
Total Parental Behaviors	416.5±43.92	339.8±25.47	309.1±62.95	377.6±29.19	436.8±23.10	348.0±27.32	323.4±42.76	408.6±18.64	347.5±49.11	363.7±28.32	422.5±18.35	371.6±36.52
Exploration	40.86±23.67	28.53±7.57	26.96±8.64	31.87±8.76	23.36±7.79	56.45±16.11	39.13±12.82	27.85±7.23	66.23±37.40	35.89±6.94	10.16±1.56	35.80±14.83
Feeding	0.00±0.00	0.64±0.64	0.65±0.65	1.39±1.39	0.00±0.00	0.00±0.00	0.00±0.00	1.03±1.03	0.38±0.38	2.26±2.26	0.96±0.96	0.13±0.13
Grooming	13.63±10.41	7.38±2.06	5.51±3.29	17.05±12.09	4.09±0.96	15.25±10.95	18.42±10.86	3.83±1.93	6.51±3.57	12.32±8.70	5.69±2.43	14.02±5.84
Sniffing	28.23±10.58	7.32±1.33	23.83±14.25	13.25±3.12	23.83±7.62	19.06±3.72	11.42±2.83	9.86±2.11	20.82±5.35	10.79±1.99	6.79±1.88	16.48±4.70
Total Non-parental Behaviors	82.71±35.88	43.87±7.35	56.95±22.71	63.55±19.19	51.28±12.29	90.76±19.32	68.97±22.63	42.58±10.06	93.93±43.63	61.26±12.64	23.60±4.21	66.43±17.29
Moving	4.43±3.11	0.80±0.50	0.93±0.46	0.66±0.30	1.78±0.78	0.59±0.29	6.19±4.86	2.09±0.84	4.06±2.17	0.91±0.28	0.61±0.61	1.25±0.63

Table 3.4. Parental and Non-Parental Behaviors in Adult Offspring (Mean±SEM [sec])

Group	CO		SSPI		SSPS		LSPI		LSPS	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
<b>Elevated Plus Maze Test</b>										
N	9	16	6	6	4	7	3	6	4	5
Open arms [sec]	113.67±33.34	145.19±26.06	121.50±35.63	267.17±56.20	67.00±20.05	133.29±42.87	115.00±63.98	188.00±28.10	121.75±14.59	123.00±35.84
Closed arms [sec]	359.78±48.33	314.56±27.50	322.17±35.55	235.83±38.09	340.00±44.70	370.71±44.83	316.00±82.12	270.00±34.17	291.75±29.69	319.40±53.67
Entries to Open Arms	6.05±2.52	10.31±2.74	7.00±1.37	8.83±2.57	4.50±1.04	5.14±1.50	5.00±1.53	8.33±1.89	5.25±0.48	6.60±2.06
Entries to Closed Arms	10.00±2.77	16.38±2.84	12.83±2.27	11.17±2.27	9.00±2.16	9.29±1.87	11.00±1.53	14.50±3.25	17.00±3.14	10.60±1.40
Total Entries	16.05±4.98	26.69±4.66	19.83±2.97	20.00±4.79	13.50±2.90	14.43±2.72	16.00±2.31	22.83±4.69	22.25±2.87	17.20±3.35
<b>Open Field Test</b>										
N	8	13	4	8	5	7	6	5	5	6
Duration in Central Area [sec]	6.10±1.44	6.17±0.98	4.55±2.69	9.59±1.14	9.16±2.62	4.90±0.82	6.82±0.49	4.36±1.26	6.12±1.07	5.95±1.09
Entries to Central Area	12.50±4.85	14.30±4.93	9.25±5.31	19.88±4.38	10.80±3.32	7.00±2.51	10.33±1.82	6.00±2.17	4.60±0.81	7.67±3.96
Crossings within Central Area	13.75±5.56	15.81±5.85	11.25±6.84	23.88±5.08	13.60±4.06	6.86±2.18	13.00±2.39	6.80±2.65	6.60±1.29	10.17±5.34
Crossings within Periphery	139.00±30.18	176.74±31.39	136.75±42.40	174.63±28.67	112.60±22.40	99.00±19.26	160.50±31.27	112.60±27.08	97.80±20.65	116.17±33.83
Total Crossings	178.75±44.84	222.07±42.33	167.50±56.75	238.88±40.68	148.60±30.00	120.86±26.14	195.17±37.11	132.20±33.39	114.20±23.37	142.67±46.03
<b>Forced Swim Test</b>										
N	7	13	5	9	5	7	6	5	5	5
Immobility [sec]	238.43±34.59	161.54±30.66	127.40±24.13	183.78±29.15	132.20±29.88	174.86±33.21	117.33±34.83	201.20±29.99	154.20±55.15	104.40±51.15
Struggling [sec]	203.57±40.97	263.08±32.96	235.40±38.33	228.67±39.74	238.8±100.2	283.00±35.94	228.83±78.35	146.20±58.27	162.60±49.69	276.80±71.37

