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Family life and its consequences: insights from the monogamous prairie vole (*Microtus ochrogaster*)

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Family life and its consequences: insights from the monogamous prairie vole (*Microtus ochrogaster*)

By

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B.A., Oberlin College, 2002

Advisor: Larry J. Young, Ph.D.

An abstract of

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Abstract

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By Todd H. Ahern

Family life exerts a profound influence on both adults and children. For humans, family life is marked by a rich set of dynamic social interactions, including adult pair bonds, maternal- and paternal-infant bonding and nurturing, and parental coordination. Differences in the structure, stability, and quality of these family dynamics influence physiology, behavior, and mental health throughout life, yet little is known about the neurobiology that mediates this influence. Manipulations of early environment and mother-infant care in traditional animal models, such as rats and mice, have helped fill this gap, but their uniparental family structure precludes the study of other family dynamics, such as partner loss, shifts in family structure, paternal care, and parental coordination. The goal of this dissertation was to examine how these other types of family dynamics affect behavioral and neurobiological outcomes using monogamous prairie voles (*Microtus ochrogaster*). I began by examining the effects of pair bond disruption. Male prairie voles exhibited increased passive-coping following partner loss and this effect was dependent on the activation of both known corticotropin-releasing factor (CRF) receptors: CRF1 and CRF2. I then characterized the biparental family dynamics of primiparous prairie voles under laboratory conditions and examined the effects of altering family structure through the removal of the father. Under biparental (BP) conditions, prairie vole parents of both sexes engaged in high levels of coordinated pup care. Removal of the father resulted in single-mother (SM) family units and a decrease in pup care that had a striking effect on offspring behavior in adulthood, including changes in social bonding and two types of parenting behavior. I then examined the effects of family structure on socially-relevant neuropeptide systems and found significant differences in hypothalamic oxytocin (OT) content and CRF2 densities. Based on parallels in the action and responses of these two systems to stress and social environment, I ended with an investigation of the anatomical link between OT and CRF2. Overall, these findings establish prairie voles as a promising animal model for the study of family dynamics and offer new insights into how differences in family life can affect behavior, emotion, and neurobiology.

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TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

CHAPTER 1

Family life and its contributions to variation in behavior, emotionality, mental health, and vulnerability to psychopathology: a human and animal review

INTRODUCTION

Family life profoundly affects who we are. It takes every characteristic that distinguishes us as individuals and blends them into a dynamic system that leaves none of us the same as when we first entered it. Whether good or bad, our experience of family life shapes us, and research in a variety of fields is beginning to define how, and to what extent, specific aspects of family life contribute to our development, our mental health, our individual differences in behavior, and our vulnerabilities to psychopathology. Unfortunately, little is known about the neurobiological factors that drive and mediate these effects. To help fill this gap, I propose the use of prairie voles (*Microtus ochrogaster*) as a new model organism for studying family dynamics and present data throughout this dissertation in support of this proposal.

Here, I first briefly survey the influence of family life on human behavioral, psychological, and neuroendocrine outcomes; I then identify and discuss the traditional animal models that have contributed to our neurobiological understanding of how family life influences individual outcomes; and finally I examine the current impact and long-term promise of less traditional animal models, like prairie voles, to further our insight. I end with a brief outline of the aims of this dissertation.

What is family?

"Family" is a nebulous term: It holds a variety of meanings for a variety of people, and it defies rigid definitions with countless exceptions. Even in research disciplines that study families, the term is often broadly understood. For example, the American Psychological Association currently describes the *Journal of Family Psychology* as a

"premiere family research journal [that] is devoted to the study of the family system, broadly defined…" (American Psychological Association, 2010). This diversity in meaning arises, of course, from the diversity in reality. Indeed, throughout the animal kingdom the evolution of mating and family structures, particularly in mammalian orders, is remarkably varied (Kleiman, 1977; Clutton-Brock, 1989; Fuentes, 1998). Even within species, there is often variation in family composition, structure, and participation based on genetic, geographic, seasonal, and resource constraints (e.g., Getz et al., 1992a; Low, 2007; Fortunato and Archetti, 2010). This variety is perhaps most pronounced in humans. Based on cross-cultural comparisons in pioneering ethnographic and anthropological work by GP Murdoch and others (e.g., Murdoch, 1967, 1981), humans boast one of the richest, most diverse sets of mating and family practices in the animal kingdom (Low, 2007). Polygyny, monogamy, polyandry, adoption, step-families, homosexuality… the list is long and they all have representation.

Competently addressing such a diverse range of definitions and structures of family life is well beyond the scope of this dissertation. My more limited focus here is to understand relatively small groups of individuals that, by choice, law, biological disposition, or necessity form a strongly interconnected and relatively exclusive social unit aimed (to some degree) at reproduction and care for the next generation of offspring. This perspective is narrow enough to allow meaningful study, but broad enough to encompass, and allow comparisons between, common differences in family life. It is also sufficiently broad to consider small groups of *nonhuman* individuals that, by biological disposition or necessity, form strongly interconnected and relatively exclusive social units aimed at reproduction and care for the next generation to be families as well. I focus

on this understanding of "family" because these types of social units contribute much to the development and well-being of not only humans, but also of many animal species. Such parallels may be helpful in understanding ourselves better.

Why families are important

Over the last several decades, researchers from a variety of fields, from demography to pediatric medicine, have started to quantitatively define how differences and shifts in family dynamics affect a wide range of outcomes, including behavior, emotional balance, physical and mental health, and child development. Their work has identified factors such as structure, composition, and stability, as well as the quality and quantity of familial interactions, as important regulators of a wide range of behavioral and developmental outcomes in children and adults. From a psychological perspective, an individual's experience of family life can significantly influence how that individual experiences almost everything else. Yet little is known about the neurobiological factors that mediate this influence. Identifying which aspects of human family life affect behavioral and psychological outcomes may suggest new approaches for the use of animal models to help fill this gap.

HUMAN FAMILY RELATIONSHIPS

The dynamics and structure of human families

There are many ecological (Low, 2007), social (Schuiling, 2003), legal (Low, 2007) and resource (Fortunato and Archetti, 2010) based pressures that sculpt the great diversity of human mating and family structures, but they all depend on the ability of

both males and females to form long-term selective social bonds with related (e.g., parents, offspring) and unrelated (e.g., mates) conspecifics, as well as the dual maternal and paternal interest in the care of offspring (Low, 2007). The exercise of these abilities creates a family environment that is far more complex than the family environment of most uniparental species. Furthermore, it creates environments in which multiple types of relationships must be maintained simultaneously. Thus, the quality of one type of relationship (e.g., mother-father) can significantly influence the quality of others (e.g., father-child or sibling-sibling) in a dynamic fashion. This integrated social network has been likened to an organism in which the mental health and behavior of individual members (or cells) cannot be fully understood except in the context of the whole system (or organism)—a system that exists in dynamic equilibrium (Cox and Paley, 1997). With this perspective, adding, removing, or altering the quality of any of the selective family bonds could have a profound effect on the family's dynamic equilibrium and therefore on the development, behavior, and well-being of each member of that family. Indeed, this hypothesis has been at the heart of an enormous body of research.

Below I illustrate three types of family dynamics and how they affect outcomes in humans: adult-adult selective social bonds, family structure and child outcomes, and the quality of maternal and paternal care on child development. I then turn to a survey of the animal models that have, or may, provide insight into the neurobiological factors that mediate these effects.

Adult-adult selective social bonds and their impact on adult outcomes

Human families often begin with the coupling of two unrelated individuals. In addition to the joy and euphoria of romantic love (Fisher, 2004), there are important changes in behavior that accompany this coupling. Couples become more exclusive in their social interactions and couples spend much of their time together. Often, couples eventually settle into a cooperative social (family) bond such that the two are, in many respects, no longer independent (Sbarra and Hazan, 2008). The depth of this interdependence is perhaps most vividly observed when the bond is disrupted by death, abandonment, or divorce. The effects of bereavement on behavior and mental health are often dramatic and can occasionally turn pathological (Bowlby, 1982; Biondi and Picardi, 1996; Shear and Shair, 2005; Sbarra and Hazan, 2008).

Bereavement is a common, highly disruptive experience that usually leads to what writers, poets, and artists have long depicted as the "broken heart" (Biondi and Picardi, 1996). It affects everyone, regardless of socioeconomic status, age, sex, or ethnicity. The loss is crushing and is met with denial and disbelief. Bereaved individuals become socially withdrawn and display searching behaviors. With time, bereaved individuals are overcome with acute anguish, somatic distress, and yearning; social withdrawal and impaired motivation become more pronounced (Zisook and Shuchter, 1985; Biondi and Picardi, 1996; Bonanno, 2004; Maciejewski et al., 2007). A month or two after the event, many bereaved individuals begin to rebalance emotionally and reorganize behavioral patterns, forming new social bonds and finding new activities that provide enjoyment. Unfortunately, between 20-40% of bereaved individuals do not. Instead, they suffer from extended bouts of bereavement-induced depression (Biondi and Picardi, 1996; Zisook et

al., 1997; Shear and Shair, 2005) and/or complicated grief (Prigerson et al., 1995b; Prigerson et al., 1995a; Shear and Shair, 2005). These pathological forms of grief can still be present two years after the initial loss, and the effects can compound. For instance, the occurrence of bereavement-induced depression increases the likelihood of recurrent bouts of major depression, as well as deterioration of physical health over time (Prigerson et al., 1995b; Prigerson et al., 1995a; Zisook et al., 1997; Shear and Shair, 2005). Interestingly, these pathological forms occur at higher frequency in younger individuals and are more pronounced in men (Biondi and Picardi, 1996).

A similar, but more complicated, story emerges for persons who have experienced abandonment, separation, or divorce. Humans end or abandon relationships for a variety of reasons, and in some cases they do so to protect themselves from continued emotional or physical harm, but the general findings suggest that, on average, the untethering process has long-term mental and emotional health consequences. Divorced and nondivorced individuals often exhibit marked differences in levels of happiness, psychological distress, well-being, health factors, number of negative life events, and even mortality that are not explained by pre-divorce issues such as antisocial personality traits, depression, or a history of psychological problems (Amato, 2000). It is important to note that these outcomes do not pertain to every situation or individual, but coupled with the bereavement data, they suggest that shifts in family structure can play an important role in the regulation of behavior and mental health (Sbarra and Hazan, 2008). Unfortunately, little is known about how these changes in family life are altering neurobiology, nor how they are interacting with potential risk factors for disease and psychopathology.

Family structure and child outcomes

Evidence supporting a modulatory influence of family life on behavioral and mental outcomes is even more pronounced when differences in family structure and interactions are studied in relation to the development of children. Newborns and young children pass through several developmental "critical periods" (from language (Huttenlocher, 1998) to weight gain (Cottrell and Ozanne, 2008) to affective regulation (Steinberg, 2005)) within the social context of family life. While children demonstrate a high degree of biological robustness (i.e., some sort of mental, cognitive, and emotional development occurs regardless of environment), their development may be particularly sensitive to differences and shifts in family environment.

Over the last 50 years, there has been a dramatic shift in the prevalence of a wide range of family systems. While the "traditional" (mom, dad, and the kids, forever) family structure dominated the demographic charts in the earlier parts of the 20th century, divorce, separation, out-of-wedlock births, adoption, remarriage, step-families, gay parents, and grandparent-headed homes have all become more common (Schor, 2003). In fact, a few may unseat the "traditional" family as the most common family type. For example, in the US, nearly 50% of all marriages are dissolved or disrupted during child-bearing and child-rearing years due to separation and divorce (McLanahan and Sandefur, 1994; Bumpass et al., 1995; Gottman, 1998; Joshi et al., 2003). Practically, this results in fractured, non-cohabitating families, single-parent-headed homes, and second marriages that are occasionally accompanied by step-siblings. Interestingly, second marriages are dissolved at an even higher rate than first marriages (Bumpass et al., 1995). Out-of-

wedlock reproduction has also become more prevalent (McLanahan and Sandefur, 1994; Gottman, 1998; Schor, 2003). In the US, nearly 40% of all births now occur outside of marriage, with rates as high as 72% in some ethnic populations (Hamilton et al., 2009). This trend results in a large number of single-parent families from the time of birth (Joshi et al., 2003; Schor, 2003), and greatly influences the degree of paternal involvement and maternal child-rearing attitudes (Brunelli et al., 1995).

Many investigators have studied the impact of these changes in family life and found differences in child outcomes. For example, children of divorced and nondivorced families differ significantly in such domains as academic achievement, psychological adjustment, behavioral conduct, social competence, and even long-term health (McLanahan and Sandefur, 1994; Amato, 2000). Similar differences are seen between children reared in two-parent versus single-parent homes (McLanahan and Sandefur, 1994; Schor, 2003). While not without criticism, the effects are generally stable and consistent across a broad range of studies from several different decades (McLanahan and Sandefur, 1994; Amato, 2000; Schor, 2003). Qualitatively, many of investigators have concluded from the available data that children of single-parent and divorced families have, on average, worse outcomes than children of stable two-parent homes (e.g., increased teen pregnancy, higher school drop-out rate, lower rates of normal behavioral and social competence, etc.). This is perhaps most clearly stated by EL Schor and the Task Force on the Family: “The evidence is overwhelming that, in general, children do best when they are living with 2 mutually committed parents who respect and support one another, who have adequate social and financial resources, and who both are actively engaged in their upbringing” (see Schor, 2003, pg. 1545). While such qualitative

assessments are important for public policy and ultimately for the identification of children who need intervention, my interest is more on the simple finding that differences and shifts in family dynamics result in different outcomes. Such differences suggest a differential development and/or regulation of neurobiological systems that could be assessed using animal models, where qualifications of better or worse are less clear (e.g., Meaney, 2001).

Maternal and paternal investment and child outcomes

Child development is also affected by parental investment and quality of care. Offspring of biparental species, such as humans, receive care from two independent sources that make distinct contributions. Just as there is variation in family structure, there is a wide spectrum in the degree and quality of maternal and paternal care.

At one end of this spectrum is the presence or absence of physical and emotional abuse. The effects of abuse on the mental and physical development of children are profound. Even discounting the physical dangers, children who are emotionally, sexually, or physically abused exhibit higher incidences of major depression, post-traumatic stress disorder (PTSD), attention-deficit/hyperactivity disorders, and other behavioral problems (Heim and Nemeroff, 2001; Sanchez, 2006). There is also a greater risk of suicide and physical disease such as obesity and diabetes (Heim and Nemeroff, 2001; Meaney, 2001). Less extreme variations in parental investment also modulate emotional and behavioral outcomes.

Paternal absenteeism and authoritarian (or demanding) paternal discipline both increase aggressive tendencies in male offspring (Dubowitz et al., 2000; Kentner et al.,

2009), as well as eating disorders, borderline personality disorders, and self-esteem issues in daughters (Frank and Paris, 1981; Zanarini and Frankenburg, 1997; Maccoby, 2000; Zanarini and Frankenburg, 2007; Enten and Golan, 2009). Likewise, maternal neglect and overprotection result in increased occurrences of behavior problems, psychological maladjustments, and adulthood risk for psychological disorders such as depression (Leckman and Herman, 2002). In contrast, maternal and paternal affection and involvement are related to increased cognitive competence, psychological adjustment, emotional problem solving, and self-regulation across a broad range of socioeconomic backgrounds (Cabrera et al., 2000; Maccoby, 2000; Meaney, 2001; Leckman and Herman, 2002; Flouri and Buchanan, 2003; Flouri, 2008; Flouri and Tzavidis, 2008; Enten and Golan, 2009; Kentner et al., 2009).

It is important to note that differences in behavioral and mental outcomes are mediated by both parents. In many respects, basic social and emotional needs can be met equally well by either parent, but they are not completely redundant (as ME Lamb has argued about paternal involvement (Lamb, 2004)). Each parent fills a parenting niche that makes a unique contribution to child development (Cabrera et al., 2000; Flouri and Buchanan, 2003; Lamb, 2004). There is also the contribution of parental cooperation—how the two parenting styles mesh to create a "united front". Behavioral and emotional disunity has been tied to differences in the level and quality of parental attachment of offspring (Gable et al., 1994; Barnett et al., 2008), suggesting that parental cooperation could account for some child outcomes better than either examining parent alone.

Overall, estimates suggest that family structure and parenting variables account for between 20% to 50% of the variance in child outcomes (Maccoby, 2000). These

family variables interact with genetic factors, and many investigators have noted the importance of appropriate parenting strategies to counteract genetic predispositions to behavior problems (Maccoby, 2000). Likewise, poor-quality parenting can exacerbate genetic risks. For example, abuse and negelect are known to exacerbate the genetic effect of the short-form polymorphism of the serotonin transporter For example, abuse and negelect are known to exacerbate the genetic effect of the short-form polymorphism of the serotonin transporter gene on depressive outcomes (e.g., Caspi et al., 2003).

Understanding families as networks

Together, these three sections illustrate and support the notion that families are dynamic social networks (Cox and Paley, 1997) have important behavioral and developmental consequences. Unfortunately, our understanding of which brain systems mediate the effects of family life on behavior and mental balance is sparce at best. One of the most promising approaches to gaining a fuller understanding of the relevant neurobiology is through the use of rich, well-developed mammalian models that recapitulate to some degree the dynamics of human family systems.

BEHAVIORAL AND NEUROBIOLOGICAL INSIGHTS FROM TRADITIONAL ANIMAL MODELS

The use of traditional rodent and nonhuman primate models such as rats (*Rattus norvegius*), mice (*Mus musculus*), and rhesus macaques (*Macaca mulatta*) have provided remarkable insight into how diversity in family life affects neurobiology and behavior

throughout the life-span. However, based on their predominantly uniparental family structure of these animal models, they have also had their limitations.

Maternal effects on the development of offspring behavior

Rodent handling and maternal separation

Investigators employing traditional, uniparental animal models to study the effects of early life experience have focused on mother-infant family interactions and noted the importance of the mother-infant relationship in humans. In rodents, some of the most studied family manipulations have involved periods of handling (brief 15 min daily maternal separations), non-handling (no human interaction during the preweaning period), extended maternal separation (MS; 3 to 6 hours daily) and maternal deprivation (24 hour periods without maternal contact) (Pryce and Feldon, 2003). The results have been impressive (Lehmann and Feldon, 2000; Meaney, 2001; Pryce and Feldon, 2003; Millstein and Holmes, 2007). Comparisons across groups reveal a wide-range of long-term effects on offspring. For example, maternal separation and deprivation increases acute stress response (e.g., puff of air, restraint, or acoustic startle), general anxiety (in the elevated plus maze and open field), and drug addiction susceptibility, as well as induces abnormal latent inhibition and decreased learning and memory (for review, see (Lehmann and Feldon, 2000; Meaney, 2001; Pryce and Feldon, 2003; Plotsky et al., 2005; Kosten et al., 2006; Millstein and Holmes, 2007)). While there are inconsistencies in magnitude of the effects and the appropriateness specific groups as controls, the findings seem to have some general relevance to situations of human maternal neglect,

since similar types of outcomes are characteristic of human children that have been neglected.

Nonhuman primate maternal abuse

Another approach has been to observe the effects of naturally occurring child-abuse in nonhuman primates (Maestripieri and Carroll, 1998; Sanchez, 2006). In humans, child abuse occurs at relatively high rates (2-10%) and it tends to occur in pedigrees. A similar phenomenon, with strikingly similar rates of occurrence and association with matrilines, has been described in rhesus macaques. In situations of intermingled abuse and neglect, rhesus macaque mothers reject, drag, and hit their young, which greatly alters their growth and development patterns of the offspring (Sanchez, 2006). Abused offspring often exhibit abnormal socioemotional development in the form of increased signs of distress and irritability, as well as delayed social development (Maestripieri and Carroll, 1998; Sanchez, 2006), that have several noted parallels in humans (Heim and Nemeroff, 2001; Sanchez, 2006).

Rodent neglect and nonhuman primate abuse experimental systems are helpful, but these types of familial interactions are at one extreme in the diversity of family life. This has induced some investigators to ask: what are the effects of less severe treatments on the development of offspring? Moreover, how do social interactions at one level of the family network influence the nature and outcomes of other interactions? In macaques, research projects aimed at addressing these questions are still in their infancy. In rodents, investigators have already gained some insight using subtler approaches to test the effects of family life on long-term outcomes. Two strategies have been particularly productive.