Table 3.5. Emotionality Tests in Adult Offspring (Mean±SEM)

### **3.2.4. Partner Preference**

There were no differences between C and CU animals. Thus these two groups were combined as control (CO). Overall, females were found in the partner cage more often than males ( $F_{(1, 80)}=7.86$ ,  $p=0.006$ ), while no sex differences were found in the time spent in neutral or stranger cages. Previous studies have shown that prairie voles exhibit sexual dimorphism in formation of pair bond. Thus, males and females were analyzed separately in the following analyses.

#### **3.2.4.1. Previous Experience**

Infanticidal and non-infanticidal animals did not differ in their partner preference. There was no effect of emotionality tests in the time spent in partner and neutral cages. However, animals who experienced forced swim were found in a stranger cage significantly less often than those who were tested for open field and elevated plus maze ( $F_{(2, 80)}=5.11$ ,  $p=0.008$ ). No interaction of separation and type of emotionality test was found.

#### **3.2.4.2. Overall Preference**

For both males and females, total frequency of being in the partner, neutral, or stranger cages did not differ among groups with different types of separation. However, groups differed when individuals were categorized according to the most preferred cage (the cage with the largest number of observations for each animal). Percent of animals that preferred partner, neutral,

or stranger cages were differently distributed among groups (Figure 3.6;  $X^2_{(8)}=64.1, p<0.001$ ).

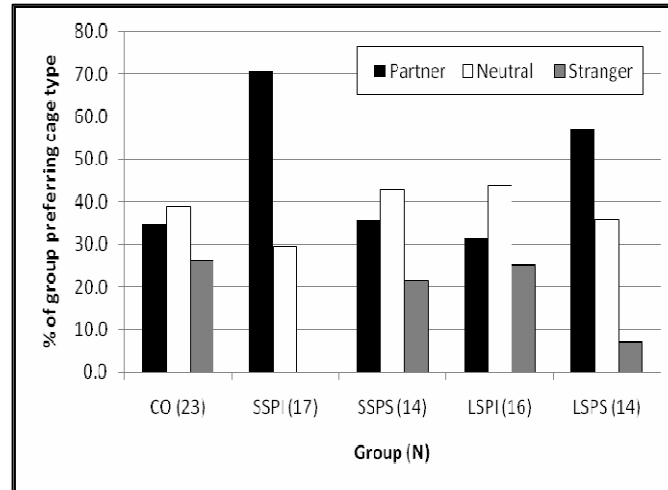
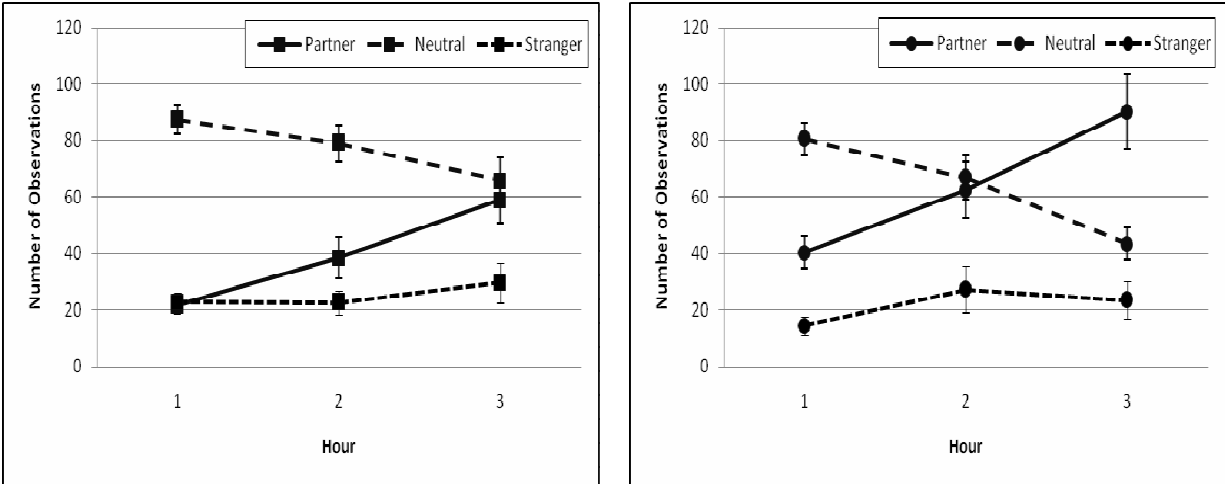