The consequences of subtler variations in maternal care

One strategy examines natural variations in maternal care and compares the effect of high- versus low- licking and grooming and arch-backed nursing (LG-ABN) rat mothers on offspring development (for review, see Meaney, 2001). The other approach manipulates the family environment of mice by increasing the absolute number of caretakers through single-mother or communal rearing (Sayler and Salmon, 1969; Branchi and Alleva, 2006; D'Andrea et al., 2007; Branchi, 2008; Curley et al., 2009). In both situations, pups receive adequate maternal care, with no exposure of active abuse (e.g., being stepped upon, handled roughly, etc.), yet each situation produces robust, quantifiable differences in the quantity of social contact and care pups receive.

Comparisons of offspring reared under high- versus low- LG-ABN conditions reveal striking differences in behavioral responses to stressful and anxiety-inducing situations, play behaviors, and even maternal care toward the next generation (Meaney, 2001; Parent and Meaney, 2008). These data are particularly striking because cross-fostering can completely reverse the effects, indicating sociobehavioral mode of intergenerational phenotypic transmission rather than a strictly genetic mode (Meaney, 2001). Some investigators have noted procedural concerns—for example, about the whether the type of cross-fostering (partial, rather than whole litter; see Pryce and Feldon, 2003) are appropriate and the possibility of genetic segregation—but the conclusions are clear: subtler variations in mother-pup interactions have long-term consequences.

Altering the complexity of the family environment, in the case of communal versus single-mother rearing in mice, also has significant long-term effects on offspring

development. While early studies showed modest differences in maturational trajectories (Sayler and Salmon, 1969), more recent findings have emphasized significant differences in anxiety, depressive-like, and sociobehavioral domains. Communally reared mice exhibit higher levels of thigmotaxis (or "anxious" wall-hugging) in the open-field test for anxiety, increased "depressive-like" immobility behavior in the forced swim test for depression, and increased rates of social interaction and hierarchy establishment, in comparison to single-mother reared counterparts (Branchi, 2008; Curley et al., 2009). In the case of both high/low LG-ABN rats and communal/single-mother rearing in mice, these behavioral effects can be perpetuated across generations (Meaney, 2001; Branchi, 2008; Curley et al., 2009).

Whether the product of abuse, neglect, or variations in maternal care, there is a clear consensus that differences in or changes to the mother-offspring family relationship matters in the long run. As in humans, the data from mammalian models suggest that family life exerts a long-term influence by nudging offspring to one or the other end of the behavioral spectrum and that in some cases the effect can be extreme, resulting in a pathological behavioral profile.

Maternal effects on the developing brain

As helpful as animals have been in allowing experimental manipulation and observation throughout the life span—in some cases across multiple generations—perhaps the greatest advantage of these animal models lies at the level of the brain. At this level, investigators have started to connect the dots between family environment and behavioral outcomes through specific neural systems.

Regulation of the HPA axes

Broadly understood, stress is a perturbation of homeostasis. Many early investigators of familial manipulations predicted that, since each mammalian species tended to exhibit species-typical forms of familial interactions in natural and laboratory environments, perturbations to these forms would "communicate" different levels of environmental stress that may exert long-term effects on the developing stress axes of offspring (Meaney, 2001). Since stress axes were known to regulate emotional, social, and cognitive functions in adulthood, they appeared were well-positioned to connect differences in early environment with differences in the behavioral outcomes of offspring. Indeed, their predictions proved fruitful. In the case of both macaques and humans, abuse leads to a significant dysregulation of several stress-related neuroendocrine factors—increased hypothalamic production of cortiotropin-releasing factor (CRF), altered adrenocorticotropin hormone (ACTH) release, and increased basal, circulating levels of cortisol (or corticosterone in rodents; CORT) accompanied by a flattening of the normal circadian cycle (Heim and Nemeroff, 2001; Meaney, 2001; Sanchez, 2006). Similar neuroendocrine changes have been observed in MS rats (Meaney, 2001; Pryce and Feldon, 2003; Plotsky et al., 2005). In rat studies, investigators also had relatively easy access to the brain where they found increases in CRF receptor densities in the paraventricular nucleus of the hypothalamus (PVN), locus coreuleus (LC), and frontal cortex—brain regions that mediate both HPA and sympathetic responses to stressors (Plotsky et al., 2005).

Importantly, the brain's response to stress is not unidimensional; it must also mediate a return to homeostasis once the stressor has passed. CORT acting at glucocorticoid receptors (GR) is one of the central elements in this process. MJ Meaney, PM Plotsky, and others have described several manipulations of early family environment, including natural variations in LG-ABN maternal behavior, that significantly alter GR expression densities in regions, such as the hippocampus, that are important for this negative feedback (Meaney, 2001). Rats from high-LG-ABN mothers have higher hippocampal GR densities and tighter control of HPA axis function compared to low-LG-ABN counterparts (Meaney, 2001). Interestingly, these effects are mediated by epigenetic modifications of the GR gene and subsequent changes in transcriptional efficiency—epigenetic modifications that have been found not only in rats, but also recently in humans (Meaney, 2001; Weaver et al., 2004; Szyf et al., 2007; McGowan et al., 2009). Again and again, the HPA stress axis has been identified as an important biological mediator of family experiences on behavioral, emotional, and neurobiological outcomes in offspring.

Other brain systems

The HPA axis is not the only system modified by early family interactions. Abuse interacts with the serotonin (5-HT) system to mediate long-term psychopathological responses in humans (Caspi et al., 2003) and monkeys (McCormack et al., 2009). In rats, MS treatment increases frontal 5-HT concentrations as well as several 5-HT receptor subtypes; it also decreases in alpha2-adrenoreceptors in the locus ceuroleus, by which norepinephrine modulates its own release (Holmes et al., 2005). Likewise, GABAergic

receptors exhibit differences in subunit composition and sensitivity (Meaney, 2001). Dopamine receptors show little change in response to rodent MS, but it does increase dopamine transporter binding in the striatum, including the nucleus accumbens (NAcc). This change in dopamine transporter in the NAcc may help explain increased addiction susceptibility observed in MS treated animals (e.g., Meaney et al., 2002; Kosten et al., 2006).

Factors that regulate the growth and maintenance of brain circuits are also modified. Branchi and colleagues have found that the socially enriched environment of the communal nest alters a wide range of neurotrophic growth factors, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF; Branchi, 2008). Interestingly, this effect is not mediated entirely by the presence of more mothers; it depends also on increased peer-peer interactions as well (Branchi, 2008).

Differences in mother-infant interactions modify brain systems that directly regulate maternal and social behaviors as well. In mice, FA Champagne and colleagues have found epigenetic modifications of the estrogen receptor alpha gene that are mediated by maternal LG-ABN (Champagne and Curley, 2008). These modifications alter gene expression and thus estrogen sensitivity, ultimately modifying adult maternal behavior toward offspring. The effects of early life experience on brain development extend to socially- and parentally-relevent neuropeptides as well. Oxytocin (OT) and arginine-vasopressin (AVP) are nonapeptides that act centrally through the oxytocin and vasopressin 1a receptors (OTR and V1aR), respectively (Young and Wang, 2004; Donaldson and Young, 2008; Ross and Young, 2009). Variations in LG-ABN alter the long-term expression densities of these neuropeptide receptors in rats (Francis et al.,

2000; Francis et al., 2002), and human women who have been abused show altered peripheral OT concentrations (Heim et al., 2009). The OT and AVP systems are of special interest because their role in modulating species-typical social behaviors appears to be evolutionarily conserved across a wide-range of species (Donaldson and Young, 2008). Moreover, they regulate behaviors such as selective bonding, maternal care, and paternal care, all factors that are central to the formation and maintenance of family life. Indeed, if the OT and AVP systems are consistently susceptible to differences in early family life—a highly social environment—these systems may help explain some of the adult differences in social behavior (e.g., play, social hierarchy, social withdrawal, etc.) that have been noted by a number of investigators in rats and mice (Meaney, 2001; Branchi, 2008; Parent and Meaney, 2008). Since social behavior lies at the heart of maintaining a functional family system, a deeper understanding of how differences and shifts in the family environment affect human social and family behaviors may come from a more thorough analysis of these two neuropeptide systems. Such investigations, however, may be limited in traditional animal models due to a uniparental family structure.

Limitations

The findings made in traditional animal models have provided invaluable insight into the neurobiological changes that can occur in response to differences in family dynamics, but the focus has been relatively narrow. Mother-infant interactions are no doubt a core feature of human family life, but it is not the only feature. As illustrated previously, family members at all stages are influenced by a wide variety of family

factors, including the adult pair bond, paternal care, and parental coordination. Traditional animal models can provide little insight into the effect of these factors, since they cannot be used to test the effects of disrupting selective social bonds or how coordinated biparental care of offspring can affect development. They also lack the paternal dimension. To gain a fuller understanding of how differences and shifts in family dynamics—factors that occur much frequently than abuse and neglect—an alternative, but complementary approach, seems necessary.

A COMPLEMENTARY APPROACH: NON-TRADITIONAL ANIMAL MODELS OF FAMILY SYSTEMS

As noted at the outset, selective social attachments and biparental care of offspring are rare traits among mammalian species (Kleiman, 1977; Clutton-Brock, 1989). Most of these species, if they have been studied in depth at all, have been studied predominately in the context of population dynamics or for insight into the evolutionary pressures that drive differences in social organization. A few investigators, however, have started to look beyond these naturalistic investigations and are now using a hand full of these species—particularly from the rodent order—to answer questions related to the neurobiology of selective bonding, paternal care, and family dynamics.

Expanding our view of selective family bonds

Similar to humans, family life for species capable of social monogamy begins with the sociosexual coupling of two mates (Kleiman, 1977; Fernandez-Duque et al., 1997; Fisher, 2004). This pairing induces a variety of behavioral changes, such as mate-

directed grooming, tail entwining, nest sharing, and mate guarding, that have obvious parallels in humans (Mendoza and Mason, 1986; Carter et al., 1995; Fernandez-Duque et al., 1997; Young and Wang, 2004). This pair bonding is, in a sense, the beginning of family life for these species.

Perhaps the best studied rodent model of pair bonding is the monogamous prairie vole (*Microtus ochrogaster*; Getz et al., 1981; Carter et al., 1995; Getz and Carter, 1996; Young and Wang, 2004; Donaldson and Young, 2008; McGraw and Young, 2009). After pairing, prairie voles exhibit rapid (~24 hr) onset of quantifiable partner preferences (Williams et al., 1992a; Williams et al., 1992b; Carter et al., 1995; Young and Wang, 2004; **Appendix 1**). Pharmacological manipulations of these preferences have clearly demonstrated the importance of several neuromodulatory systems, including OT, AVP, dopamine, and even CRF, for the initiation of bond formation (Aragona et al., 2003; Young and Wang, 2004; Aragona et al., 2006; Lim et al., 2007; Donaldson and Young, 2008). Likewise, specific brain regions have also emerged as important players, including the lateral septum, NAcc, and ventral pallidum (Young and Wang, 2004). Situated in the central and ventral forebrain, these regions are well known for their roles in reward and reinforcement behaviors, supporting claims that selective social bonding is driven in part by the rewarding nature of the bond (Fisher, 2004; Young and Wang, 2004). The involvement of these systems in selective bonding is not exclusive to monogamous rodents. Monogamous titi monkeys (*Callicebus moloch*) show high activity in the NAcc after being paired (Bales et al., 2007a), and intra-nasal oxytocin induces greater partner-seeking during a partner preference test in the monogamous black-pencilled marmosets (Smith et al., 2010).

Once paired, prairie voles undergo a gradual shift in behavior that results in increased aggressiveness toward unfamiliar, opposite-sex conspecifics, which helps maintain the bond (Aragona et al., 2006). Interestinlgy, this shift has been associated with changes in the density of dopamine receptors throughout the striatum. It is likely that many of these systems, pathways, and changes are occurring in other species capable of selective social bonds between mates, such as humans.

But what happens when the bond is disrupted? Do bonded mammals, such as prairie voles, show behavioral and physiological distress in response to partner loss in a way that may parallel human greiving? If so, might they be used to study the neurobiology of shifts in partner availability, potentially approximating bereavement in human couples and families? Unfortunately, only a few investigators have attempted to address these questions.

In the mid-1980s, JN Crawley identified a "separation syndrome" in monogamous Siberian dwarf hamsters (*Phodopus sungorus*). Male hamsters separated from a female partner exhibited marked increases in food consumption and body weight, as well as decreases in social interactions and exploratory behavior (Crawley, 1984b, 1984a, 1985). Noting behavioral and physical parallels with human depression, Crawley treated the animals with antidepressants, finding some more effective (tranylcypomine) than others (monoamine oxidase inhibitors [MAOIs]) in reversing these responses (Crawley, 1984b, 1984a, 1985). A follow up to this work a decade later revealed that these separation profiles are accompanied by increased HPA axis function (i.e., higher corticosterone) and decreased sympathetic tone (i.e., decreased peripheral norepinephrine; Castro and Matt, 1997). Interestingly, human subjects experiencing pathological grieving and

bereavement-induced depression have long-term increases in HPA axis activity (Biondi and Picardi, 1996) and the antidepressants used to treat abnormal bereavement exhibit varying degrees of efficacy (Hensley, 2006). To our knowledge, no one has investigated the neurobiological changes resulting from dwarf hamster partner loss, nor the specific brain regions involved.

Responses to partner loss have also been noted in prairie voles, but here the data are even less developed. Prairie voles separated from their partners for 24 hrs had increased plasma CORT levels, which decrease to baseline when reunited (reviewed in Carter et al., 1995).

In monogamous titi monkeys, a 1 hr forced mate separation elicits significant increases in behavioral agitation (vocalizations and hyperactivity; Mendoza and Mason, 1986). Longer separations have been performed to test pair bond durability, but unfortunately no behavioral measures were taken during the time of separation (Fernandez-Duque et al., 1997).

Considering the wealth of human literature demonstrating the dramatic changes to behavior and mental state that accompany family bonding and bereavement (Zisook and Shuchter, 1985; Prigerson et al., 1995b; Prigerson et al., 1995a; Biondi and Picardi, 1996; Zisook et al., 1997; Bonanno, 2004; Shear and Shair, 2005), a more vigorous interest in these animal models may prove extraordinarily useful for biological psychiatry. They may help identify the brain systems that are altered by the formation of selective social bonds, and how these systems become unbalanced—sometimes pathologically—in response to the addition, loss, or shift in such a bond. It may also help our understanding

of how dramatic shifts in the relationship between partners can influence the behavior and mental health of all the members of a family.

Expanding our view of family relationships

As promising as the use of non-traditional animal models might be for understanding the neurobiology of behavioral and emotional changes that accompany the formation, maintenance, and disruption of pair bonds, there is perhaps even greater promise in the examination of how a richer set of family factors, such as parental coordination, paternal care, and family structure can affect each member of the family unit, particularly offspring.

As noted previously, biparental rearing creates an inherently complex social network. But do animals capable of monogamy and biparentism in fact display a rich set of family interactions? Behavioral ecologists have characterized family interactions, at least to some degree, in a wide range of monogamous and biparental species, including prairie voles (*Microtus ochrogaster*; Hartung and Dewsbury, 1979; Getz et al., 1981; Wang and Novak, 1992; Solomon, 1993; Getz and Carter, 1996; McGuire et al., 2007), social voles (*Microtus socialis guentheri*; Libhaber and Eilam, 2004), pine voles (*Microtus pinetorum*; Oliveras and Novak, 1986), mandarin voles (*Lasiopodomys mandarinus*; Tai and Wang, 2001; Tai et al., 2001; Smorkatcheva, 2003), California mice (*Peromyscus californicus*; Gubernick et al., 1993; Bester-Meredith et al., 1999; Gubernick and Teferi, 2000; Frazier et al., 2006), Mongolian gerbils (*Meriones unguiculatus*; Wynne-Edwards and Reburn, 2000; Wynne-Edwards and Timonin, 2007; Gromov, 2009), Siberian dwarf hamsters (*Phodopus sungorus*; Crawley, 1984b; Wynne-Edwards and Reburn, 2000; Wynne-Edwards and Timonin, 2007), trumpet-tailed rats

(*Degus octodon*; see Wilson, 1982; Ovtscharoff et al., 2006), titi monkeys (*Callicebus moloch*; Mendoza and Mason, 1986; Fernandez-Duque et al., 1997) and marmosets (e.g. species in the genus *Callithrix*; Ziegler, 2009; Smith, 2010). While some have made greater in-roads as biomedically relevant animal models than others, each one has the potential to offer generalizable mechanistic insight into the neurobiology of family dynamics, as well as how differences and shifts in family dynamics change behavior, emotional regulation, and vulnerability to psychopathology. They offer not only the ability to manipulate and observe differences in a greater number of family situations, they can also be used to test the effects family manipulations on social cognition and social behavior—deficits that are found in a number of psychopathologies (McGraw and Young, 2009).

As with pair bond disruption, few systematic investigations of the effects of biparental family structure, composition, and interactions in relation to behavioral, emotional, and developmental outcomes have been attempted from a biomedical perspective. This is especially surprising considering the plethora of natural variations in family structure in wild-populations that seem to parallel human family structures. For example, in wild prairie vole populations, offspring are often reared under three conditions: communal groups, biparental pairs, and single-mothers (Getz et al., 1992a; Getz and Carter, 1996). Moreover, in the studies that have examined differences in family structure or paternal influence, investigators have found significant effects on offspring development and behavior. For example, in semi-natural environments, Z Wang and M Novak found that single-mother (SM) reared offspring exhibit differences in alloparenting (babysitting) behavior as juveniles (Wang and Novak, 1992)—although this

may have been confounded by the continued presence or absence of the father during juvenile alloparental observations. In pioneering work in the monogamous California mouse by Marler and colleagues, direct paternal care, in the form of pup retrievals, was found to influence the development of aggressive tendencies in male offspring (Bester-Meredith et al., 1999; Bester-Meredith and Marler, 2003; Frazier et al., 2006), while removal of these fathers alters the performance of adult offspring in at least two cognitive tasks (Bredy et al., 2004).

More recently, a few investigators have started identifying which neural systems may be influenced by family structure and paternal investment, and how these variations may drive variation in the adult behavior of offspring. Marler's team has examined how paternal behavior and early family environment regulate AVP expression and adult aggression. In trumpet-tailed rats (*Degus*), pups that are SM-reared exhibit differences in spine densities and dendritic arborization not only in the somatosensory region, but also in the orbitofrontal cortex—a region of the brain in humans that regulates executive function and future planning (Ovtscharoff et al., 2006).

Offspring, of course, are not the only ones influenced by differences in family structure; adults also respond to changes in family life. KE Wynne-Edwards and others have spent several years examining on how males within monogamous species change in response to pairing and the birth of offspring (Wynne-Edwards and Reburn, 2000; Wynne-Edwards and Timonin, 2007). While effects found in rodents have not always been perfectly recapitulated in humans (such as testosterone fluctuations), these types of studies using nontraditional animal models have helped identify endocrine and neural systems that can be the focus of future human research.

Indeed, whether the effects are in infants, fathers, or mothers, the findings born from research in nontraditional animal models are likely to have a two-fold impact. Since most of these species are outbred they will have ethological relevance; for example, the studies may help explain how diversity in family structure leads to greater behavioral diversity, which in turn informs adaptive significance. But there is also the potential impact on biomedical and psychological research. Identification of brain mechanisms that drive and mediate the effects of family life on adult social, behavioral, emotional, mental, cognitive, and physical well-being may suggest important avenues of human study and intervention.

Cautious Optimism

Caution, of course, must be taken that studies in "monogamous" species are not over-interpreted or excessively anthropomorphized. Each species arose to fill a particular environmental niche and must be considered, in a sense, unique. Caution also needs to be taken in how generalizable any particular finding is; for example, as just noted, testosterone fluctuations in the transition to fatherhood is different across biparental species. Moreover, just because there are similarities in the observed behaviors does not necessarily indicate identical—or even similar—mechanisms. Still, caution should not negate the potential benefits. Rats, mice, macaques and humans have their points of convergence; they also have their points of divergence, many of which are often overlooked. Yet situated in a framework of mammalian evolution, mechanistic studies in these traditional animal models have yielded enormous insight for our understanding of human development and disease.

The same is likely to be true of monogamous species of voles, mice, gerbils, and monkeys. Despite substantial face validity, they will diverge on specific points. But they will also have important points of convergence. It is at these points that tremendously valuable information is likely to be garnered. The importance of this perspective should not be overlooked. Prairie voles are used far less often than most traditional animal models. Yet insights from research in these highly social animals are making important contributions to studies in humans. Built upon a solid foundation of evolutionary conservation of the OT and AVP neuropeptide systems (Donaldson and Young, 2008), findings such as the pro-bonding effects of OT in female prairie voles and the association between sociality and variation in *avpr1a* gene promoter microsatellite in male prairie voles, have driven new basic and clinical research in humans that relates to social cognition, human bonding, and even social deficits in autism (Heinrichs et al., 2003; Donaldson and Young, 2008; Walum et al., 2008; Guastella et al., 2009; McGraw and Young, 2009; Meyer-Lindenberg et al., 2009; Andari et al., 2010). Finding points of convergence in relation to how family dynamics are influencing behavior, mental health, and vulnerability to psychopathology, may bear similar fruit.

AIMS FOR THIS DISSERTATION

This thesis aims to develop prairie voles (*Microtus ochrogaster*; **Figure 1.1**) as an animal model that can be used to study how differences and shifts in biparental family dynamics affects behavior, emotion, and (potentially) vulnerability to psychopathology.

Figure 1.1 - A prairie vole family
Above is depicted a biparental prairie vole family, consisting of a mother (left), father (right) and five pups.

In **Chapter 2**, I aim to examine how male-female pair bonding can affect behavioral and neurobiological outcomes by studying pair bond disruption in prairie voles. In **Chapter 3**, I propose to establish prairie voles as an informative, nontraditional, animal model of family interactions that can be studied in a quantitative and repeatable manner under laboratory environments and examine the effects of ethologically relevant variations in family structure on parental behavior and pup care. In **Chapter 4**, I examine how the ethologically relevant manipulations of prairie vole family structure established in Chapter 3 can significantly influence the diversity of social and emotional behaviors in adult offspring. In **Chapter 5**, I attempt to establish a neurobiological link between ethologically relevant differences in family structure (observed in Chapter 3) and the adult behavioral differences (observed in Chapter 4) by focusing on variation in the

expression of neuropeptides and neuropeptide receptors that have previously been associated with social behavior in these in animals. In **Chapter 6**, I examine the anatomical relationship between corticotropin-releasing factor receptor 2 (CRF2) and oxytocin (OT), as a possible mechanistic link between stress and sociality that may be relevant to both early life experience and adult social interactions. Finally, in **Chapter 7**, I review the main findings, place these findings within the context of the literature, and propose future experimental projects that will extend this work and further establish prairie voles, and other monogamous, biparental rodents, as a relevant approach to guiding human research.

CHAPTER 2

The effects of pair-bond disruption in monogamous prairie voles

Adapted from:

Bosch OJ, Nair HP, **Ahern TH**, Neumann ID, Young LJ (2008) The CRF system mediates increased passive stress-coping behavior following the loss of a bonded partner in a monogamous rodent. *Neuropsychopharmacology* (34) 1406-1415

PREFACE

The chapter that follows is adapted from Bosch et al. (2009), which was published in *Neuropsychopharmacology* (Bosch et al., 2009). While I am not the first author, I made several important contributions to this paper. I also helped research and write it. The preface serves to distinguish what was completed prior to my entering the laboratory and what as completed by me and with my assistance. I also briefly discuss how participation in this research project served as a foundation for the rest of the research presented in this dissertation.

OJ Bosch and HP Nair conceived the idea to test pair bond disruption in prairie voles (*Microtus ochrogaster*) and began a collaboration. OJ Bosch had already conducted Experiments A-D when I started on the project (see **Figure 2.1**). He had already established the behavioral paradigm and shown several important behavioral and neuroendocrine responses to pair bond disruption. This included passive-coping in the forced swim test and tail suspension test, no change in the elevated plus maze test for anxiety, and increases in plasma corticosterone (CORT). He had also just shown that the passive-coping response could be blocked by intracerebroventricular (icv) infusion to of the non-selective corticotropin-releasing factor (CRF) receptor 1 and receptor 2 (CRF1 and CRF2, respectively) nonselective antagonist, d-Phe-CRF. While this had shown the importance of the CRF system in the onset of a depressive-like profile, it was unclear which CRF receptor was mediating the effect.

This is where I came in. MM Lim had shown that activation of both CRF1 and CRF2 were necessary for CRF-induced partner preference formation after a shortened cohabitation (Lim et al., 2007). Since both receptor types were involved in stress-related

responses to bonding, it was possible that the same situation may hold for bond disruption. To test this, I needed to infuse selective CRF1 and CRF2 antagonists separately and measure their effects on behavior. At that point only the CRF1 antagonist, CP-154,526, had been used in voles. While highly selective in other rodents (Schulz et al., 1996), a selectivity curve had not been generated in vole tissue. Since ventricular infusion results in an unknown concentration in the brain, I needed to know that it would be highly selective over a broad range. We also needed to find a selective CRF2 antagonist and generate a similar selectivity curve. Using receptor autoradiography, I established the specificity of both CP-154,526 (the CRF1 antagonist) and Astressin-2B (the CRF2 antagonist) in vole tissue (**Figure 2.2**).

Following this advance, I planned, organized, and prepared for Experiment E (see **Figure 2.1**). I also helped perform surgeries to implant the minipumps that were attached to the icv cannulae. I also set up video-feeds from the testing room to a video-monitoring room, so that I could rate all the behavior completely blind. (Up to this point, OJ Bosch had a handled all the animals and scored the behavior live. This created the potential for rater bias. The blind scoring added to the objectivity of the analysis and negated any potential concern over accidental bias.) After data collection, I helped write and edit the entire manuscript for publication, providing many of the insights from the human literature based on my extensive work I performed in writing and re-writing an NIH RO1 grant (that we obtained: MH77776). I also helped put together many of the graphical representations of the data.

In addition to the work I put into the manuscript, I also used the process as an opportunity to think about my research interests in general. While prairie voles had been

used to study the neurobiology of selective pair bonding, little work had focused on how these selective social interactions changed the brain. The series of experiments presented in **Chapter 2**, which were spearheaded by Dr. Bosch, had shown that the bonding process had a profound impact and disrupting that bond had important emotional and behavioral consequences. From this work grew my interest in how the social network of the family—and its dynamics—influence behavior, mental state, and potentially vulnerability to psychopathology. The bulk of the work directed and performed entirely by me is presented in **Chapters 3-6**.

ABSTRACT

Social relationships significantly influence physiology and behavior, including the hypothalamo-pituitary-adrenal axis, anxiety, and mental health. Disruption of social bonds through separation or death often results in profound grieving, depression and physical illness. As the monogamous prairie vole forms enduring, selective pair bonds with the mating partner, they provide an animal model to study the physiological consequences of bond formation and bond disruption. Male prairie voles were paired with a novel female or male sibling. After 5 days, half of the males of each group were separated from the partner. Elevated plus maze, forced swim, and tail suspension tests were used to assess anxiety-like and passive stress-coping behaviors. Following 4 days of separation from the female partner, but not a male sibling, males displayed increased passive stress-coping. This effect was abolished by long-term intracerebroventricular infusion of a non-selective corticotropin releasing factor (CRF) receptor antagonist without disrupting the bond itself. Further analysis revealed that CRF type 1 and 2 receptors were both involved in the emergence of passive stress-coping behavior. Furthermore, pairing with a female was associated with elevated CRF mRNA in the bed nucleus of the stria terminalis, and partner loss elicited a pronounced increase in circulating corticosteroid and adrenal weight. We speculate that the CRF system may mediate an aversive affect following separation from the female partner, which may facilitate proximity seeking between the pair-bonded individuals. Hence, the prairie vole model may provide insights into brain mechanisms involved in the psychopathological consequences of partner loss.

INTRODUCTION

There is increasing evidence that social bonds have a positive impact on health and buffer against stressors (Kikusui et al., 2006). In contrast, the absence or sudden disruption of those bonds increases susceptibility to diseases (House et al., 1990; Kirschbaum et al., 1995; Biondi and Picardi, 1996; Uchino et al., 1996; Capitanio et al., 1998; DeVries et al., 2003; Grippo et al., 2007a), including depression (Zisook et al., 1997; Watanabe et al., 2004; Grippo et al., 2007a; Grippo et al., 2007c; Grippo et al., 2007b). In humans, bereavement is a highly disruptive experience that is usually followed by a painful period of acute grief (Shear and Shair, 2005). Thus, understanding the neurobiological consequences of partner loss, particularly with respect to increased susceptibility to depression, may be informative for developing strategies for coping with the loss of a loved one.

Prairie voles (*Microtus ochrogaster*) have served as an excellent animal model for examining the neurobiological mechanisms underlying social bonding (Carter and Getz, 1993; Carter et al., 1995; Getz and Carter, 1996; Aragona and Wang, 2004; Shear and Shair, 2005) and for investigating the physiological consequences of social loss (Grippo et al., 2007a; Grippo et al., 2007c; Grippo et al., 2007b). Unlike 95% of all mammalian species, prairie voles are socially monogamous, forming enduring and selective pair bonds with their mates (Carter and Getz, 1993; Getz and Carter, 1996). While the formation of pair bonds is thought to be based on brain circuitries mediating reward and reinforcement (Aragona et al., 2003; Young and Wang, 2004; Aragona et al., 2006; Nair and Young, 2006), an aversion to prolonged partner separation may help preserve the bond over time by inducing proximity seeking behaviors. Thus, we predicted that even a

short separation from a partner may be aversive and lead to an alteration in emotionality as reflected by increased passive stress-coping or anxiety-like behavior in male voles. In fact, recent publications have shown that 4 weeks social isolation leads to increased passive coping in female prairie voles (Grippo et al., 2007c; Grippo et al., 2007b).

The behavioral consequences of disrupting a pair bond could be associated with both the brain corticotrophin releasing factor (CRF) system and hypothalamo-pituitary-adrenal (HPA) axis. Chronic up-regulation of the brain CRF system is thought to play an important role in the pathogenesis of various psychopathologies, including anxiety and depressive disorders (for review Holsboer and Barden, 1996; Nemeroff, 1996; Keck, 2006). Reduced anxiety- and passive-coping related behaviors have been shown after blocking CRH receptors in rats (Liebsch et al., 1995; Bakshi et al., 2002; Hodgson et al., 2007) and in mice lacking CRH receptors (Behan et al., 1996; Timpl et al., 1998; Kishimoto et al., 2000). Social support reduces the basal activity of the HPA axis as well as cortisol responses to psychological stressors in humans and in other species (Elliot, 1989; Kirschbaum et al., 1995; DeVries et al., 1997; Sachser et al., 1998; Thorsteinsson and James, 1999) and, thus, may also affect well-being (Elliot, 1989; Kikusui et al., 2006) by minimizing long-term exposure to increased levels of glucocorticoids (DeVries et al., 2002).

To examine the consequences of disruption of a pair bond and the potential involvement of the CRF system, we measured passive stress-coping and anxiety-like behavior in male prairie voles following a 3 to 5 day separation from a female partner or a male sibling. We then tested whether separation from a female partner increases plasma ACTH and glucocorticoid levels or brain CRF mRNA. Finally, we investigated the role

of CRF receptors (CRF1 and CRF2) in the behavioral changes precipitated by the loss of a bonded partner.

MATERIALS AND METHODS

Animals

All animals were sexually naïve adult male and female prairie voles (70-100 days of age) from the laboratory breeding colony originally derived from field-captured voles in Illinois, USA. After weaning at 21 days of age, subjects were housed in same-sex sibling pairs or trios under standard laboratory conditions (14:10h light-dark cycle, lights on at 0600 h; 20°C, 60% humidity and free access to water and Purina rabbit chow). All behavioral tests were performed between 0800 h and 1200 h. In an attempt to minimize stress during the experimental paradigm, a careful cage change regimen was followed: animals were only exposed to new cages at the beginning of the pair housing and separation periods. Furthermore, approximately 1/3 of the bedding from an animal's previous cage accompanied it to the new cage. The animal studies were conducted in accordance with the guidelines of the National Institute of Health and were approved by Emory University's Institutional Animal Care and Use Committee.

Experimental protocol

Male voles were paired with either unfamiliar females or male-siblings. In the male-siblings group, only those siblings were paired which were housed separately from each other since weaning (49-79 days without contact). After 5 days, half of the voles of each group were separated. Twenty-four hour of cohabitation with a female – even without mating – is sufficient for the induction of a partner preference, which is a

laboratory proxy for pair bond formation (Williams et al., 1992b). Prairie vole females do not display regular ovarian cycles, but are reliably induced into estrus 24-48 hours following cohabitation with a male as a consequence of exposure to male urine. Females will mate for a 24-hr period during estrus (Roberts et al., 1998a), and mating does not occur after this period. Therefore, the stage of the estrous cycle was not assessed in the females prior to cohabitation with the male. Each vole underwent only one of the experiments A – E. The details for each experiment are illustrated in **Figure 2.1**. We first sought to determine whether disruption of a pair bond in male prairie voles would result in changes in passive stress-coping (Experiment A) and anxiety-related behavior (Experiment B). In Experiment C we investigated whether the pairing and separation alters basal plasma levels of stress hormones. We then determined whether the CRF system is linked to partner loss-induced passive stress-coping by non-selective blocking brain CRF receptors (Experiment D). Finally, Experiment E employed selective CRF1 and CRF2 antagonists to identify which CRF receptor type underlies this link.

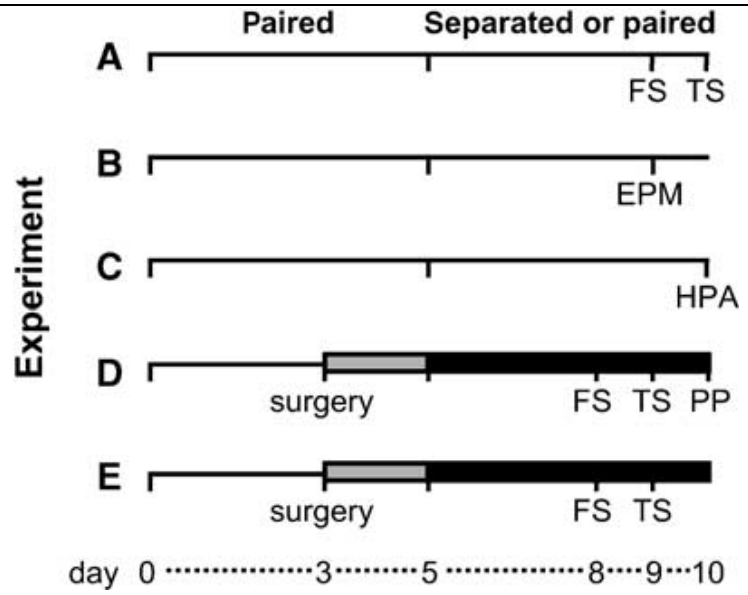

Figure 2.1 - Pair bond disruption testing and manipulation schedules
Testing-schedules of the five experimental approaches (**A** - **E**). Male prairie voles were co-housed and allowed to form a partner bond. After 5 days, half of the males of each group were separated from the partner; animals from paired groups stayed with their partner. On the 9th day after pairing, i.e. the 4th day after separation (**A**, **B**) or on the 8th day after pairing, i.e. the 3rd day after separation (**D**, **E**), testing for behavioral effects started, or voles were left undisturbed until the 10th day after first pairing, i.e. the 5th day after separation in the particular groups, to take blood samples and brains under basal conditions (**C**). In Experiments **D** and **E**, osmotic minipumps were implanted after 3 days of pairing delivering Ringer's solution until day 5 of pairing (grey bars) when half of the animals were separated followed by CRF receptor antagonist lasting until the end of the experiment (black bars). EPM = elevated plus-maze; FS = forced swim test; HPA = hypothalamo-pituitary-adrenal axis; PP = partner preference test; TS = tail suspension test.

Forced swim test (FST). Male voles were exposed to the FST in a single test session as this has been shown to reveal differences in passive stress-coping in rats (Liebsch et al., 1999; Overstreet et al., 2004; Overstreet et al., 2008) and mice (Borsini and Meli, 1988; Oshima et al., 2003; Cryan and Mombereau, 2004; Cryan et al., 2005). Animals were forced to remain for 5 min in a 4L glass beaker (15 cm in diameter) filled to a height of 20 cm with tap water (23 ± 1°C). The behavior of the voles was scored by a

trained observer blind to the animals' treatment. The following behaviors were recorded according to our previous studies in rats (e.g., Ebner et al., 2005) using an automatic timer software package (*Stopwatch+*, Center for Behavioral Neuroscience, Atlanta, GA: downloadable at http://www.cbn-atl.org/research/behavioralcore.shtml): (1) struggling, defined as movements during which the forelimbs break the water's surface; (2) swimming, defined as movements of the fore and hind limbs resulting in forward motion without breaking the water surface, including diving, and (3) floating, defined as the behavior during which the animal uses limb movement to maintain its equilibrium without any movement of the trunk.

Tail suspension test (TST). The procedure used was similar to that described by Steru et al. (Steru et al., 1985). Male voles were suspended by their tail using adhesive tape to an aluminium stick (diameter 1 cm) and hung in the middle of a black covered box (40 x 40 x 40 cm^3) approximately 80 cm above the ground. The duration of immobility (passive hanging) during a 5-min test period was recorded using *Stopwatch+* software (see above).

Elevated plus-maze (EPM). In the EPM test, a conflict situation is created between the animal's exploratory drive and its innate fear of open and exposed areas as demonstrated in rats (Pellow et al., 1985; Liebsch et al., 1998) and voles (Insel and Hulihan, 1995; Hendrie et al., 1997; Pitkow et al., 2001). The plus-maze consists of an elevated (height: 100 cm) plus-shaped aluminium platform with two closed (40 cm high walls out of dark PVC, < 20 lux) and two open arms (each 60 x 10 cm, 80 lux), connected at the centre by a neutral zone (10 x 10 cm). Before a vole was placed on the EPM, the surface of the maze was cleaned with water containing a low concentration of a

detergent and dried. The vole was placed in the neutral zone with its head facing a closed arm. The following parameters were recorded with a video/computer system (Plus-maze V2.0, Ernst Fricke, Germany) by a trained observer blind to the animals' treatment during the 5-min exposure according to studies in rats (Neumann et al., 1998; Bosch et al., 2005): (I) percentage of time spent on the open arms vs. total time on all arms, (II) percentage of entries into open arms vs. entries into all arms, and (III) the number of entries into closed arms.

Partner preference test. To reveal whether our pharmacological manipulation of the CRF system might influence the expression of a partner preference after 5-days of cohabitation, we tested the males from Experiment D (non-selective CRF receptor antagonist) in the partner preference test as previously described (Williams et al., 1992b; Winslow et al., 1993). Numbers of cage entries were counted to reveal differences in locomotor activity. On the next day, voles were euthanized and the brains collected for confirmation of cannula placement. The injection site was verified by injecting ink via the implanted brain infusion kit targeting the lateral ventricle, removal of the brain, and a vertical cut through the brain.

Detection of ACTH and corticosterone

Between 0900 h and 1100 h, male voles were briefly anesthetized with isoflurane, and immediately decapitated using scissors within 2 min after removal from the cage. Trunk blood (~ 0.2 ml) was collected on ice in EDTA-coated tubes complemented by aprotinin (10 µl/tube; Trasylol, Bayer AG, Leverkusen, Germany). The blood was centrifuged at 4°C, 5000 rpm for 5 min, and plasma was aliquoted and stored at -80°C

until assay. Plasma ACTH and corticosterone were measured in 50 µl and 10 µl plasma samples, respectively, using commercially available kits (ICN Costa Mesa, USA) according to the respective protocols.

In situ hybridization for CRF Mrna

In situ hybridization for CRF was performed using a rat CRF ^{35}S riboprobe as described previously (Ressler et al., 2002). In a pilot study, slides were exposed to x-ray film and a densitometric analysis was performed for the central nucleus of the amygdala, lateral bed nucleus of the stria terminalis, medial bed nucleus of the stria terminalis (mBNST), and paraventricular nucleus (PVN) of the hypothalamus. As differences in signal between groups was detected only in the mBNST, we performed a more quantitative analysis of CRF expression in this area by counting silver grains over cells of emulsion dipped slides. The slides were exposed to photographic emulsion (Kodak NTB-2) for four days and counterstained with cresyl violet prior to coverslipping. Silver grains in the mBNST were quantified using the AIS Image Analysis software's grain counting feature. Images of the area of interest were taken through a 40X objective, and digitized with a MTI CCD72 camera. The numbers of grains in approximately 15 clusters over cells in the mBNST were counted using a 40 pixel diameter circular cursor setting for each cluster. Grain counts from a proportionate area in the caudate putamen were taken as a background reading. For the mBNST and background readings, bilateral grain counts from 3 adjacent sections were taken and averaged. The background reading for each section was subtracted from the reading for the mBNST.