Figure 3.6. Percent of Animals in Each Group That Preferred Partner, Neutral, or Stranger Cages

### 3.2.4.3. Hour by Hour Preference

In both males and females, cage preference changed hour by hour during the 3h partner preference test, although such hourly changes in cage preference did not differ among groups with different types of separation. Frequency of males found in a partner cage increased every hour, regardless of separation treatment (Figure 3.7a;  $F_{(2, 90)}=13.59, p<0.00001$ ). Frequency in a neutral cage decreased ( $F_{(2, 90)}=5.83, p=0.004$ ), and post-hoc tests (Fisher's LSD) revealed that males were found in the neutral cage more often during the first than third hour. No changes were found in preference of stranger cage from hour to hour. Similarly to males, frequency in partner cage increased every hour for females (Figure 3.7b;  $F_{(2, 60)}=10.29, p=0.0001$ ), while time spent in the neutral cage

decreased every hour ( $F_{(2, 60)}=16.64$ ,  $p<0.0001$ ). Females were found in the stranger cage more often in the second than first hour ( $F_{(2, 60)}=3.57$ ,  $p=0.034$ ).

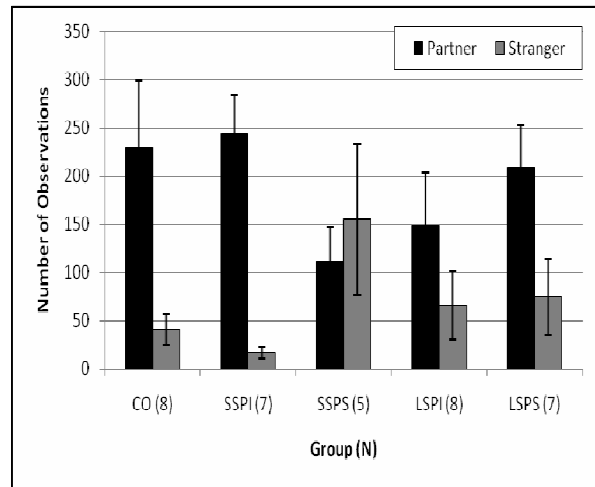
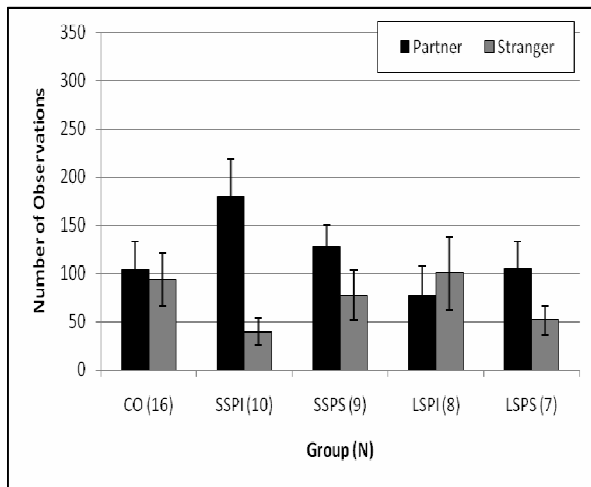


Left: Figure 3.7a. Male Cage Preference per Hour (Mean±SEM)  
 Right: Figure 3.7b. Female Cage Preference per Hour (Mean±SEM)

#### 3.2.4.4. Partner vs. Stranger within Group

For males, only SSPI group showed significant preference for a partner over stranger cage (Figure 3.8a;  $t_{(9)}=3.09$ ,  $p=0.013$ ). In females, SSPI (Figure 3.8b;  $t_{(6)}=5.29$ ,  $p=0.0018$ ) and CO ( $t_{(7)}=2.43$ ,  $p=0.046$ ) animals were found in the partner cage more often than the stranger cage. Differences in frequencies between partner and stranger cage during the third hour were also analyzed for each group. Only LSPS ( $t_{(6)}=2.64$ ,  $p=0.039$ ) and SSPI ( $t_{(9)}=3.48$ ,  $p=0.007$ ) males were found in the partner cage more often than the stranger cage in the third hour. In females, CO ( $t_{(7)}=2.37$ ,  $p=0.049$ ) and SSPI ( $t_{(6)}=4.05$ ,  $p=0.007$ ) animals remained in the partner cage significantly more often than the stranger cage.





Left: Figure 3.8a. Preference of Partner to Stranger in Males (Mean±SEM)  
 Right: Figure 3.8b. Preference of Partner to Stranger in Females (Mean±SEM)

### 3.2.5. Behavioral Response to a Five-minute Forced Swim Stressor

There was no difference between C and CU, thus these two groups were combined as a control (CO).