CRF receptor antagonist selectivity

Since the specificity of the CRF1 and CRF2 antagonists CP-154,526 and Astressin-2B has not been previously established in the prairie vole model, we performed competitive receptor binding experiments to examine specificity of these compounds for the prairie vole CRF receptors. Fresh frozen prairie vole brains were cut on a cryostat in 1:6 series of 20 µm thick coronal sections. Slides were thawed at room temperature until dry, briefly fixed in 0.1 % paraformaldehyde-PBS solution (2 min, pH 7.4), and then rinsed twice in 50 mM Tris base (pH 7.4) solution for 10 min each. Slides were then incubated in tracer buffer for 2 h. The tracer consisted of 50 mM Tris base, 10 mM MgCl, 0.1 % bovine serum albumin, and 0.2 nM [^{125}I-Tyr^{0}]-sauvagine (PerkinElmer/NEN, Boston, MA), a ligand with high affinity for CRF1 and CRF2 receptors. In the competition assays, unlabeled CP-154,526 or Astressin-2B was also included in the tracer buffer. Post-incubation, slides were rinsed with 50 mM Tris-base/10 mM MgCl (pH 7.4) for 4 x 5 min, plus 30 min with stirring on a magnetic stir plate. Finally, slides were dipped in dionized H_2O, dried with cool air, and apposed to Kodak MR film for 89 h. [^{125}I]-microscale standards (GE/Amersham Biosciences) were included to allow quantification.

To demonstrate that CP-154,526 and Astressin-2B have high affinity for the CRF1 and CRF2 receptors, respectively, we co-incubated these compounds (50 nM) separately (**Figure 2.2b-c**) and together (**Figure2.2d**) in the [^{125}I-Tyr^{0}]-sauvagine binding buffer. To determine the specificity of the CRF receptor antagonists, competition binding assays were performed. Brains from two groups of animals (n = 4-5) were used to generate antagonist specificity curves. To analyze CRF1 receptor specificity, the tracer

buffer contained 0.2 nM [^{125}I-Tyr^{0}]-sauvagine plus 0, 0.5, or 25 μM of cold CP-154,526, a selective CRF1 antagonist (Schulz et al., 1996). The selectivity of Astressin-2B was determined by incubating the sections in tracer buffer containing 0.2 nM [^{125}I-Tyr^{0}]-sauvagine plus 0, 0.05, 0.5, 5, or 25 μM of cold Astressin-2B. CRF1 receptor binding was quantified in the cingulate cortex, while CRF2 binding was quantified in the choroid plexus. Binding density was quantified as described previously (Lim et al., 2005). Optical density measurements were taken bilaterally and averaged for each brain region across two or three sections. All density readings were converted to nano-curries per milligram tissue (nCi/mg) based on a known set of standard values ([^{125}I]-microscale; GE/Amersham Biosciences). The means for each brain region were then averaged across 3-5 voles for each of the antagonist concentrations. Finally, mean binding densities across antagonist concentrations were normalized such that the regional densities obtained in the presence of 0 μM of antagonist serve as 100 % binding for each brain region (**Figure 2.2e-f**).

CP-154,526 is selective for CRF1 receptors since 50 nM eliminates binding at CRF1 binding sites (Cing.; factor CP-154,526 concentration: $F_{2,11}$ 202.6, $p < 0.001$; **Figure 2.2e**) but does not decrease tracer binding at CRF2 binding sites even at 25 μM (C.Plexus;1-way ANOVA, factor CP-154,526 concentration, $F_{2,11}$ 1.77, $p = 0.216$). Astressin-2B is selective for CRF2 since 50 nM of this compound significantly reduces radioligand binding in the choroid plexus (C.Plexus), but does not significantly reduce binding in the cingulate (Cing.) CRF1 receptors until 5 □M. (C.Plexus (CRF2); 1-way ANOVA, factor Astressin-2B concentration: $F_{4,15}$ 153.9, $p < 0.001$; **Figure 2.2f**). These

results indicate that CP-154,526 and Astressin-2B have similar specificities at the CRF1 and CRF2 as previously reported in the rat.

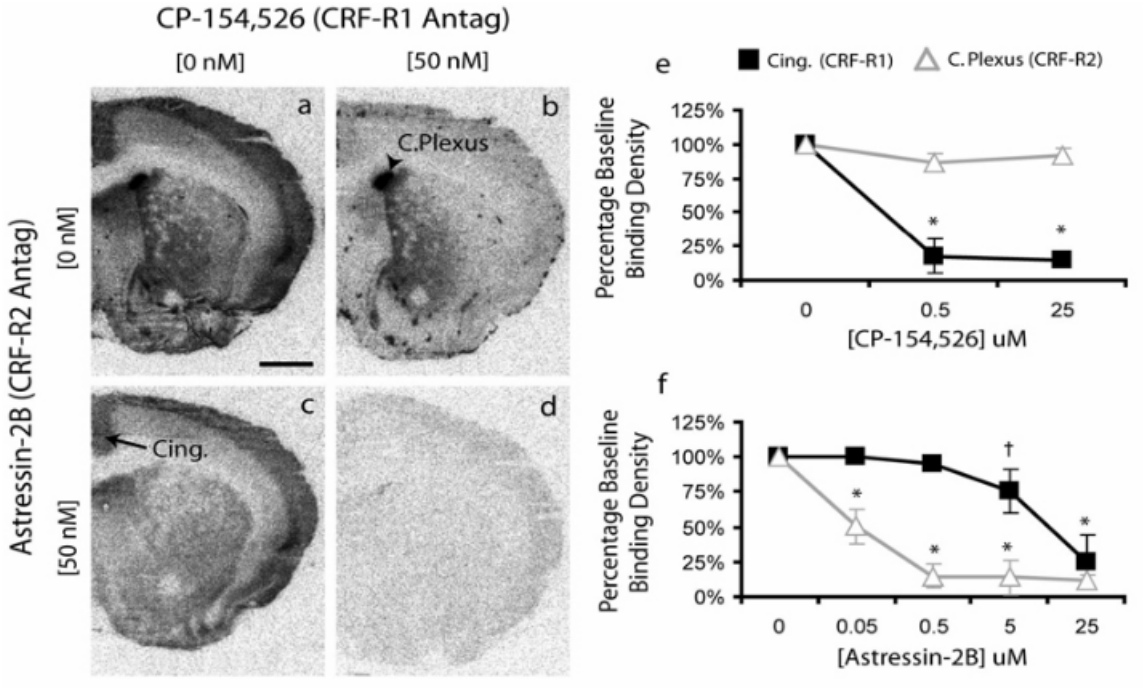

Figure 2.2 - Selectivity of the selective CRF receptor antagonists CP-154,526 (for CRF1) and Astressin-2B (for CRF2)

Competitive receptor autoradiography to show the selectivity of CP-154,526 and Astressin-2B for prairie vole CRF1 and CRF2 receptors, respectively. (a) Total CRF1 and CRF2 binding after incubation with 0.2 nM of [^{125}I-Tyr^{0}]-sauvagine. (b) CRF2 binding after competition at CRF1 receptors by 50 nM of cold CP-154,526. (c) CRF1 binding after competition at CRF2 by 50 nM of cold Astressin-2B. (d) [^{125}I-Tyr^{0}]-sauvagine binding after competition at both CRF1 and CRF2 by 50 nM of CP-154,526 and 50 nM Astressin-2B. (e, f) Dose-response curve for CP-154,526 (e) and Astressin-2B (f) in both the cingulate (Cing.; black square) and the choroid plexus (C.Plexus; gray triangle). Group size n = 3-5. Data are mean±SEM. * $p < 0.001$ vs. 0 nM binding density; † $p < 0.05$ vs. 0 nM binding density. Scale bar = 1 mm.

Long-term intracerebroventricular (icv) administration of CRF receptor antagonists

On the 3rd day of pairing an icv osmotic minipump (Model 1007D, infusion flow rate: 0.5 µl/h; Brain Infusion Kit 3; fixant Loctite 454; Alzet Osmotic Pumps, Cupertino, CA, USA) was stereotaxically implanted (coordinates for the lateral ventricle: nose bar -2.5 mm, AP -0.6 mm, ML -1.0 mm, DV −3.0 mm) under isoflurane anaesthesia (Novaplus, Hospira Inc., Lake Forest, IL, USA) as described before (Torner et al., 2001). The cannula was connected to the osmotic minipump via PE-20 tubing filled with either Ringer's solution or Ringer containing (1) the non-specific CRF receptor antagonist d-phe-CRF (14.1 pmol/h; Bachem, Montreal, Canada; Experiment D) or (2) the selective antagonist for CRF1 (CP-154,526; 137 pmol/h; generously provided by Prof. Michael Owens, Emory University, Atlanta, USA) or CRF2 (Astressin-2B; 12.4 pmol/h; Sigma-Aldrich, St. Louis, USA; Experiment E). Infusion of each antagonist was timed such that the treatment actually began 48 h after surgery, the day that half of the animals were separated. To accomplish this, the PE-20 tubing extending from the osmotic minipump was filled with enough Ringer's solution to last for up to 44 h (from surgery to the end of the 5-day cohabitation period). To prevent diffusion between this Ringer's solution and the CRF receptor antagonist, a small air bubble separated the two solutions in the tubing. At the end of the surgery, animals received 0.01 ml i.p. analgesia (buprenorphine; Buprenex, Henry Schein Inc.). Afterwards, all male voles were placed on a piece of cotton wool in the original home cage with the female partner. Recovery of the animals was monitored until animals were fully awake. At the end of the experiment animals

were euthanized and cannula placement was verified by infusion of black ink into the cannula and adrenals were removed and weighed.

Statistics

Data are presented as mean+SEM. Either a one-way ANOVA (factor CRF receptor type 1 or 2 antagonist), two-way ANOVA (factors partner type (sibling / female); separation condition (paired / separated); treatment (vehicle / antagonist)) or three-way ANOVA (factors separation condition; treatment; female type (partner / stranger)) was performed for all experiments. All interactions were followed up by Newman-Keuls *post hoc* tests for pair-wise comparisons. Significance was accepted at $p < 0.05$. All statistics were performed using GB-Stat 10.0 (Dynamic Microsystems, Silver Springs, USA).

RESULTS

Isolation from a female partner induces passive stress-coping behavior

Experiment A. During the FST, the time spent floating differed among groups (partner type [female or male sibling] x separation condition [paired or separated] interaction: $F_{1,34}$ 9.38, $p = 0.004$; **Figure 2.3a**). Males separated from a female partner displayed significantly more floating behavior ($p < 0.01$) relative to males that remained with their female partner, and males that either remained with or were isolated from their sibling partner. In the TST, we found a significant interaction between partner type and separation condition ($F_{1,33}$ 18.1, $p = 0.0002$; **Figure 2.3b**). Males isolated from a female displayed significantly more immobility ($p < 0.01$) compared with males in the other treatment groups.

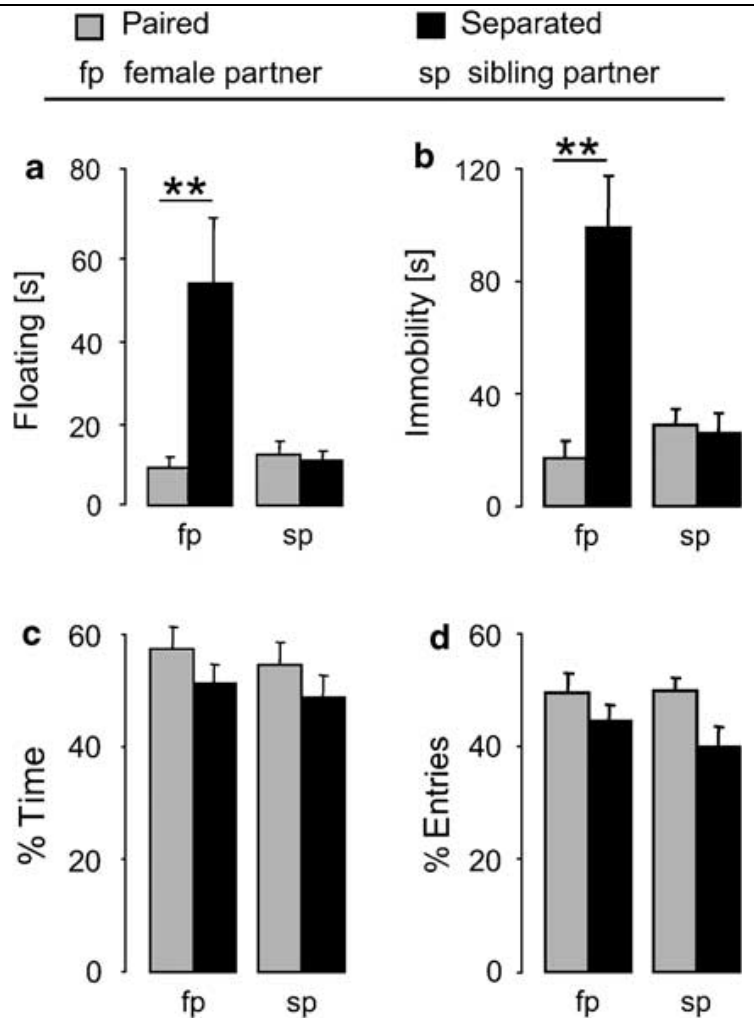

Figure 2.3 - Effect of partner separation on passive-coping and anxiety behaviors
Effect of partner separation on passive stress-coping behavior in **(a)** the forced swim test and **(b)** the tail suspension test as well as **(c,d)** anxiety-like behavior on the elevated plus-maze. Male voles were paired for 5 days with a female (fp) or male-sibling (sp). Half of the males were then separated from their cage-mate (black bars) while the remaining half remained pair housed (grey bars) for 4 to 5 days before behavioral testing (from Experiment A **(a, b)** or Experiment B **(c, d)**; see **Figure 2.1** for details. Passive stress-coping behavior is reflected as the amount of time the animal spends inactive, i.e. floating **(a)** or immobility **(b)**. Anxiety-like behavior is reflected as percentage of time spent in and percentage of entries into the open arms vs. all arms (c,d). Numbers of animals included in the statistics were **(a)** female partner: n = 9 in each group; sibling partner: n = 10 in each group; **(b)** female partner: n = 9 in each group; sibling partner paired: n = 9; separated: n = 10; **(c, d)** female partner paired: n = 10; separated: n = 12; sibling partner: n = 12 in each group. Data are expressed as mean + sem. ** $p<0.01$ vs. all other groups.

Experiment B. On the EPM, separation from the partner altered only one parameter related to anxiety-related behavior, i.e. the percentage of entries into the open arms of the EPM decreased in the separated groups independent of the sex of the partner (two-way ANOVA; factor partner: $F_{1,42}$ 5.48, $p = 0.02$; **Figure 2.3d**). However, the post-hoc test revealed no further differences. Separation had no effect on the percentage of time spent on the open arms (partner x separation interaction: $F_{1,42}$ 0.02, $p = 0.90$; **Figure 2.3c**). There was no difference in the locomotion as reflected by entries into closed arms (female partner, paired: 12±2; separated: 13±1; male partner, paired: 12±1; separated: 12±1; $F_{1,42}$ 0.30, $p = 0.59$) between the groups.

Isolation from a female partner increases basal corticosterone levels

Experiment C. Basal plasma corticosterone levels significantly differed among groups (partner type x separation condition interaction: $F_{1,35}$ 4.65, $p = 0.04$; **Figure 2.4a**). Males separated from a female partner had higher corticosterone levels than males that remained with the female partner ($p < 0.05$). In the sibling-paired groups, no differences were found, paralleling recent findings by Grippo et al. (2007b).

No significant differences were found for ACTH due to a high deviation in the female-paired, separated group (**Figure 2.4b**).

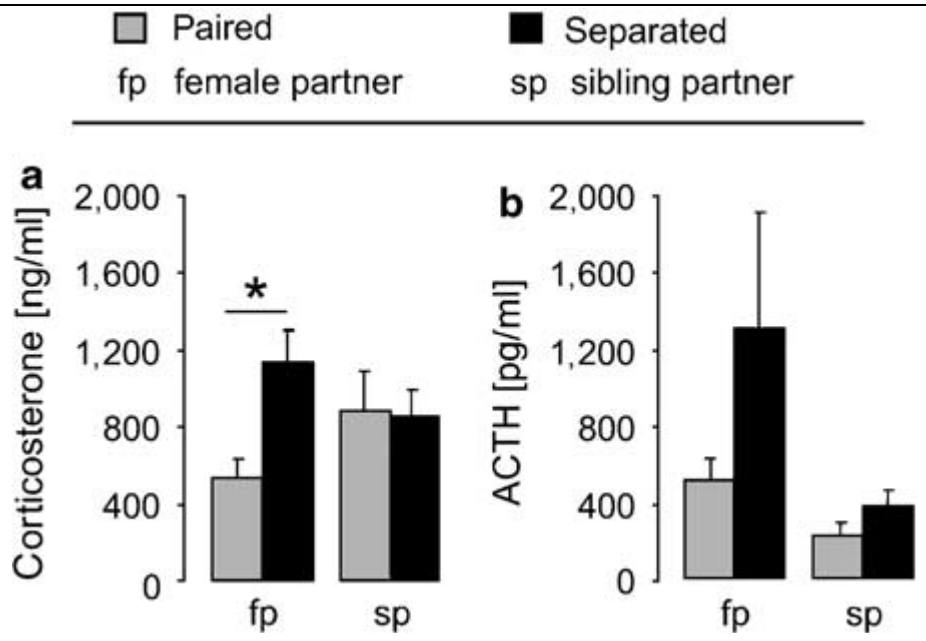

Figure 2.4 - Effect of partner separation on the HPA axis
Effect of partner separation on basal plasma levels of corticosterone **(a)** and ACTH **(b)**. Male voles were paired for 5 days with a female (fp) or male-sibling (sp). Half of the males were then separated from their cage-mate (black bars) while the remaining half remained pair housed (grey bars) for 5 days (Experiment C; see **Figure 2.1** for details). The voles were left undisturbed until the initial separation from the partner and no behavioral tests were performed. Numbers of animals included in the statistics were 10 per group. Data are expressed as mean + sem. * $p < 0.05$ vs. respective female-paired group.

Pairing with a female increased CRF mRNA in the mBNST

In a pilot study (data not shown), we performed *in situ* hybridization for CRF mRNA in males paired or isolated from females or male siblings. Relative optical density measurements from *in situ* hybridization films revealed that among the central nucleus of the amygdala, lateral bed nucleus of the stria terminalis, medial bed nucleus of the stria terminalis (mBNST), and paraventricular nucleus (PVN) of the hypothalamus, the only region showing differences across groups was the mBNST. To further verify this finding,

brains of male voles from Experiment C were removed and processed for CRF mRNA *in situ* hybridization with emulsion dipping for grain counting. Grain cluster density and photomicrograph of a representative section in the mBNST are illustrated in **Figure 2.5 a-b**. Two way analysis of variance for partner type and separation condition revealed a main effect of partner type ($F_{1,29}$ 6.68, $p< 0.02$), such that males paired with a female or isolated from a female showed significantly higher CRF mRNA grain counts in the mBNST relative to males paired or isolated from a sibling (**Figure 2.5c**). Thus both groups of male with previous sexual experience displayed elevated CRF mRNA grain counts.