#### 3.2.5.1. Previous Experience

Infanticidal and non-infanticidal animals did not differ in the duration of immobility during the 5min forced swimming. Also, type of emotionality test did not influence the duration of immobility. However, animals subjected to partner preference test (PPT) showed significantly shorter immobility than those who was not subjected to PPT ( $F_{(1,118)}=6.15, p=0.015$ ).

### 3.2.5.2. Effect of Separation

Overall, no effect of separation or sex, or an interaction of sex and separation was found in the duration of immobility. However, effects of separation were found within the animals that did not experience PPT ( $F_{(4,53)}=2.72$ ,  $p=0.039$ ). Post-hoc analyses (Fisher's LSD) revealed that CO animals were immobile longer than SSPI, SSPS, and LSPS animals (Figure 3.9). Animals with the experience of PPT did not differ in the duration of immobility among separation treatments.

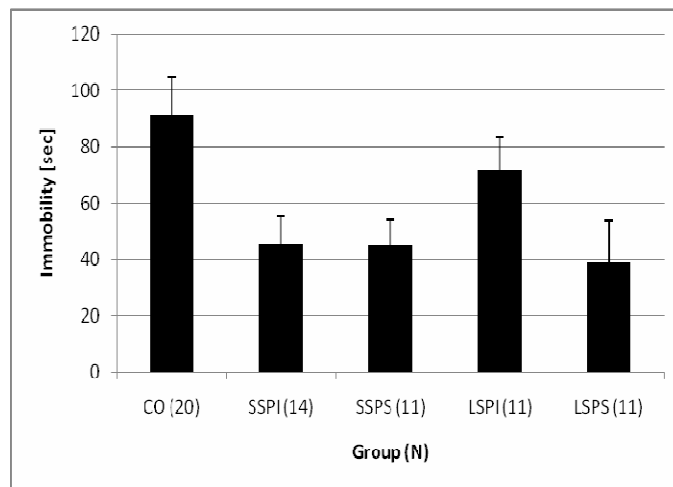


Figure 3.9. Duration of Immobility during 5min Forced Swimming in Animals Not Subjected to Partner Preference Test (Mean±SEM)

## **CHAPTER 4**

### **DISCUSSION**

Previous studies have shown that parent-pup separation alters physiology and behavior. However, these studies tend to focus on specific systems, often without providing multiple measures of behavioral outcomes together. The present study was conducted to provide an integrative perspective of the effects of parent-pup separation on prairie voles, which exhibit social monogamy. Both parents and adult offspring that were subjected to separation exhibited altered behaviors.

#### **4.1. Parental Responsiveness**

##### **4.1.1. Parental Responsiveness in Parents**

In parents, separation did not influence parental behaviors but did alter non-parental behaviors (grooming and exploration) after 10 days of separation treatment.

No effect of separation was found on parental behaviors. This finding is different from previous studies in rats, which show reduction in parental responsiveness after prolonged daily separation (Boccia and Pedersen, 2001; Caldji et al., 1998; Liu et al., 1997; Pryce et al., 2001). This discrepancy may be explained by the presence of a partner. Persistent contact with a partner may reduce prolonged stress responses and maintain social motivation and affiliative behaviors in each animal. As a result, a parent displays unchanged parental behaviors towards pups. A compensated effect of prolonged separation was also

found in rats. Hyperemotionality in adult offspring was prevented by providing foster pups to parents during separation (Huot et al., 2004). In this study, a partner might take the same role as those foster pups, resulting in no differences in parental behaviors among groups. Bales et al. (2006) reported sexually dichotomous changes in parental behaviors after exposure to a forced swim stressor, supporting previously presented hypotheses that stress facilitates parental responsiveness only in male but not in female prairie voles. In the current study, parental behavior did not differ between dams and sires. The testing procedure used in this study (habituation to testing cage for 30min in isolation) may not be stressful enough to induce sexual dimorphism in parental behaviors.

Interestingly, a sex difference was found in the total duration of grooming. Specifically, CU (undisturbed during separation paradigm) and LS (separated 6h daily) dams showed longer grooming than sires in the same group, while SS (separated 15min daily) and C (detached from pups without separation) animals did not show significant sex differences. In many rodents, increased grooming has been observed when subject is exposed to anxiogenic environment, such as novelty and open space (Eguibar et al., 2003; Kalueff et al., 2004; Kametani, 1988; Spruijt et al., 1992). Because grooming does not correlate with other indications of anxiety, such as freezing and defecation, Spruijt et al. (1992) argued that animals habituate to novelty during grooming. In the present study, increased grooming may be evoked by isolation, a novel experience for a subject, during the parental behavior test. Unlike daily pup separation, during which a parent remained in the home cage with its partner, each parent was transferred

into clean testing cages individually. Although subjects were allowed to habituate to the testing cage for 30min, this procedure may induce sexually dichotomous reactions to isolation in grooming. Differences in grooming found in LS animals suggest that prolonged daily separation, which is predicted to be more stressful than brief separation, results in sex differences in parents. Similar findings in CU animals may be related to habituation to daily handling. Both C and CU parent experienced no separation. However, unlike CU animals, who were never disturbed until PND11, C animals were handled daily during PND1-10. Habituation to handling may prevent a sexually dichotomous stress response in C animals.

The duration of exploration during parental behavior test shows somewhat contradictory results. CU animals explore longer than C and LS animals, regardless of sex. The definition for exploration in the present study is “moving around the cage and rearing while being away from pups.” Such a definition fails to distinguish exploration driven by anxiety from that driven by novelty-seeking, nest-seeking or other behaviors related to parental behavior.

#### **4.1.2. Parental Responsiveness in Adult Offspring**

In adult offspring, differences in the duration of licking and total parental behavior were found. Although there was no effect of separation in licking, an interaction of sex and separation treatment was found. Only undisturbed (CU) animals exhibited sexually dimorphic licking. Similarly, interaction of sex and separation treatment was found in the duration of total parental behavior. Unlike parents, no sex and group differences in the duration of non-parental behaviors.

Exposure to neonatal separation, regardless of its duration and housing condition, did not result in different degree of licking. This finding is different from the prediction that brief separation would facilitate and prolonged separation would reduce licking in adulthood. In rats, the amount of licking is directly transmitted from dam to daughter, regardless of genetic predisposition (Champagne et al., 2001). In this study, separated animals did not differ in parental behaviors. This may be because prairie vole parents did not differ in their parental behaviors. Another explanation for this finding is that procedures in the previous rat studies were different from present study. In this study, observation of licking was conducted during a parental behavior test with unrelated pups. It is possible that behaviors displayed during the test were influenced by handling and the novelty of the testing environment, including clean testing cages with less bedding, isolation, and novel pups. Parental care towards pups in their home cage would be more comparable to rat studies to examine the amount of licking exhibited by parents and their adult offspring.

Although an effect of separation was not found, an interaction of separation and sex was observed in the duration of licking. CU males licked unrelated pups longer than CU females, whereas other groups did not show sex differences. CU animals experienced no manipulation except daily lid openings. Interestingly, rat dams lick anogenital region of male pups longer than that of female pups (Moore and Morelli, 1979). To date, such preferences have not been examined in prairie voles. It can be predicted, however, that if such a preference exists in prairie voles, its effects are more likely to be observed in the CU group

rather than in others, since parenting is unlikely to be disturbed by the experimenter. It is important to note that there are no sex difference in body licking (Moore and Morelli, 1979), and that the transmission of maternal responsiveness to pups in rats is mediated by the total amount of body and anogenital licking (Champagne et al., 2003). An experiment examining sex difference in anogenital and body licking would provide insight into mechanism of licking behavior in prairie voles.

An interaction of separation and sex was also found in the duration of total parental behavior. In the SS (15min) group, isolated females displayed longer parental care than females kept with siblings and isolated males. Housing condition during separation was correlated with parental behaviors in those experiencing daily brief separation. Detailed group differences and interpretation are found in the section of general discussion.

## **4.2. Emotionality**

### **4.2.1. Anxiety-Like Behaviors**

In the open field test (OF), percent entries to the central area differed among groups, whereas total time spent in the central area was different in adult offspring. In the elevated plus maze test (EPM), no effect of separation was observed both in parents and adult offspring.

In parents, anxiety-like behavior, measured by percent entries into the central area of OF, was different among groups. Although significant differences were not found between each group and each sex in post-hoc tests, SS (15min)

dams were more anxious than LS (6h) and control dams, while sires showed an opposite pattern. Habituation to daily separation might explain these results. During 10 days of separation, SS sires might have learned that their pups would be returned shortly after the separation, and as a result, they showed less anxiety-like behaviors. On the other hand, LS sires learned that once separated, their pups would not be back for a long period, resulting in increased anxiety-like behaviors. Control sires also exhibited heightened anxiety-like behaviors. For them, emotionality tests were their second time of being isolated from their partner and pups. The lack of previous experience of separation might stimulate strong emotional response in control sires. An opposite response pattern in dams can be explained by their altered sensitivity to stimuli that evoke emotional responses. In lactating rats, separation from pups increased anxiety-like behaviors (Ohl et al., 2001). This increase was found only in SS dams. Less anxiety-like behaviors in control and LS dams are possibly reflections of pup-seeking behaviors. Since the interpretation of the entries into the central area can vary, further study would be important to fully understand what causes such sexually dichotomous responses in the open field test.