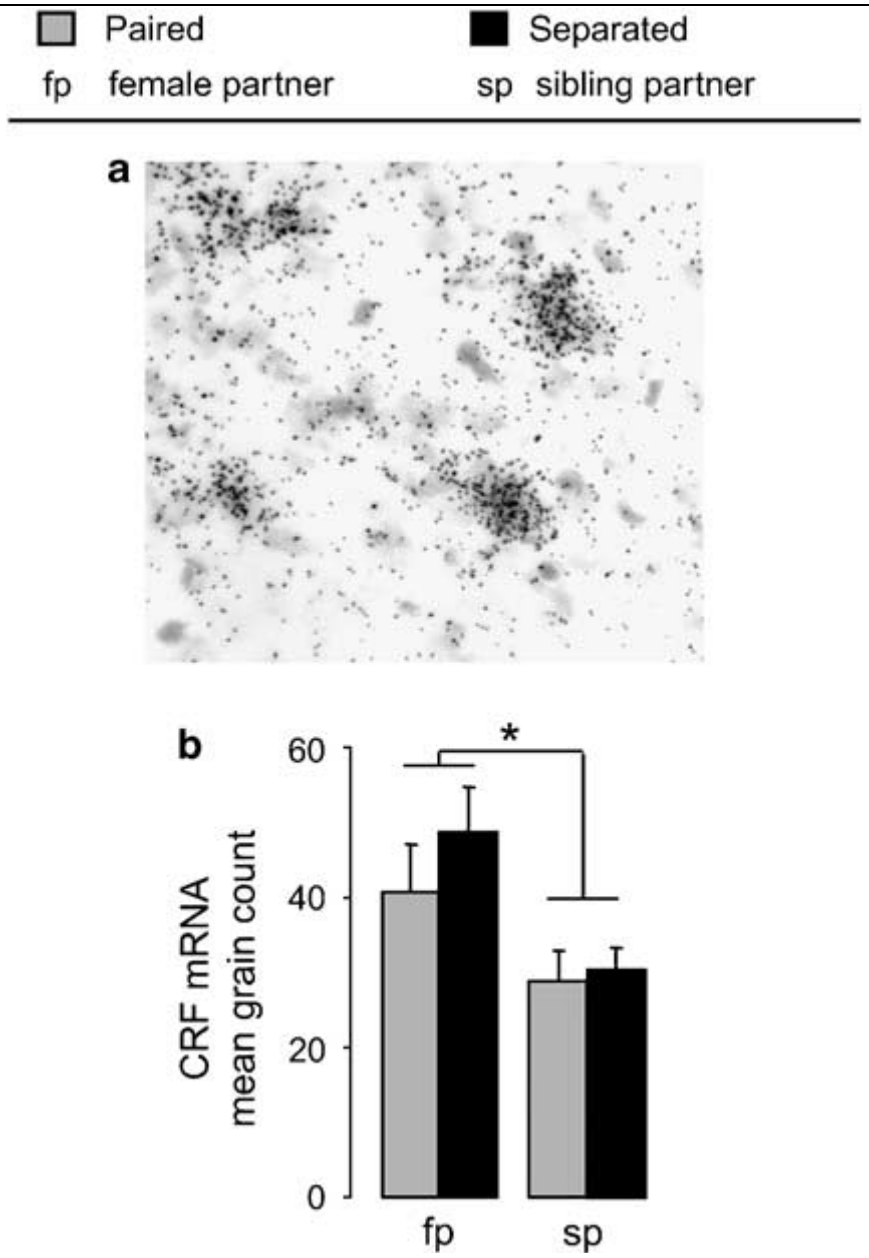

Figure 2.5 - Effect of pairing and separation on CRF in the mBNST
Approximate level of the mBNST **(a)**; depicted on the right side is a representative picture indicating CRF mRNA expression. Clusters of grains in the region of the mBNST **(b)**. Males voles (Experiment C; see **Figure 2.1** for details) paired with a female (grey bars) or separated from a female (black bars) demonstrate higher CRF mRNA grains in the mBNST relative to males paired or separated from a sibling male (c). Data are expressed as mean + sem. * $p < 0.05$.

Infusion of d-phe-CRF during the isolation period blocks separation-induced passive-coping behavior

Experiment D. Continuous icv infusion of the non-selective CRF receptor antagonist, d-phe-CRF, which began on the day of isolation, resulted in differences among groups in the FST (factors separation x treatment: $F_{1,34}$ 9.38, $p = 0.004$; **Figure 2.6a**) and TST ($F_{1,33}$ 18.1, $p = 0.0002$; **Figure 2.6b**). D-phe-CRF treated male voles separated from their female partner showed reduced floating ($p < 0.01$) and immobility ($p < 0.01$) in the FST and TST, respectively, relative to vehicle controls (**Figure 2.6a-b**). There was no effect of the d-phe-CRF on floating or immobility in males that were not separated from their female partner.

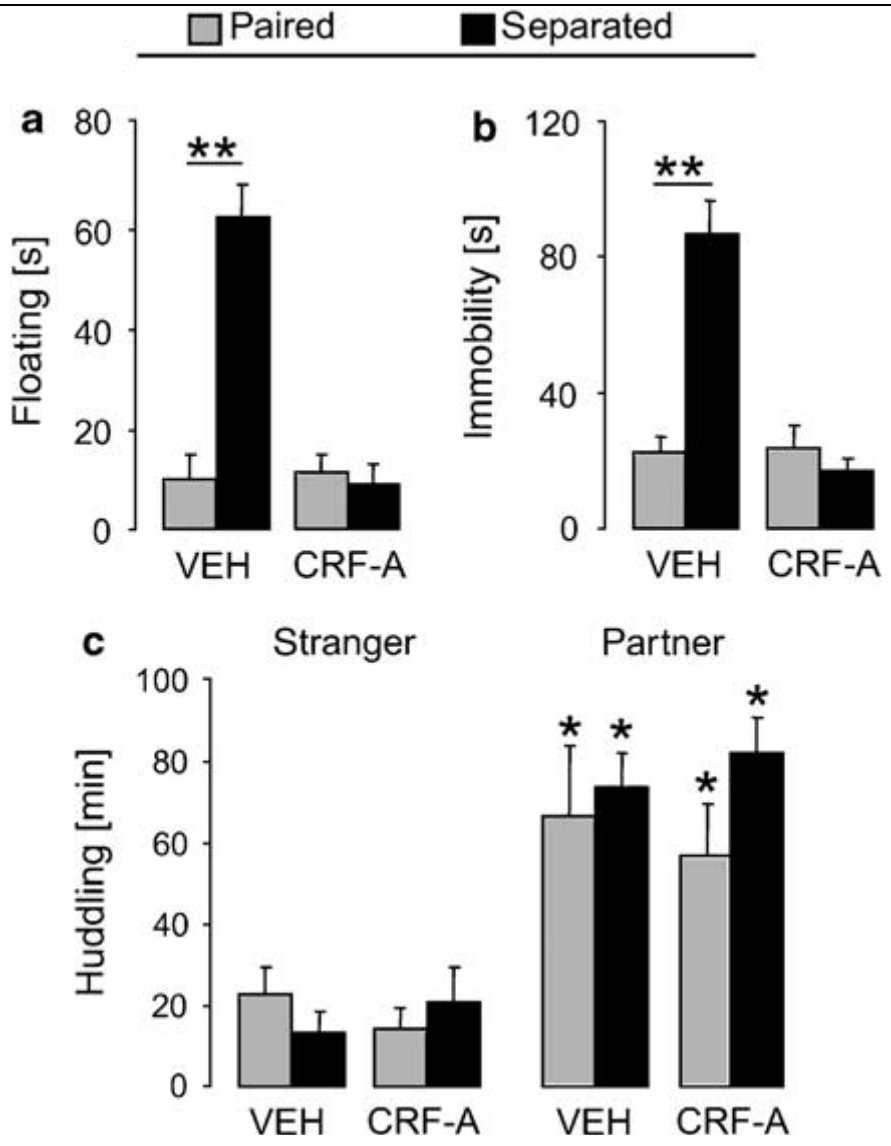

Figure 2.6 - Effect of nonselective CRF receptor blockade on partner separation induced passive stress-coping

Effect of icv CRF receptor antagonist (d-Phe CRF) treatment unspecific for type 1 and 2 on passive stress-coping in **(a)** the forced swim test, **(b)** the tail suspension test, and **(c)** the partner preference test in female-paired male voles from Experiment D (see **Figure 2.1** for details). Male voles were group-housed for 5 days with a female and constantly infused with CRF-A the following days while being still with the partner (grey bars) or separated 3 to 5 days (black bars). The amount of time the animals spend on passive stress-coping strategy, i.e. floating **(a)** or immobility **(b)**. The partner preference **(c)** is represented by the time the male voles spent on huddling with either a female stranger or the bonding partner. Numbers of animals included in the statistics were paired VEH: 5; CRF-A: 4; separated VEH: 7; CRF-A: 6. Data are expressed as mean + sem. ** $p<0.01$ vs. all other groups. ## $p<0.01$, # $p<0.05$ vs. huddling with stranger in same group.

The partner preference test revealed that male voles paired with a female spent more time with the original female partner than with an unknown female (three-way ANOVA; main effect of partner type: $F_{1,36}$ 54.9, $p < 0.0001$; **Figure 2.6c**). There were no treatment ($F_{1,36}$ 0.05, $p = 0.94$), separation condition ($F_{1,36}$ 1.08, $p = 0.30$), or interaction effects, indicating that, once a pair bond is established, partner preferences are not disrupted by subsequent CRF antagonist treatment. Locomotor activity as reflected by the number of cage entries was similar between the groups independent of the treatment (paired VEH: 130±15; d-phe-CRF: 156±22; separated VEH: 177±35 mg; d-phe-CRF: 121±20; two-way ANOVA: $F_{1,21}$ 2.08, $p = 0.17$).

The weight of the adrenal glands was higher after separation from the female partner (paired VEH: 148±10 mg; d-phe-CRF: 146±5 mg; separated VEH: 196±11 mg; d-phe-CRF: 186±7 mg; two-way ANOVA; factor separation: $F_{1,36}$ 26.0, $p<0.0001$) regardless of the treatment with d-phe-CRF (factor treatment: $F_{1,36}$ 0.48, $p=0.50$).

Both CRF receptor subtypes (CRF1 and CRF2) are involved in mediating passive stress-coping behavior after separation

Experiment E. Competitive receptor binding studies revealed that CP-154,526 is selective for the prairie vole CRF1 while Astressin-2B is selective for the prairie vole CRF2, as has been previously reported in the rat (see Supplementary Material). Both the CRF1- and the CRF2-selective antagonists resulted in differences among groups in the FST (factors separation x treatment: $F_{2,57}$ 6.36, $p = 0.003$; **Figure 2.7a**) and TST ($F_{2,58}$ 6.42, $p = 0.003$; **Figure 2.7b**). Male voles separated from their female partner showed reduced floating in the FST when treated with icv CRF1 antagonist ($p<0.05$) or CRF2

antagonist ($p<0.01$) as well as reduced immobility (CRF1 antagonist: $p < 0.01$; CRF2 antagonist: $p < 0.01$) in the TST, relative to the separated vehicle control (**Figure 2.7b**). There was no effect of the CRF receptor antagonists on floating or immobility in males that were not separated from their partner.

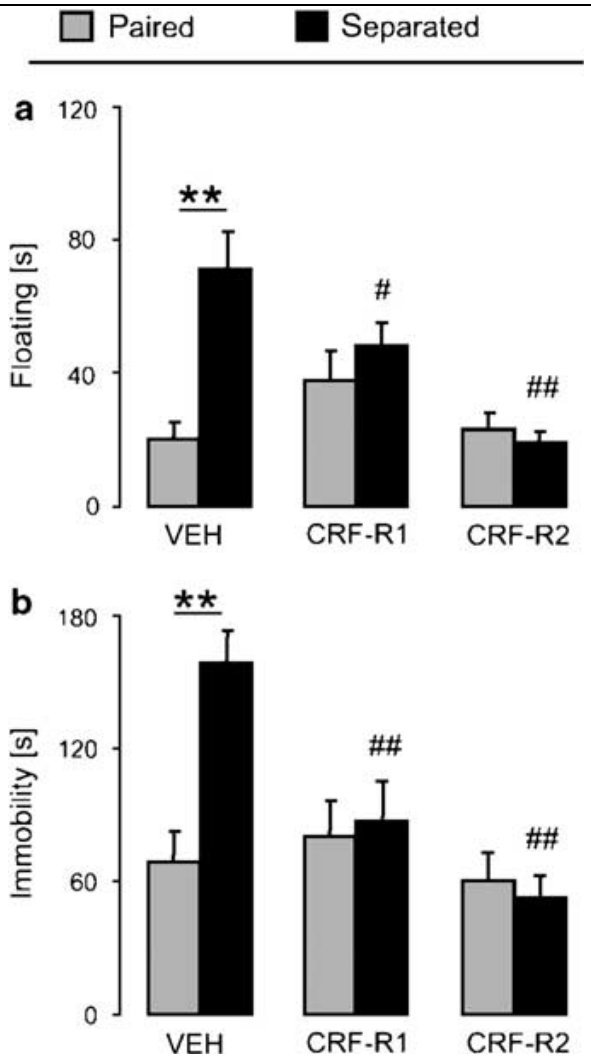

Figure 2.7 - Effect of selective CRF1 or CRF2 blockade on partner separation induced passive stress-coping

Effect of icv CRF1 (CP-154,526) or CRF2 (Astressin-2B) antagonist treatment on passive stress-coping in (a) the forced swim test and (b) the tail suspension test in female-paired male voles from Experiment E (see **Figure 2.1** for details). Male voles were group-housed for 5 days with a female and constantly infused with CRF1 or CRF2 antagonist the following days when still with the partner (grey bars) or separated 3 to 4 days (black bars). The amount of time the animals spend on passive stress-coping strategy, i.e. floating (a) or immobility (b). Numbers of animals included in the statistics were paired VEH: 10; CRF1: 9; CRF2: 9; separated were 10 per group. Data are expressed as mean + sem. ** $p<0.01$ vs. respective female-paired group; ## $p<0.01$, # $p<0.05$ vs. vehicle-treated separated group.

DISCUSSION

In humans, long-term separation from the partner, e.g. due to its death, has been shown to increase the risk for depression (for review Biondi and Picardi, 1996; Zisook et al., 1997; Shear and Shair, 2005). Here we show for the first time that male prairie voles separated from a female partner display increased passive stress-coping strategy in the FST and the TST, indicative of depressive-like behavior in rodents (Porsolt et al., 1977; Armario et al., 1988; Martí and Armario, 1993). Importantly, this effect was not present in males that remain paired with a female, paired with a male-sibling unknown since weaning, or isolated from such a male-sibling and, thus, emphasize the significant consequences of the loss of a bonded partner for male prairie voles. It is important to note that previous studies have shown that in sexually naïve female prairie voles long-term social isolation (4 weeks) increases passive stress-coping behavior (Grippo et al., 2007a; Grippo et al., 2007c). However, the present study is the first one to suggest a selective effect of loss of a bonded partner, as opposed to mere social isolation, on passive stress-coping behavior. Interestingly, separation from a partner, regardless of its sex, tended to increase anxiety-related behavior on the elevated plus-maze in male prairie voles, consistent with previous findings after a 24 h separation (Stowe et al., 2005).

There are two caveats of the present study that must be considered in the interpretation of this data. First, it is important to note that the present study does not differentiate the effects of pair bond disruption in general versus loss of a potential sexual partner. However, under the pairing conditions used in this study, mating begins 24-48 hours following pairing and extends for only 24 hours (Roberts et al., 1998a), therefore mating would have ceased by the time of the separation. We did not quantify mating

during the 5 day cohabitation period, but it should be noted that even 24 hours of cohabitation without mating is sufficient for the development of a partner preference (Williams et al., 1992b). Thus, this suggests that it is the disruption of the pair bond, and not the loss of a potential sexual partner, that is altering the coping behavior. Future studies can address this issue by pharmacologically blocking partner preference formation and examining coping behavior following separation from the partner. A second caveat is that the males in the paired group cohabitated with the females for several days longer than the males in the separated group. However, since mating does not occur after the fourth day of cohabitation, and there were no differences in coping behavior between the males that remained paired with their female partner and the male either paired or separated from a sibling partner, it is unlikely the mere difference in duration of cohabitation with the female resulted in the increase in passive coping behavior.

Evidence from both clinical and basic research indicates that the development of depression may arise from a dysregulation of the CRF system (Holsboer and Barden, 1996; Nemeroff, 1996; Keck, 2006). Animal models of stress-induced anhedonia show increased CRF content in the BNST (Stout et al., 2000), while passive coping behaviors in the FST can be reversed with CRF receptor antagonists (Griebel et al., 2002; Bale, 2005). Interestingly, there is growing evidence supporting the use of CRF1 antagonists as antidepressants (Holsboer, 1999; Zobel et al., 2000; Bale, 2005), but see (Binneman et al., 2008).

Based on these data, we predicted that the passive-coping behaviors arising from separation from a female partner may be linked to CRF activity. Constantly blocking

CRF1 and/or CRF2 during the isolation period prevented the emergence of passive coping behavior, supporting our hypothesis. Interestingly, these treatments did not decrease passive stress coping in voles that remained with the female partner, which is probably due to the fact that in these groups the time spent on floating and immobility in the FST and the TST, respectively, was already low. More importantly, despite the fact that partner preference formation is facilitated by exposure to stressors (DeVries et al., 1996) or central infusions of CRF (DeVries et al., 2002; Lim et al., 2007), CRF receptor antagonist given after partner preference formation did not disrupt partner preferences (Experiment D). The preservation of the partner preferences demonstrates that social memory of the partner was not affected by the CRF receptor antagonist treatment (Heinrichs, 2003).

We had initially predicted that CRF mRNA content might also be the highest in animals separated from a female. However, this was not the case –males that remained with a female and males that were isolated from a female both showed increased CRF mRNA content in the mBNST relative to males paired or separated from a male sibling. This finding suggests that pairing with a female partner, including sexual experience, alters the CRF tone, perhaps priming the brain to quickly respond to social stressors. Also, a lack of increased CRF mRNA expression in other brain regions emphasizes the selectivity of the effects on CRF expression.

The up-regulation of the CRF mRNA in the mBNST in pair-bonded males and the lack of increased passive stress-coping behavior in separated males with long-term CRF receptor antagonism together provide solid evidence that the CRF system plays an integral role in the passive stress-coping behavior following isolation from an opposite

sex bonded partner. Which CRF ligands (CRF or the urocortins) and which CRF receptor populations mediate social loss-induced passive stress-coping behavior remains to be investigated. However, our findings of a role for both CRF1 and CRF2 are consistent with the findings of Bakshi and co-workers (Bakshi et al., 2002) that showed that both CRF1 and CRF2 mediate stress responses in independent brain areas and that both CRF1 and CRF2 in the nucleus accumbens are necessary for CRF-induced partner preference formation in prairie voles (Lim et al., 2007).

HPA axis activity is used as a measure of the physiological stress status. An acute increase of these parameters is traditionally viewed as a stress response (Levine et al., 1989), whereas an elevation in basal corticosterone (Albeck et al., 1997; Zelena et al., 1999) and adrenal hypertrophy (Biondi and Zannino, 1997; Reber et al., 2006; Reber et al., 2007) are established markers for chronic stress. In support, our experiments revealed basal plasma corticosterone levels to be significantly higher in male voles separated from a female compared to all other groups. Moreover, an increased weight of the adrenal glands, the source of corticosterone and adrenaline, was found in males 5 days after separation from a female, thereby giving further evidence of a chronically upregulated basal HPA axis in male voles separated from the bonded female partner.

The finding of elevated corticosterone levels exclusively in males separated from the female bonded partner is in line with studies showing that contact with a female suppresses basal HPA axis activity in both naïve and pair bonded male voles (DeVries et al., 1997). Our results also support earlier reports that baseline corticosteroids are elevated following separation from a female partner in various species (Crawley, 1984b; Mendoza and Mason, 1986; Carter et al., 1995; Ziegler et al., 1995; Castro and Matt,

1997; Norcross and Newman, 1999), including prairie voles (Carter et al., 1995). Interestingly, in earlier studies short-term separation from a sibling was reported to increase basal levels of corticosterone in 40-day old female prairie voles (Kim and Kirkpatrick, 1996) as well as in juvenile voles of both sexes (Ruscio et al., 2007). Thus, it seems that in adult female voles the loss of social support by a conspecific partner of the same sex has strong physiological (Kim and Kirkpatrick, 1996) and emotional (Kim and Kirkpatrick, 1996; Grippo et al., 2007a; Grippo et al., 2007c) effects whereas in males only the loss of a bonded female partner results in alterations of these parameters.

Surprisingly, the elevated corticosterone in this study was not accompanied by increased CRF mRNA content in the hypothalamic PVN. One possibility is that AVP released from the parvocellular neurons of the PVN may act synergistically with CRF on ACTH-containing cells of the pituitary under stressful conditions (Gillies et al., 1982; Aguilera, 1994). In the present study, the long term separation from the female partner might act as a chronic stressor, during which AVP appears to become the predominant regulator of HPA function (Aguilera, 1994) while CRF mRNA expression can be unchanged depending on the stressor (Aguilera and Rabadan-Diehl, 2000).