In adult offspring, females isolated during short separation (SSPI) exhibited less anxiety-like behaviors than SSPI males, while females kept with siblings during short separation (SSPS) were more anxious than SSPS males. Given the fact that their parents experienced exactly the same daily treatment (15 minutes of separation from pups), housing conditions during the separation seem to have an impact on anxiety-like behaviors in a sexually dichotomous manner.



Among the voles that experienced short separation, emotionality was higher for isolated females than females kept with siblings, and vice versa in males.

Interestingly, anxiety-like behaviors in animals exposed to long separation did not differ from each other, indicating that the long separation negates the differences between being isolated and kept with siblings during separation.

Several limitations are found in this study. First, parents in this study were tested for their emotionality 4-6 hours after the parental behavior test.

Experiencing two behavioral tests in one day may have evoked a strong response and thus masked the effects of separation on anxiety-like behaviors. However, such a time gap was designed to avoid sustained stress response prior to the emotionality tests. Also, while EPM did not show differences among groups, anxiety-like behaviors differed when measured in OF. The timing of emotionality tests in parents seems valid in this study. Second, the timing of emotionality tests differed between parents and adult offspring. This may contribute to different results in parents and adult offspring. Last, although both EPM and OF were used to assess anxiety-like behaviors, effects of separation were found only in OF. It is possible that EPM was not sensitive enough to reveal group differences. Many studies of anxiety-like behaviors have been conducted on rats and mice, and prairie vole researchers have adapted their methods. Differences in behavioral phenotypes between common laboratory animals and prairie voles (e.g. social structure) may result in different sensitivity to these emotionality tests. In addition, prairie vole life history, in which they live in burrows and emerge to forage may make the saliency of open field and elevated plus maze test cues

different from that experienced by rats. Interestingly, previous exposure to OF changed behaviors observed in EPM (Lister, 1987; Pellow, 1985), indicating influence of previous experience on the outcome of EPM. In this study, such concern was avoided by using a between-subject design. Prior to the emotionality test, all subjects experience the same event at the same time in this study. Different anxiety-like behaviors in prairie voles correlate with separation treatment.

#### **4.2.2. Depression-Like Behaviors**

In parents, daily handling influenced the duration of immobility in the forced swim test (FST). However, the duration of separation (none vs. brief vs. long) did not affect immobility and struggling in the forced swim test, in both parents and adult offspring.

In postpartum female rats, Boccia and Pedersen (2007) reported that prolonged daily pup separation induced depression-like behaviors. In the present study differences were not seen among animals that experienced various durations of separation. Surprisingly, however, parents that were handled daily during the separation paradigm exhibited longer immobility than those who were not handled, suggesting conditioned helplessness in handled animals. Although these parents were never exposed to forced swimming previously, they were picked up by an experimenter for 10 consecutive days. The lack of handling in CU parents resulted in longer mobility during FST.

Unlike parents, adult offspring did not show group differences. This suggests that repeated daily manipulation is disruptive only on parents, and that prolonged separation does not induce depression-like behaviors in pups. Interestingly, a trend of interaction between sex and duration of separation was found in adult offspring. Although it did not reach statistical significance, CO females exhibited longer immobility than separated (SS and LS) females, while males did not differ among groups. Correlation of depression-like behaviors between parents and their offspring needs to be further investigated.

In addition to the duration of immobility, duration of struggling was measured in this study. Struggling was defined as vertical swimming and diving into the water. We originally thought that the struggling would reflect the motivation to escape from water, inducing a longer struggle in animals that have greater drive for survival. However, effects of separation on struggling were not observed in parents or adult offspring. This can be explained if all animals were equally motivated to escape. The other possible explanation is that prairie voles may be adapted to swimming. In their natural habitat, floods may occur occasionally, and thus prairie voles may have evolved a strategy to float comfortably. In our observations, this appeared to be the case. The FST was a stressor, as indicated by an increase in corticosterone levels measured 30 minutes after the FST (Gill, et al., unpublished data).

### **4.3. Pair Bond**

#### **4.3.1. Partner Preference**

Effects of separation and sex were found in partner preference. Overall, females were found in their partner's and neutral cages more often than the stranger's cage, whereas males stayed in the neutral cage more than other two cages. Both males and females preferred their partners to strangers. When analyzed according to separation, females and males showed different patterns of partner preference.