In conclusion, in the monogamous male prairie vole, 4-5 days of separation from the bonded female partner only, but not social isolation *per se* as seen in female voles (Grippo et al., 2007a; Grippo et al., 2007c), results in the emergence of passive stress-coping behavior as well as increased HPA axis activity. While it is difficult to interpret increased passive stress-coping behavior in voles, anti-depressants reduce this behavior in the FST and TST in other species, suggesting that it is relevant to depressive-like behavior in these species. The combined increase in passive stress-coping behavior and in

HPA axis activity in males in the present study are, however, consistent with the hypothesis that separation from a bonded mating partner is aversive in prairie voles, as it is in humans. While the emergence of passive stress-coping behavior and increased HPA activity following the loss of a bonded female partner is intuitively maladaptive, we speculate that this phenomenon may actually be the by-product of an adaptive bi-dimensional integrative emotional system (e.g. reward/aversion) that is fundamental to the formation and preservation of enduring bonds (Panksepp et al., 1997). Although CRF receptor activation is apparently not necessary for the display of a partner preference, activation of this system following separation from the mating partner may act to preserve the established pair by maintaining contact. Thus, the CRF system may compliment the reinforcing effects of other neuropeptides systems (e.g. oxytocin and vasopressin) which are thought to be critical to pair bond formation. Based upon our results we believe that prairie voles represent an important rodent model that will provide us with unique insights into the neurobiology of the loss of a bonded partner which may be relevant to grieving and bereavement in humans.

Disclosure / Conflict of interest

The authors declare that, except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service others than stated below and that there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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CHAPTER 3

Parental division of labor and the effects of family structure on parenting in monogamous prairie voles

Adapted from:

Ahern TH, Hammock EAD, Young LJ (*to be submitted*) Parental division of labor and the effects of family structure on parenting in monogamous prairie voles (*Microtus ochrogaster*)

PREFACE

Chapters 3-5 examine prairie vole family structure and how changes to that family structure affect offspring development. During these studies 5 different cohorts of animals were used. Below is Table 3.1, which presents each cohort and how it was used. It also presents which chapters and which publications (Ref) contain (or will contain) data from each cohort.

Table 3.1 - Prairie vole family structure studies by breeding cohort and age

Assays by breeding cohort & age				
Cohort	**Neonatal Period** (PND1-15)	**Weaning** (PND22)	**Adulthood** (PND60-90)	**Ref**
Cohort 1 (Chpt 3)	Family observations - Coordination - Licking & Grooming - Nursing Posture		(Offspring used in Hammock & Young (2005; data not included here).	A
Cohort 2 (Chpt 3&5)	Family observations - Coordination - Licking & Grooming - Nursing Posture Pup weights	Weanling weights	Plasma Corticosterone (CORT) Brain - Oxytocin mRNA - Oxytocin Receptor (OTR) Binding - Vasopressin 1a Receptor (V1aR) Binding - CRF Receptor 1 (CRF1) Binding - CRF Receptor 2 (CRF2) Binding	A,B
Cohort 3 (Chpt 4)	Pup weights	Weanling weights	Open-field test Adult weights (PND65) Elevated plus maze Alloparental behavior test Adult weights (PND90)	B
Cohort 4 (Chpt 4)	Pup weights	Weanling weights	Adult weights (PND90) Mating Partner preference test - post 24 hr cohabitation - post 48 hr cohabitation - post 1 wk cohabitation	B
Cohort 5 (Chpt 4)		Weanling weights	Adult weights (PND90) Same group male/female pairing - BP-reared male + BP-reared female - SM-reared male + SM-reared female Partner preference test - post 24 hr cohabitation (females only) Gestational Measures Family observations (PND1-7) - Coordination; Licking & Grooming	A

(A) Ahern TH, Hammock EAD, Young LJ (*to be submitted*) Parental division of labor and the effects of family structure on parenting in monogamous prairie voles (*Microtus ochrogaster*)

(B) Ahern TH, Young LJ (2009) The impact of early life family structure on adult social attachment, alloparental behavior, and the neuropeptide systems regulating affiliative behaviors in the monogamous prairie vole (*Microtus ochrogaster*). *Frontiers in Behavioral Neuroscience* 3:17

In Ahern & Young (2009), animal cohorts 2, 3, and 4 for **Table 3.1** above correspond to cohorts 1, 2, and 3, respectively, within the publication.

ABSTRACT

Differences in the mother-infant relationship influence a wide range of developmental outcomes, including vulnerability to psychopathology, yet little is known about how biparental rearing influences the brain. Prairie voles (*Microtus ochrogaster*) are socially monogamous and biparental and have emerged as an excellent animal model for studying the neurobiology and genetics of social bonding and social cognition. In this series of studies, I examined prairie vole family dynamics in a laboratory environment that is amenable to biomedical manipulation. In the first study, I found that primiparous prairie vole mothers and fathers both exhibit robust parental care. I also found that the parents coordinate nest attendance, licking and grooming (LG), and even nursing posture. In the second study, I examined how parental behavior would change in response to the removal of the male—creating single-mother family units. Single-mothers (SM) showed little behavioral compensation (e.g., no increases in pup-directed care) at any point during the two week observation period. Situated in the context of human family dynamics and family psychology, these data lay the foundation for future studies aimed at understanding the neurobiology of family life and how variations can affect development of offspring.

INTRODUCTION

Human families are dynamic social systems (Cox and Paley, 1997) that are often marked by a strong social tie between adult partners and biparental care of offspring (Maccoby, 2000; Schor, 2003). Whether driven evolutionarily by ecological (Low, 2007) or inheritance (Fortunato and Archetti, 2010) or cultural pressures (Schuiling, 2003), socially monogamous, biparental family environments are common, and research from a variety of disciplines suggests that differences and shifts in these family systems make significant contributions to the psychological and behavioral development of children. One striking example is the finding that children participating in stable, affectionate two-parent family groups tend to have different cognitive, social, and behavioral profiles than children in distressed, unstable family groups (Maccoby, 2000; Meaney, 2001; Schor, 2003; Sarkadi et al., 2008). Unfortunately, children from the latter family environment are faced with increased vulnerability to depression, anxiety, drug abuse, attachment disorders, and other psychopathologies. At present, little is known about the neurobiology that underlies these findings.

One approach to address this deficiency is to use animal models. Most traditional animal models, however, such as rats, mice, and rhesus macaques, do not form selective social attachments between mates, nor do they rear their offspring biparentally. To accurately address the question of how family dynamics affect offspring development, I need another approach.

Over the last several decades, monogamous prairie voles have emerged as an excellent model species for studying the neurobiology and genetics of sociality, with a special focus on social bonding (Carter et al., 1995; Young and Wang, 2004; McGraw

and Young, 2009). Prairie voles do more than bond, however; they exhibit a range of family related behaviors such as long-term nest sharing, partner preference, preferential mating, and mate guarding, as well as spontaneous alloparental and biparental care. Ethologically, there is a wide range in the expression of these behaviors, similar to humans. In the wild, prairie vole pups are often found being reared by large extended family, two parents, and even single-mothers (Getz and Carter, 1996).

Before these animals can be used to address questions regarding the interplay between family dynamics and neurobiology, however, there needs to be a better understanding of their family dynamics. In this series of studies, I aimed to more fully characterize prairie vole family dynamics in a laboratory environment and then test how prairie vole parenting would change in response to ethologically relevant manipulations.

In the first study, I characterized primiparous biparental care within standard laboratory breeding cages of cohort 1 animals and tested the hypotheses that (1) both primiparous parents participate in pup care, (2) parents coordinate nest occupancy even as first-time parents, and (3) parental interactions with the pups are dynamic (i.e., pup-directed care differs as a function of nest attendance by each parent). Data for this study were obtained from a pre-existing data set, of which only a small portion had been used previously (Hammock and Young, 2005). Our focus here is on the large unused portion.

In the second study, I used a new cohort of animals (cohort 2) to examine the effects of altering the family environment by creating single-mother (SM) units. I hypothesized that SMs would eventually compensate for the lack of the father to some degree, but that pups would receive less direct parental care (e.g., higher rates of exposure, and lower rates of licking and grooming).

STUDY 1

Do primiparous prairie vole breeders show long-term parental coordination under laboratory conditions?

MATERIALS AND METHODS (STUDY 1)

Animals

Twenty-five 4-5 month old, cohort 1, sexually naïve female prairie voles (*Microtus ochrogaster*) from our colony were paired with an equal number of age- and experience-matched prairie vole males in large polycarbonate cages. Our colony is derived from wild-caught Illinois stock, is maintained at Emory University, and remains consistently outbred. Pairs were housed with a 14:10 light:dark schedule at 22-25 C, with *ad libitum* access to food (PMI Nutrition International, Brentwood, MO) and water. Cages were lined with bed-o-cob and one nestlet; cages were changed weekly. All protocols were approved by the Institutional Animal Care and Use Committee of Emory University (IACUC) and are in accord with national guidelines.

Because prairie voles are induced-ovulators (Carter et al., 1980), the 25 pairs were initially separated by a perforated polycarbonate divider. This setup aimed to standardize receptivity and pregnancy. After 5 days, dividers were removed and mating occurred in all pairs within 1 hr. Approximately 18 d post-mating, all males were shaved dorsally to ease identification. Twenty-one pairs (84%) delivered pups between 23-30 d after

removal of the perforated divider. Litters born within 6 to 8 hours of each other were cross-fostered.

Family unit observations

Prairie vole gestation is approximately 21-22 d, so breeder pairs were checked several times daily starting d 20 post-mating. On the day the first pair gave birth, behavioral observations for all family units began. Observation sessions occurred twice per day for one-hour each. The first occurred during the first hour of lights ON (0500-0600 hr) and the second occurred during the first hour of lights OFF (1900-2000). Dark-phase observations were made under low-light (1-2 lux) conditions. All 25 family units were observed regardless of whether they had pups or not. Observations of pair prior to delivering pups was used as the post natal day 0 (PND0). For each family unit, PND1 marked the day that that family unit first had pups. 15 of the 21 pairs that had pups were observed for at least 15 PNDs; these were used for statistical analyses.

Behavioral data were collected for each family unit 10 times per hour, twice per day, using a "spot-check" method. Each cage was briefly observed (or spot-checked) and the behaviors of the mother and father. Behavioral observations of the pups were used to establish a developmental timeline. Spot-checks for any given cage were separated by 5-6 min. The behavioral log was divided into three basic categories: (1) nest occupancy, (2) pup-directed licking and grooming (LG), and (3) type of nursing. For nest occupancy, animals were either ON or OFF the nest. If both parents were ON the nest, then they were either huddling or not. Each parent was either engaged in pup-directed LG or not.

Nursing postures included active nursing (arch-backed or crouching), passive nursing (lying on her side), or no nursing.

Data analysis

The visual spot-check method resulted in 10 data points per hour for each behavior for each adult and litter. If the behavior occurred during a spot-check, it received a 1; if it did not occur, it received a 0. I then calculated the frequency of each behavior for each PND.

To simplify the data set, and yet faithfully convey changes over time, behavioral frequencies were either averaged across 3-day bins (i.e., PND 1-3, 4-6, 7-9, 10-12, 13-15, designated PND bins 1-5, respectively). In some cases, frequencies were averaged over the entire post-natal observation period.

For most analyses, separate mixed repeated measures ANOVAs were used to test both within subjects effects (time-of-day, PND, and certain behaviors [e.g., nursing posture]) as well as between subjects effects—sex differences. *A priori* Least Significant Difference(LSD) *post hoc* analyses were performed when appropriate. In cases where averages were taken over the entire post-natal observation period, independent or paired t-tests were employed depending on the nature of the comparison. $P < 0.05$ was accepted as significant and was adjusted (Bonferroni) for multiple comparisons when necessary.

RESULTS (STUDY 1)

I characterized the frequency of parenting behaviors of 15 primiparous female and male prairie vole pairs over the first 15 d postpartum. I also tested the hypothesis that the two parents coordinate their behavior to minimize pup exposure. Such behavioral coordination can be established if parents display different levels of behavior depending on the presence or absence of the other parent on the nest.

Nest Occupancy

Mothers and fathers were, in total, on the nest at roughly equal rates, with both showing a decrease in nest occupancy over time (**Figure 3.1A**; Two-factor ANOVA; PND: $F(1,4) = 5.487$, $P < 0.001$, Sex: $F(1,28) = 0.185$, $P = 0.671$, PND x Sex Interaction: $F(1,4) = 1.594$, $P = 0.181$). Mothers and fathers huddled on the nest far more often than they were alone on the nest (Huddling > BP-Mother Alone: $P < 0.001$; Huddling > BP-Father Alone: $P < 0.001$) and they were alone on the nest at essentially equal frequencies (BP-Mother Alone vs BP-Father Alone: $P = 0.621$). Huddling on the nest occurred at the highest frequency on the days following birth and then decreased over time, while the frequency of observations spent alone on the nest increased (marginally) over time.

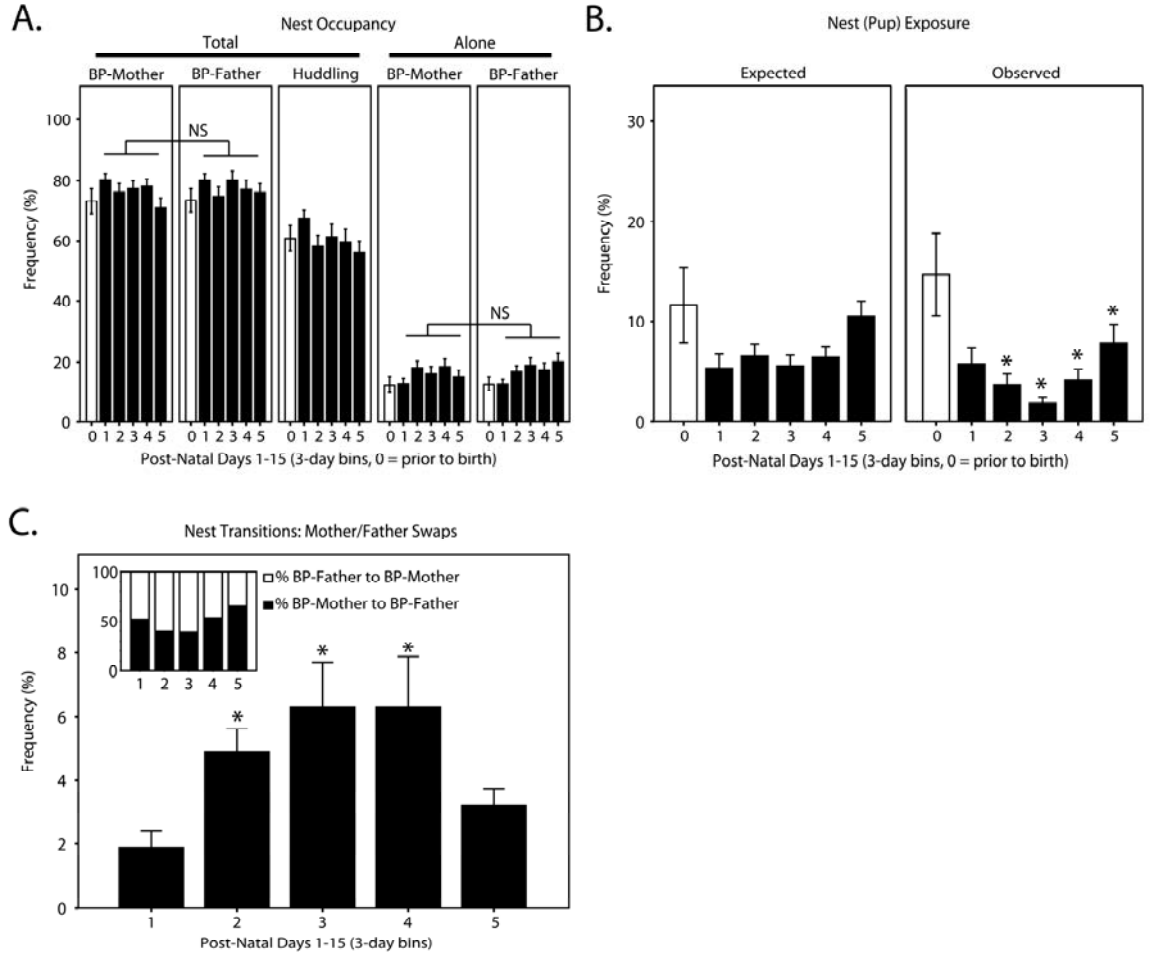

Figure 3.1 - Nest occupancy and coordination under biparental conditions
(A) Mothers and fathers attend to the nest (and pups) at a high frequency—most often together. They are alone on the nest at indistinguishable frequencies. NS = not significant. **(B)** The nest and the pups are rarely exposed, and the "observed" exposure is significantly less than "expected" ([mother-off] x [father-off] = [expected nest exposure]), suggesting behavioral coordination; * $P < 0.05$, paired test, expected exposure vs observed. **(C)** Parents increase the frequency of total nest swaps (mother-alone to father-alone and vice versa) from PND bins 2-4 (or PND 4-12) compared to PND bin 1, again suggesting coordination; * $P < 0.05$, paired tests. **(C inset)** Fluctuations in father-to-mother and mother-to-father swap frequency over time. N = 15 family units. Bars represent Mean ± SEM.

Pup exposure

Primiparous prairie vole parents left the nest (and pups) exposed at a very low rate (**Figure 3.1B, "Observed"**)—often less than 5% of the time. Nest exposure first decreased then increased (One-factor ANOVA; PND: $F(1,4) = 12.525$, $P < 0.001$) (I am not sure that it is legit to use ANOVA on these frequency data, its not a continuous variable, I may need to consult a statistician about this). Nest exposure leaves the pups vulnerable and two parents could in theory coordinate their behavior to decrease nest vulnerability.

Parents that do not coordinate nest occupancy should show an observed nest exposure frequency equal to a theoretical "expected" exposure frequency, which is calculated by multiplying the probability of finding each parent off the nest: (mother-off frequency) x (father-off frequency) = (expected nest exposure frequency). If parents coordinate to decrease the nest vulnerability, then the observed frequency should be significantly lower than the expected frequency.

Here I observed nest exposure frequencies that were significantly lower than expected (**Figure 3.1B**; Two-factor ANOVA: PND: $F(1,4) = 6.992$, $P < 0.001$, Obs-vs-Exp: $F(1,14) = 34.351$, $P < 0.001$, PND x Obs-vs-Exp Interaction: $F(1,4) = 2.967$, $P = 0.027$). *Post hoc* paired comparisons revealed observed frequencies that were significantly lower than expected for each of the PND bins in which pairs were caring for pups ($P < 0.05$) (this cant be true for bins 1 or 5, see graph, need to double check these stats). There was no evidence of nest occupancy coordination in pairs prior to the birth of pups (PND0; Observed vs Expected paired comparison: $P = 0.327$) nor in pairs that did not have pups; in fact, pairs that lost pups within the first 3 PNDs (N=)had significantly

greater observed nest exposure frequencies than expected (Observed > Expected: $P < 0.05$ for PND bins 2-4; data not shown).

Nest occupancy swaps

To gain greater insight into behavioral coordination, I examined the frequency of nest occupancy swaps. A swap consists of having one parent alone on the nest at one observation and then having the other parent alone on the nest at the next observation. The total frequency of such swaps was low during the first 3 PNDs, but then significantly increased for PNDs 4-12 and then dropped off again as pups matured (**Figure 3.1C**; One-way ANOVA; PND: $F(1,4) = 6.910$, $P < 0.001$). Of these swaps, 9 were directly observed: on 3 occasions, the father started to run to the nest as the mother started to leave the nest; on 4 occasions, one partner dragged the other partner to the nest; and on 2 occasions partners fought/wrestled before one remained with pups. While there were no sex differences in the frequency of swaps, there were fluctuations in the percentage of each type of swap (**Figure 3.1C inset**).

Licking and grooming

During the first two weeks post birth, both mothers and fathers LG pups (**Figure 3.2A**). Pups received LG from mothers at a higher frequency than from fathers and, in both cases, pup-directed LG waned over time (Two-way ANOVA; PND: $F(1,4) = 7.538$, $P < 0.001$, Sex: $F(1,28) = 82.568$, $P < 0.001$, PND x Sex Interaction: $F(1,14) = 2.926$, $P <$

0.001). Averaged over the entire observation experiment and normalized to nest occupancy, LG behavior of both mothers and fathers was affected by the presence of their partner on the nest, but the effect was sex-dependent (**Figure 3.2B**; Two-way ANOVA; Partner Presence: $F(1,1) = 140.181$, $P < 0.001$, Sex: $F(1,28) = 25.523$, $P < 0.001$, Partner Presence x Sex Interaction: $F(1,1) = 16.043$, $P < 0.001$). When alone on the nest, mothers and fathers LG at statistically indistinguishable rates (*Post hoc* Bonferroni t-test: $P = 0.378$). When together on the nest, both parents LG pups less than when alone (*Post hoc* Bonferroni paired t-test: $P < 0.001$ for both), but fathers show a significantly greater drop than mothers (*Post hoc* Bonferroni t-test: $P < 0.001$).

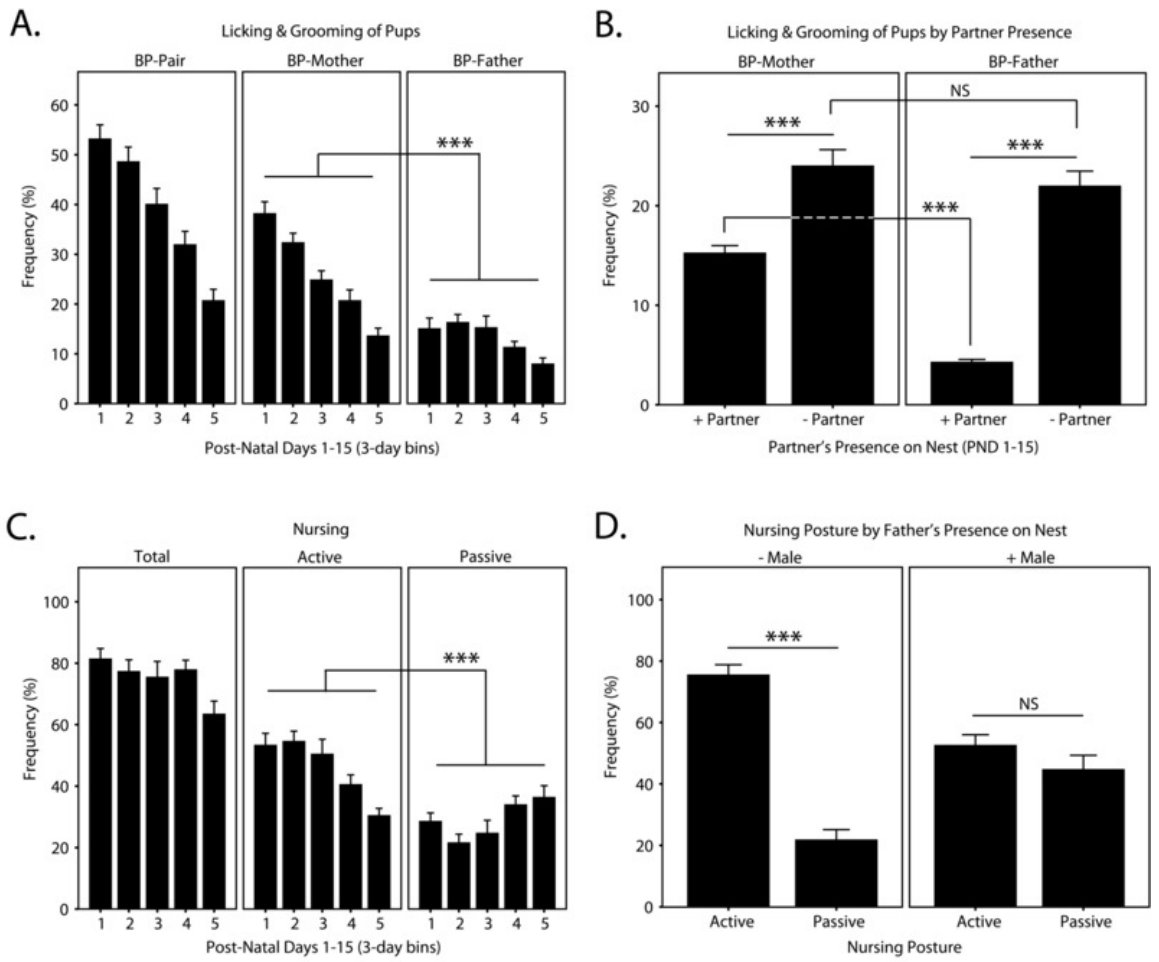

Figure 3.2 - Licking and grooming (LG) and nursing under biparental conditions **(A)** Mothers and fathers both LG pups, with mothers displaying a higher frequency than fathers. **(B)** Normalized to nest occupancy, mothers and fathers LG at equal rates when attending to the pups alone and both decrease when huddling together; fathers decrease more. **(C)** Mothers nurse (or have pups attached) at high rates, initially with a high rate of active nursing, then toward the end a higher rate of passive nursing. **(D)** The father's presence on the nest influences nursing posture. Bars represent Mean ± SEM. *** $P < 0.001$, NS = not significant.

Nursing

I also examined nursing and nursing postures. Prairie vole pups have milk teeth and therefore are attached to the mother nearly all of the time she is on the nest. This

prevents direct observation of nursing itself. Still, I quantified the frequency that pups were attached and, for brevity, refer to this as nursing.

Overall, nursing occurred at a high rate (**Figure 3.2C**), essentially mirroring the mother's nest occupancy (**Figure 3.1A**). Pups continued to nurse throughout the entire 15 day observation study. Mothers spent more time in the active nursing posture than the passive, although passive tended to dominate at the end of the second week post-birth (**Figure 3.2D**; Two-way ANOVA; PND: $F(1,4) = 6.057$, $P < 0.001$, Nursing Posture: $F(1,1) = 9.991$, $P < 0.001$), PND x Nursing Posture Interaction: $F(1,1)$: 7.105, $P < 0.001$).

As with LG, the nursing posture frequency was partner-dependent. When the male was present on the nest, active and passive nursing occurred at essentially equal rates (*Post hoc* Bonferroni paired t-test: $P = 0.261$); when the father was absent, the mother adopted the active nursing posture significantly more frequently (*Post hoc* Bonferroni paired t-test: $P < 0.001$). The father's presence did not affect the nursing rate overall.

Pup maturation and parental pup-retrievals

The offspring became independently mobile around PND9 and parents began to show retrieval behaviors at that time (**Figure 3.3A-B**). Both mothers and fathers retrieved pups, but males tended to retrieve wandering pups more often, especially as the pups matured. At first (PND9-11), parents retrieved the majority of wandering pups, but the number of unretrieved pups steadily increased over time (**Figure 3.3A**).

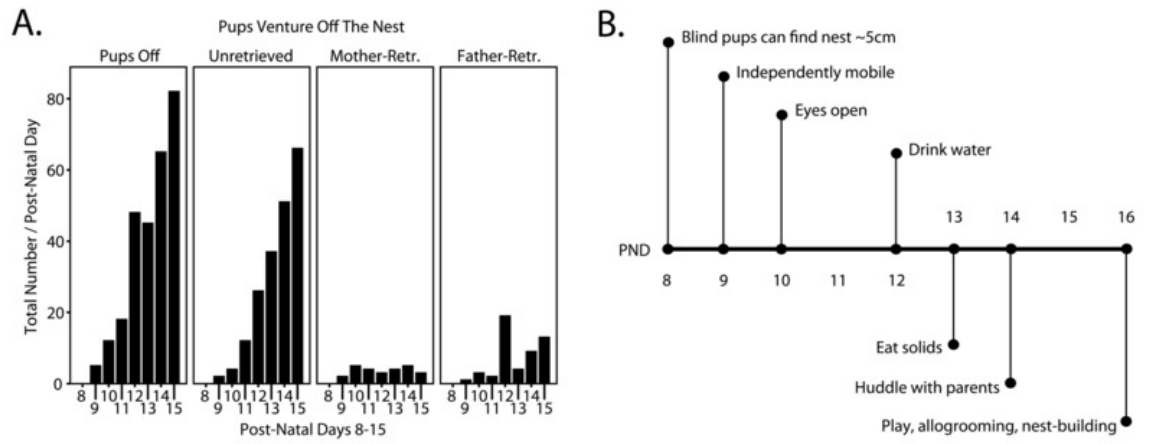

Figure 3.3 - Pup maturation and parental retrievals under biparental conditions
(A) Raw number of pups that independently ventured off the nest per PND, coupled with raw counts of how many were retrieved by each parent or left unretrieved. **(B)** Approximate maturation schedule of pups.

STUDY 2

How do primiparous family dynamics change in response to the father's absence?

Study 1 revealed that the biparental rearing strategy adopted by monogamous prairie voles creates a highly dynamic and complex social environment where parents coordinate and alter behaviors in a socially dependent manner. Not all prairie vole pups are reared under biparental conditions, however; some a reared by single-mothers (Getz et al., 1992a; Wang and Novak, 1992; Getz and Carter, 1996; Roberts et al., 1998b; McGuire et al., 2007). To test how primiparous prairie vole mothers adapt to a uniparental environment throughout the post-natal period, I compared the behavior of parents under biparental (BP) and single-mother (SM) conditions in the laboratory. I predicted that SMs would either increased rates of pup-directed care in comparison to

BP-mothers, but overall, SM-reared pups would receive less direct care (e.g., higher rates of pup exposure and decreased LG).

I also aimed to replicate and extend our description of family social interactions within the BP group, with a special focus on the sex differences in pup-directed LG. I tested the hypothesis that BP-fathers shift their focus from their pups to their partners when both parents are attending to the nest. Overall, total LG rates should show no sex difference, but pup-directed LG should be higher in BP-mothers (as in study 1), while partner-directed LG should be higher in BP-fathers. The shift to partner-directed LG should entirely account for the difference in pup-directed LG.

MATERIALS AND METHODS (STUDY 2)

Animals

Twenty-one sexually-naïve, 3-4 month old, cohort 2, female prairie voles from our Emory University colony were paired with an equal number of age- and experience-matched males in large polycarbonate, ventilated cages (34 cm long x 30 cm wide x 18 cm high) without a perforated divider. On d 18 post-pairing, all males were handled and shaved dorsally. Ten of the males were removed to a separate room, where they remained for remainder of the study. This created ten single-mother (SM) family units and eleven biparental (BP) family units. Eighteen dams gave birth between 23-30 d post-pairing (SM = 9 [90%]; BP = 9 [81%]); pups were not cross-fostered. Litters were culled to equal numbers of 2, 3, and 4 pup litters in each group. Pups were weighed on the first post-natal day (PND1) and every 7 days thereafter until weaning at PND22. Weight measurements took place after the morning observation session. Two BP and two SM family units were

not included in the statistical analysis because they either lost pups or were not observed for a full twelve PNDs. N = 7 for both BP and SM groups.

Family unit observations

Observation sessions occurred three times per day (instead of two) for one-hour each. As in study 1, observation sessions occurred at lights ON (0600-0700 hrs) and lights OFF (2000-2100 hrs), and a third was added in the middle of the day (12:30-13:30). Dark-phase observations were made under red-light (15 watt) conditions, rather than low-light. Adding the third observation session allowed us to examine another time of day for time-of-day effects and increase the observational power using a smaller sample size.

Data were again collected by visual spot-checks and the behaviors of the mother, the father, and the pups were logged based on three basic categories: (1) nest occupancy, (2) activity, (3) type of nursing. In this study, the "activity" category replaced pup-directed LG. The activity category included pup-directed LG, nest building, wandering (walking or running), digging, eating, drinking, grooming self, grooming mate, gnawing, and climbing/jumping. With prairie voles as a potential model of family unit dynamics, these more completely describe the behavior of the animals. Nursing postures again included active (arch-backed or crouching) and passive nursing.

Data analysis

With three observation sessions, each family unit logged 30 data points for each PND. Behavioral frequencies were calculated and averaged over 3-day bins or averaged

over the entire post-natal observation period. Statistical analyses paralleled study 1, except that group was often added as another between subjects factor. LSD *post hoc* tests followed significant main effects. After preliminary statistical analyses, time-of-day again showed no effect and was therefore dropped from all subsequent analyses.

RESULTS (STUDY 2)

Nest Occupancy

BP-mothers, BP-fathers, and SMs were on the nest at roughly equal rates, with all three showing a decrease in nest occupancy over time (**Figure 3.4A**; Two-factor ANOVA; PND: $F(1,3) = 10.423$, $P < 0.001$; Parent: $F(1,18) = 2.478$; PND x Parent Interaction: $F(1,6) = 0.573$, $P = 0.750$). Again, BP-mothers and -fathers huddled on the nest far more often than they were alone ($P < 0.001$ for both) and they were alone on the nest at essentially equal frequencies (**Figure 3.4A**; Two-factor ANOVA; PND: $F(1,3) = 2.471$, $P = 0.072$, Parent-Alone: $F(1,18) = 273.638$, $P < 0.001$, PND x Parent-alone Interaction: $F(1,6 = 4.474$, $P = 0.001$). SMs spent a statistically indistinguishable frequency on the nest compared to BP-mothers ($P = 0.544$).

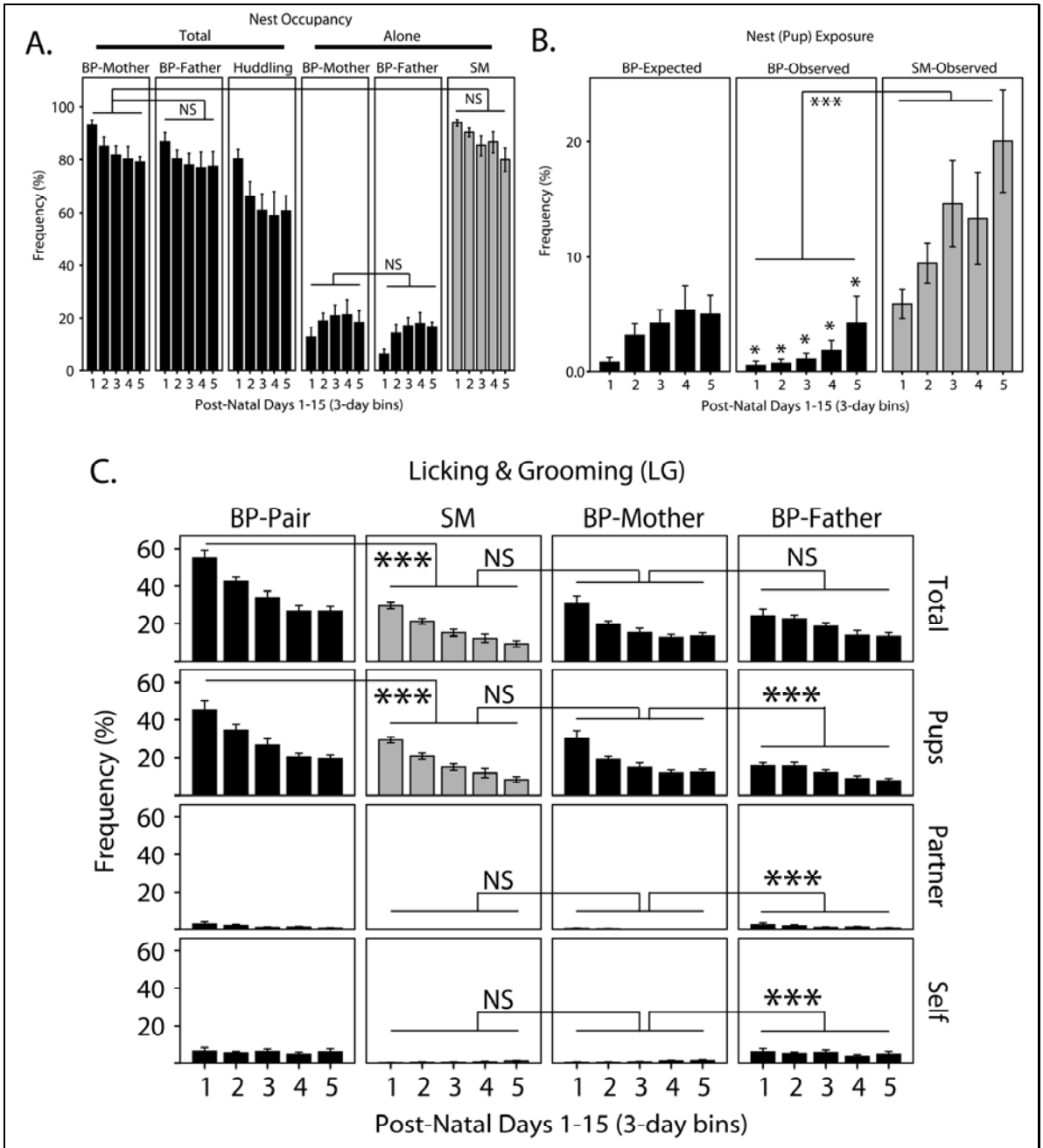

Figure 3.4 - Biparental (BP) versus single-mother (SM) nest occupancy, coordination, and licking and grooming (LG)

BP-parenting versus SM-parenting. **(A)** Parents spent approximately equal amounts of time on the nest regardless of sex or group; BP-mothers and BP-fathers were again alone with pups at approximately equal rates. **(B)** BP-parents exposed the nest less than expected for all PND bins (* $P < 0.05$) and BP pups were exposed significantly less than SM pups. **(C)** Overall, BP-mothers, BP-fathers, and SM-mothers have equal frequencies of "Total" LG, thus there is more LG in BP units than SM units; LG toward pups was the most frequent class of LG with more pup-directed LG occurring in BP units; BP-mothers and SMs LG pups with the same frequency and both LG pups significantly more than

BP-fatherst; BP-fathers LG BP-mothers more than vice versa, but they also LG themselves more. Bars represent Mean ± SEM. *** $P < 0.001$, NS = not significant.

Pup exposure

BP primiparous prairie vole parents left the nest (and pups) exposed at a very low rate (**Figure 3.4B, "BP-Observed"**)—around 2-4% of observations. The BP nest exposure rate was significantly lower than the SM nest exposure rate; both increased over time (**Figure 3.4B**; Two-factor ANOVA; PND: $F(1,3) = 2.706$, $P = 0.060$, Group: $F(1,12) = 25.992$, $P < 0.001$, PND x Group Interaction: $F(1,3) = 2.095$, $P = 0.118$).

In the BP condition, I again found parental coordination: the "observed" exposure rate was significantly lower than "expected" throughout the observation period (**Figure 3.4B**; Two-factor ANOVA: PND: $F(1,3) = 3.668$, $P = 0.032$, Obs-vs-Exp: $F(1,1) = 17.555$, $P = 0.006$, PND x Obs-vs-Exp: $F(1,3) = 5.182$, $P = 0.009$). *Post hoc* paired comparisons revealed that BP-Observed frequencies were significantly lower than expected for each of the PND bins in which pairs were caring for pups ($P < 0.05$).

Nest occupancy swaps

Study 1 had shown a significant increase in mother-father nest occupancy swaps starting a few days after birth, again suggesting parental coordination. Mother-to-father and father-to-mother swaps are only 2 of the 16 possible observation-to-observation permutations available to BP adults—compared to four for a SM. The data here again revealed an increase in parent swaps during the early neonatal period, but it also revealed several other parental dynamics. After a period of huddling, fathers tended to leave

mothers alone more often than mothers left fathers alone (Paired t-test: P = 0.09; data not shown). When left tending the nest alone, fathers were significantly more likely to then leave the pups alone than were mothers (Paired t-test: P = 0.045; data not shown). For the most part, however, fathers participated in nest occupancy swaps and transitions in a way that decreased the frequency of nest exposure.

In the SM units, nest transitions that resulted in nest exposure occurred at a significantly higher rate than in BP units. SMs exposed the nest significantly more often than either BP-mothers or BP-fathers, and SM-reared pups experienced a greater number of consecutive observations with no parent (data not shown).

Licking and grooming

Prairie voles LG pups, each other, and themselves. Since study 1 had shown that BP-males LG pups less often than BP-females, I examined whether they simply LG less or shift the focus of their LG activity. I also investigated whether SMs increase their rate of LG at any point in the postnatal period compared to BP-mothers.