Both males and females preferred their partners to strangers. However, the effect of separation on partner preference was sexually dichotomous. SSPI and LSPS males significantly preferred partners to strangers, whereas CO and SSPI females significantly preferred their partner. CO animals in both sexes were predicted to show partner preference. In this study, subjects cohabitated with opposite-sex partners for 24 hours. DeVries (1995, 1996 and 1997) reported that the formation of pair bonds is influenced by stress differently in males and females. Stress, as well as peripheral injections of corticosterone, facilitates partner preference in males and reduces it in females. In the studies by DeVries and colleagues, subjects were isolated for 2 weeks prior to pairing, while our subjects were kept with siblings until the pairing. Lack of isolation stress exposure in the present study may have failed to facilitate partner preference in CO males. Another explanation for discrepancies is that stimulus animals were not gonadectomized in our study. However, partners and strangers were age- and weight-matched, and prairie vole females ovulate in response to stimuli from

males. In our study, the reproductive quality of partner and stranger was unlikely to disturb the partner preference. Integrative interpretation of the results in partner preference is discussed in the General Discussion (page 52).

#### **4.3.2. Influence of Previous Experiences**

Effects of parental responsiveness, such as whether or not a subject attacked pups during PBT, were not observed in partner preference. Similarly, previous exposure to different types of emotionality tests did not change preference of partner and neutral cages. However, animals who experienced FST avoided the stranger cage more than those exposed to OF and EPM.

In this study, a reduced number of observed visits in the stranger cage was only found in the animals that experienced FST. Although these animals exhibited an increased stranger avoidance, their partner preference did not differ from animals that experienced OF and EPM. Exposure to a forced-swim stressor might have increased fearfulness of animals without interrupting affiliation to a partner. In parental behavior, Numan (2006) suggested that maternal motivation and pup avoidance are regulated by two distinct pathways. Similarly, avoiding a novel conspecific animal may be regulated by neural pathways distinct from pathways that regulate pair bonding.

Among the three emotionality tests, FST is considered as the most invasive test. Traumatic experience impairs social behaviors in rats (Mikics, 2008) and in humans (Pitman, 1997). A strong effect of FST may alter response to a novel animal, regardless of early-life experience in prairie voles.

## **4.4. General Discussion**

### **4.4.1. Parents**

The first hypothesis that repeated separation from pups affects parental behavior and emotionality in parents was partially supported. Parental behaviors were unchanged even after repeated separation from pups possibly because of the presence of a partner.

Anxiety-like behavior measured by open field test was influenced by separation. Dams subjected to short separation exhibited greater anxiety-like behaviors than those subjected to long or no separation, whereas sires subjected to short separation showed less anxiety-like behaviors than those subjected to long or no separation. Such a sex difference was probably due to different sensitivity to an open field between males and females. Our finding for sires was consistent with predictions. The unpredicted result in dams may be explained by lactation, during which dams become hyporesponsive to stimuli that evoke emotional responses.

Depression-like behaviors measured by forced swim test were affected by daily handling, rather than the duration of separation. Daily handling may induce learned helplessness that was exhibited as a longer immobility. Duration of separation did not correlate with the duration of immobility.

Sensitivity to pup separation differed in parental behaviors and emotionality. These findings suggest that emotionality is more susceptible to pup

separation in prairie voles, and that parental responsiveness can be maintained by stimuli from partners in addition to stimuli from pups.

#### 4.4.2. Adult Offspring

The second hypothesis that neonatal parental separation affects emotionality and physiological development in pups, and thus induces altered adult parental, emotional, and social behaviors was supported. However, predictions (Figure 1.2., page 19) were not fully consistent with the findings in this study.

Prairie voles subjected to neonatal short isolation (SSPI) exhibited longer parental behaviors (female), reduced anxiety-like behaviors (males), and stronger partner preference (both sexes) as adult. Based on these findings, I suggest a revised model for prairie voles (Figure 4.1).

This model suggests an organizational effect of HPA-axis activity during the

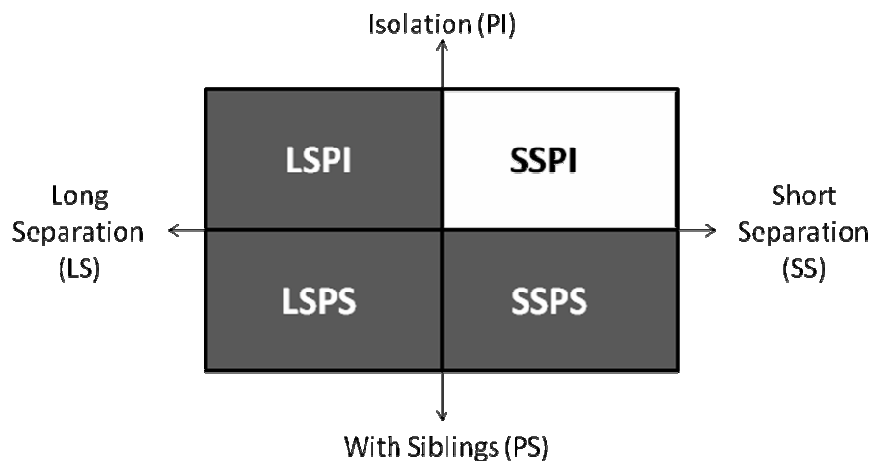


Figure 4.1. Revised Model Predicting Behavioral Changes in Prairie Vole Adult Offspring. X-axis represents duration of separation (short vs. long) and Y-axis represents housing condition (siblings vs. isolation).

neonatal period for regulation of adult behaviors. Unlike rats, prairie vole pups are not hyporesponsive to stress. Hypothetically, a rise in plasma corticosterone level is greater in isolated pups than those kept with siblings. This stress response is alleviated by parents in pups subjected to short separation, but longer separation results in more permanent neurobiological changes. Repeated increase and decrease of plasma corticosterone may influence development of neurobiological systems that regulate emotional and social behaviors in prairie voles.

In many cases, housing condition (isolated vs. with siblings) did not result in differences in animals subjected to a long separation (LS). One possible explanation is that the effect of long separation is too severe and masked the differences in housing condition. Another explanation is that experiencing both stress-induced increase and parent-induced decrease in plasma corticosterone is important for development in pups. For isolated pups in LS group, parental care provided after the 6h separation may miss the critical window to alleviate the effect of isolation. On the other hand, pups that were kept with siblings for 6 hour may not have experienced a rise in plasma corticosterone level. Interestingly, housing condition in LS animals has significant effect on the body weight on PND90, indicating that tactile stimulation has a compensatory effect on physical development, and that physical development and development of neural and endocrine pathways that regulate emotional and social behaviors can be independent from each other. SSPS animals also did not differ from LSPS animals, possibly because being kept with siblings did not induce a rise in plasma corticosterone as high as isolation.



In rats, neonatal separation affects various behaviors in adult offspring via altered maternal care in dams. In prairie voles, however, neonatal separation may alter emotional and social behaviors independently from parents.

#### **4.5. Future Directions**

Meaney and his colleagues have demonstrated that epigenetic modulation by neonatal maternal care leads to alteration of behavior in adulthood (Weaver et al., 2004; Champagne et al., 2006). These studies are particularly interesting since the amount of maternal licking directly affects maternal behaviors in the offspring by altering 1) estrogen receptor-alpha binding and subsequently, oxytocin receptor (OTR) binding in the medial preoptic area and 2) glucocorticoid receptor binding in the hippocampus (regulates HPA reactivity) and OTR expression. The present study did not show variation in licking among parents, possibly because parental responsiveness reached a threshold and was unable to differentiate individuals. Conversely, differences in the duration of licking were found in adult offspring. Examining whether or not lactating voles display different degrees of licking would lead to subsequent studies similar to those conducted on rats.

As mentioned earlier, the oxytocinergic system has been implicated in the regulation of maternal behaviors. Perinatal manipulation of oxytocin (OT) influences adult parental responsiveness and emotionality in prairie voles (Cushing et al., 2005). Although a surge of OT occurs around parturition in prairie voles, it is still unclear whether OT surges in dams affects pups perinatally. Given

that neonatal OT influences the expression of ER $\alpha$  (Kraemer, 2007) it is possible that changes in parental responsiveness occur in an estrogen dependent manner. Further studies that reveal an OT-estrogen signaling pathway and how it is related to early life experience would be interesting. As for males, vasopressin, a neuropeptide closely related to OT, is involved in regulation of paternal behaviors (Wang et al., 1994; Wang et al., 2000; Lonstein and De Vries, 2000). Variation in the vasopressinergic system in different vole species appears to correlate with expression of paternal care and monogamous behaviors. In addition to a general developmental profile of vasopressin activity, examining whether early life experience alters the vasopressinergic system in male prairie voles would advance our understanding of paternal behavior.

The HPA axis is involved in behaviors examined in the present study. Shapiro and Insel (1990) demonstrated a rise in plasma corticosterone in isolated prairie vole pups. Female prairie voles that received corticosterone during the neonatal period shows reduced parental behavior as adults (Roberts et al., 1996). These findings, as well as results from the present study, suggest an important role of HPA-axis activity in neonatal pups. Among various possible experiments, looking at glucocorticoid receptor and CRF receptor expressions, as well as distribution of these cells in cross-species comparisons would contribute further to understanding the consequences of parent-pup separation.

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