With respect to total amount of LG (pup, partner, and self), BP-fathers and SMs display essentially the same rate as BP-mothers, and this rate decreases over time (**Figure 3.4C "Total"**; PND: $F(1,3) = 46.545$, $P < 0.001$, Parent: $F(1,18) = 0.079$, PND x Parent Interaction: $F(1,6) = 1.700$, $P = 0.139$). When LG was analyzed based on the object of attention (i.e., pups, partner, or self), there were several interesting findings (Three-way ANOVA: PND: $F(1,3) = 38.587$, $P < 0.001$, Parent: $F(1,18) = 0.100$, $P = 0.905$, LG-object: $F(1,2) = 335.408$, $P < 0.001$, PND x Parent Interaction: $F(1,6) = 1.410$, $P = 0.214$, PND x LG Interaction: $F(1,6) = 34.161$, $P < 0.001$, PND x Parent x LG Interaction:

$F(1,12) = 2.551$, $P = 0.004$). *Post hoc* tests revealed that: (1) as expected, pups are the object of most LG bouts (pups > partner, $P < 0.001$; pups > self, $P < 0.001$); (2) as in study 1, BP-fathers LG pups at a significantly lower frequency than BP-mothers ($P = 0.016$), and both decrease pup-directed LG over time; (3) SMs LG at a statistically indistinguishable rate compared to BP-females throughout the post-natal period (i.e., they do not maintain a high rate of LG; **Figure 3.4C, "Pups"**; BP-mothers vs SMs: $P = 0.985$); (4) in support of our hypothesis, I found that BP-fathers LG their partner more than BP-mothers LG (**Figure 3.4C, "Partner"**; $P = 0.001$); but (5) contrary to our hypothesis, this increase in partner-directed LG did not completely account for lower pup-directed LG among BP-fathers: in addition to partner-directed LG, BP-fathers also participated in significantly more self-LG, or autogrooming, than did either BP-mothers or SMs (**Figure 3.4C, "Self"**; $P < 0.001$ and $P < 0.001$, respectively). There were no statistical differences in self-LG between BP-females and SMs ($P = 0.473$).

LG dynamics were again nest occupancy dependent. As in study 1, I found that mothers and fathers LG pups at essentially equal rates when attending to the nest alone ($P = 0.479$) and that both mothers and fathers significantly decrease pup-directed LG when the partner is on the nest ($P < 0.001$ for both), with the decrease being greater in fathers (**Figure 3.5A**). In this study, I found that neither mothers nor fathers engage in much self-LG when alone with the pups, but when together on the nest, males engage in a significantly greater frequency of partner-directed and self-directed LG than do females ($P = 0.009$ and $P < 0.001$, respectively; **Figure 3.5A**).

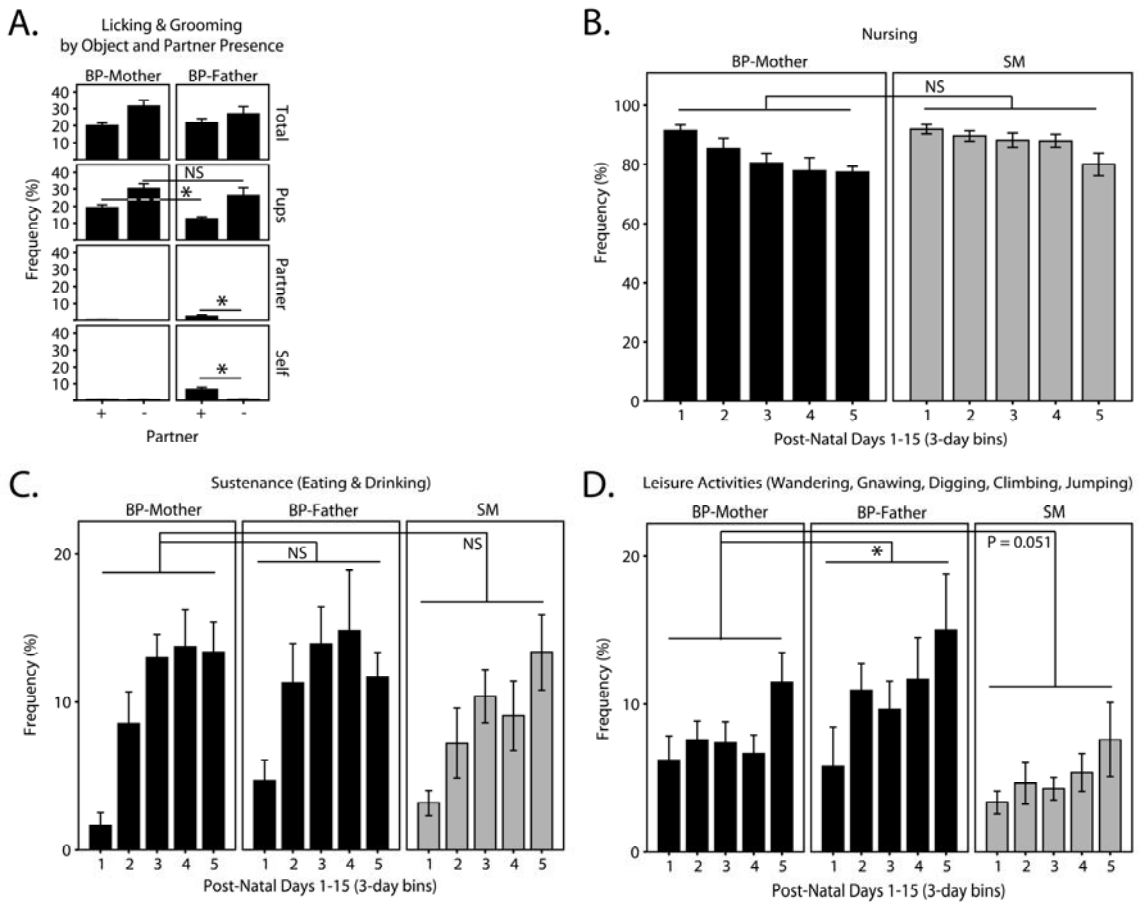

Figure 3.5 - Biparental (BP) versus single-mother (SM) nursing, sustenance, and exploratory activities

Other BP and SM Behaviors. (**A**) BP-mothers and BP-fathers again demonstrate equal pup-directed LG when attending the nest alone, and BP-fathers engage in partner- and self-directed LG predominately when huddling with the partner. (**B**) While it looks like SMs nurse at higher rates, this trend was not statistically significant. (**C**) In all three parents, eating and drinking occurs at low frequencies after birth and then increase significantly with time; there is no group difference. (**D**) BP-fathers engage in a slightly higher frequency of exploratory activities (wandering, gnawing, digging, climbing, jumping) than BP-mothers; BP-mothers have a trend toward a higher rate than SMs ($P = 0.051$). Bars represent Mean ± SEM. * $P < 0.05$, NS = not significant.

Nursing

Nursing rates decreased over time, but nursing rates between BP- and SM-family units did not differ statistically (supplemental **Fig S1-B**; Two-way ANOVA; PND: $F(1,3)$

= 5.615, P = 0.003, Group: F(1,12) = 4.474, P = 0.054, PND x Group Interaction: F(1,3) = 1.251, P = 0.305).

Sustenance

During the first 3 days post-birth, mothers and fathers had relatively low frequencies of eating and drinking, but increased over time. There were no differences in the rate of sustenance related activities (eating or drinking) between BP-fathers and BP-mothers or BP-mothers and SMs (**Figure 3.5C**; Two-way ANOVA; PND: PND: F(1,3) = 23.480, P < 0.001, Parent: F(1,18) = 1.069, P = 0.364, PND x Parent Interaction: F(1,6) = 0.770, P = 0.597; LSD *Post hoc* tests: P > 0.163 for all pair-wise comparisons).

Exploratory activities

Unlike food and water activities, there were minor differences in the frequency of time animals spent in "exploratory" activities, such gnawing, wandering, digging (likely a degraded form of tunnel maintenance, which is an indirect parenting behavior), and jumping and climbing (Two-way ANOVA; PND: F(1,2) = 3.609, P = 0.036, Parent: F(1,18) = 3.865, P = 0.037, PND x Parent Interaction: F(1,4) = 0.605, P = 0.661). BP-fathers had significantly more exploratory time than BP-mothers (P = 0.035) and BP-mothers had a near significant trend toward more exploratory time than SMs post-birth (**Figure 3.5D**; P = 0.051).

DISCUSSION

Family relationships are known to regulate the developmental trajectories of behavior, mental state, and vulnerability to psychopathology in humans (Maccoby, 2000; Meaney, 2001; Schor, 2003; Sarkadi et al., 2008), but our understanding of the neurobiology that mediates this connection is poor.

The use of traditional animal models, such as rats and mice, has made extraordinary contributions to our understanding of how severe and subtle variations in mother-infant bonding can influence offspring development (Heim and Nemeroff, 2001; Levine, 2001; Meaney, 2001; Meaney et al., 2002; Pryce and Feldon, 2003; Moriceau and Sullivan, 2005; Kosten et al., 2006; Sanchez, 2006). The impact of variations in biparental family dynamics is often overlooked. Biparental animal models may be able to fill this gap, but only if I have a proper understanding of their biparental family dynamics.

The current series of studies aimed to address this issue for the monogamous prairie vole—a highly social rodent species that has proved extraordinarily insightful for the neurobiology and genetics of pair bonding and social cognition (Carter et al., 1995; Young and Wang, 2004; Donaldson and Young, 2008; McGraw and Young, 2009). The results support several main findings: (1) primiparous prairie voles, with no previous parenting experience, participate in a rich set of familial interactions and nesting duties, such as nest occupancy, LG, and nursing, that are coordinated and dynamic over time; (2) maternal behavior is relatively resilient to removal of the father throughout the post-natal period, creating a stable, repeatable early-life manipulation that does not depend on the screening animals for certain behavioral profiles as in often required in rats (Meaney, 2001). By first more clearly testing hypotheses about how biparents coordinate, I then

was able to establish how robust certain behavioral profiles are and how they change (or do not change) in response to removal of the male. This creates an ethologically relevant manipulation that can be used to study the long-term consequences of family unit composition on the next generation (which will be the focus of Chapters 4 and 5).

Parental coordination over time

Prairie voles have been used extensively in field, semi-natural, and laboratory research environments to study the evolutionary and ecological pressures that drive divergence (and convergence) in social and mating structures (Hartung and Dewsbury, 1979; Thomas and Birney, 1979; Getz et al., 1981; McGuire and Novak, 1984; McGuire et al., 1992; Wang and Novak, 1992; Carter et al., 1995; Getz and Carter, 1996; Lonstein and De Vries, 2000; McGuire et al., 2007). Here, in study 1, I show for the first time empirical data demonstrating robust, coordinated biparental care of primiparous prairie vole parents confined to laboratory caging throughout the post-natal period. Primiparous mothers and fathers participated in all components of parental behavior (except nursing), including nest occupancy, brooding, and LG—social interactions that are important for rodents and rodent development (Kuhn et al., 1978; Meaney, 2001). These findings fit well with descriptions of experienced prairie vole parents in semi-natural and field environments (McGuire and Novak, 1984; Oliveras and Novak, 1986; Wang and Novak, 1992; Solomon, 1993; McGuire et al., 2007). Importantly, our findings demonstrate that primiparous parents not only coordinate nest attendance competently (**Figure 3.1B & 3.4B**), but also coordinate LG behaviors and nursing postures, two findings that, to our knowledge, have never been described previously in monogamous rodents.

For small rodents with altricial young, managing nest occupancy is essential. In addition to discouraging the predation of young nestlings (Getz et al., 1992b), parental contact helps maintain warmth and the concomitant LG helps convey important social and environmental information (Meaney, 2001). I had predicted that, since there are two parents, they might coordinate nest attendance in order to minimize pup exposure. Indeed, I found that pups were exposed less than would be expected if they did not coordinate (**Figure 3.1B**). I also found that, despite increases after the first 3 PNDs in sustenance and exploratory activities by both parents, pup exposure remained low and got even lower. This was accomplished, in part, by increasing the number of mother/father nest occupancy swaps (**Figure 3.1C**). In a few cases, I even observed nest coordination being actively negotiated and enforced. For example, one parent was observed dragging the other to the nest and then enforcing the swap. While type of parental negotiation and enforcement has been noted in other biparental species (Libhaber and Eilam, 2004), it had not yet been noted in prairie voles (McGuire et al., 2007).

The coordination of LG was also interesting. Grooming, which is often accompanied by licking in rodents, is an important behavioral means for conveying social information and maintaining social bonds (Sachs, 1988; Fernandez-Duque et al., 1997; Meaney, 2001); it is also associated with the behavioral and neurobiological development of offspring (Francis et al., 2000; Meaney, 2001; Francis et al., 2002; Weaver et al., 2004; Branchi, 2008; Champagne and Curley, 2008). A few investigators (Solomon, 1993; McGuire et al., 2007), but not all (Hartung and Dewsbury, 1979), have noted that prairie vole mothers LG pups more often than fathers and concluded that there is an inherent sex difference. Our data suggest that the sex difference is more subtle. I found that, overall,

total LG behavior (pup-, partner-, and self-directed) is essentially equivalent between sexes. The sex difference instead arises out of how the two parents coordinate their LG behaviors. In both study 1 and 2, BP mothers and fathers LG pups at equal frequencies when alone with the pups (i.e., no sex difference when the sole nest attendant); but they have markedly different LG profiles when attending to the nest together: mothers continue to focus most LG bouts toward the pups, while the fathers shift their LG attention toward the partner and themselves. Our data suggest there is a complex interplay of social interactions mediated by LG and that these may have important implications for maintaining partner bonds and regulating pup development.

While the nest occupancy coordination and LG data most powerfully convey the importance of studying biparental care as a dynamic system, nursing posture was also influenced in a coordinated fashion. Nursing posture is often studied because of its importance in rodent development. Here, I found that, while overall nursing frequencies are unaffected by nest occupancy dynamics, nursing posture frequencies were. Nursing was significantly more active when the mother was alone than when huddled with her partner. Whether due to the mechanics of huddling or an increase in vigilance, the presence of the partner dynamically regulates how the mother interacts with pups and thus may indirectly affect pup development. Unfortunately, little is known about whether nursing posture impacts pup development in this species.

Together, these data illustrate a substantial degree of parental coordination. It would be interesting to see if similar coordination profiles would emerge even in natural and semi-natural environments, where the father tends to become more involved in indirect parenting (e.g., tunnel maintenance; Solomon, 1993; McGuire et al., 2007), and

whether they would show the same dynamic changes throughout the post-natal period. More importantly, however, is what manipulations of these family dynamics can reveal about the neurobiology of biparental family life. Oxytocin and vasopressin are neuropeptides that are instrumental in the initiation of bonding. How would pharmacological or genetic manipulations of these systems in the parents alter coordination or the mother-father interactions? How would changes resulting from these manipulations affect the neurobiological and behavioral development of the pups? To begin to address these types of questions, I manipulated family structure in an ethologically relevant way and examined how single-mothers (SMs) would respond.

Single-mother parenting

As shown in study 1 and 2, prairie voles coordinate their behavior to decrease nest exposure, and both contribute relatively high rates of pup-directed LG. I predicted that, in response to the lack of the male, SMs would compensate by increasing, or maintaining high levels of, pup care throughout the post-natal period. Contrary to our predictions, SMs demonstrated essentially no parental compensation throughout the first two weeks of the postnatal period (**Figure 3.4**).

This corroborates and extends work by McGuire and colleagues (2007). McGuire et al. had observed BP and SM prairie vole parenting behavior in large semi-natural enclosures (1300 cm^2) for the first 3 PNDs and found no evidence for parental compensation on the part of SMs (McGuire et al., 2007). While informative, I had suspected that the increased foraging demands of a semi-natural environment and the already high levels of maternal care during the post-partum period might have obscured

long-term compensation. Our data demonstrates that even after two weeks, the only indication of compensation was a modest decrease in exploratory behavior on the part of SMs (**Figure 3.5D**).

This study clearly demonstrates a new approach to the biomedical investigation of how family environment can contribute to the development of behavior and emotional balance in the offspring. Up until now, one of the most popular methods for studying the effects of relatively subtle differences in early life experience on development has been the use of high- and low- LG rats (Meaney, 2001). In addition to the obvious drawback that only the mother-infant relationship can be investigated, it also depends on screening mothers for relative extremes in maternal behavior. The use of the biparental prairie voles in the laboratory environment addresses both of these issues: it adds the paternal component and provides an easy, reliable way of generating differences in the amount of parenting pups receive without any sort of selection bias that may simultaneously skew genetic factors.

Without compensation on the part of SMs, the result for the offspring is an overall lowering in the rate of direct parental care received by SM-reared pups. In many respects, the lack of the second adult may simply result in lost environmental enrichment. Indeed, in California mouse (*Peromyscus californicus*), another monogamous, biparental rodent, SM-rearing results in decreased cognitive development that can, in part, be compensated for by handling and thus increased maternal LG (Bredy et al., 2004). Likewise, SM rearing in the biparental trumpeted-rat (*Degus octodon*) decreases the amount of direct parental care and results in altered synapse formation in the anterior cingluate in offspring in a way that may alter emotional behavior (Ovtscharoff et al., 2006).

In other respects, however, SM rearing may change the quality of care received, not just the quantity. For example, in California mouse, paternal pup-retrievals differ in frequency and quality from maternal retrievals in a way that results in paternally mediated effects on adult aggression in offspring (Frazier et al., 2006). While, aggression has not been tested in SM-reared California mice, it is plausible that the similar types of quantitative and qualitative differences in pup care across the two rearing conditions are influencing the development of prairie vole offspring. Future research (some of which has now been conducted and is presented in chapters 4 and 5) will be needed to examine the long-term impact of differences in family structure on the development of prairie vole offspring. Of special interest will be the development of the neuropeptide systems that regulate social behavior.

Conclusions

As noted in the introduction, human family dynamics are associated with a broad range of developmental outcomes for children and adults, alike. Unfortunately, some differences and shifts in family life increase the vulnerability to certain psychopathologies, such a borderline personality disorder, eating disorders, depression, anxiety, and others (Frank and Paris, 1981; Zanarini and Frankenburg, 1997; Maccoby, 2000; Meaney, 2001; Schor, 2003; Zanarini and Frankenburg, 2007; Sarkadi et al., 2008; Enten and Golan, 2009). Many of these exhibit elements of abnormal social behavior. Now that I have tested specific hypotheses about prairie vole family dynamics, it may be possible to use them to gain greater insight into how variations in family systems influence the neurobiological and behavioral development of offspring. I predict that

studying prairie voles in this context will extend and complement the use of prairie voles as an informative model for the study of social neurobiology and genetics (McGraw and Young, 2009), and may provide biological insight into family and child psychology.

Disclosure / Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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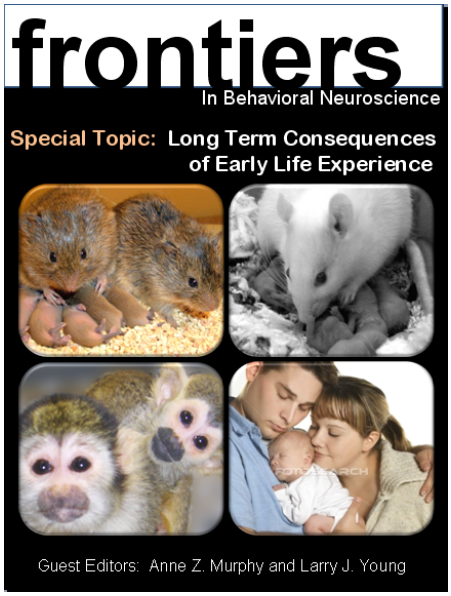
CHAPTER 4

The impact of early life family structure on adult attachment, alloparenting, and parenting in monogamous prairie voles



Adapted from:

Ahern TH, Young LJ (2009) The impact of early life family structure on adult social attachment, alloparental behavior, and the neuropeptide systems regulating affiliative behaviors in the monogamous prairie vole (*Microtus ochrogaster*). *Frontiers in Behavioral Neuroscience* 3:17

and

Ahern TH, Hammock EAD, Young LJ (*to be submitted*) Parental division of labor and the effects of family structure on parenting in monogamous prairie voles (*Microtus ochrogaster*).

ABSTRACT

Early social attachments lie at the heart of emotional and social development in many mammals, including humans. In nature, monogamous prairie voles (*Microtus ochrogaster*) experience considerable natural variation in early social attachment opportunities due to differences in family structure and dynamics (e.g., single-mothers, solitary breeding pairs, and communal groups). Previous work (see Chapter 3) has demonstrated striking differences in the frequency of direct parental care received by pups under biparental (BP) and single-mother (SM) conditions. I exploited this ethologically-relevant difference to examine how structural differences in family environment can affect the social behavior of adult offspring. In adulthood, there were striking differences: SM-reared females showed low spontaneous, pup-directed alloparental behavior ($P<0.01$) and both males and females from the SM-reared condition showed a delay in the onset of a stable partner preference formation. SM-reared offspring also interacting with their own pups differently (lower LG frequency; $P < 0.01$), suggesting the possibility for transgenerational effects. None of these differences was clearly explained by changes in stress-reactivity. Overall, these results demonstrate that naturalistic variation in social rearing conditions can introduce diversity into adult nurturing and attachment behaviors.

INTRODUCTION

Early life experience exerts a profound and persistent influence on a broad range of developmental trajectories, from emotional to physical. For example, in humans, abuse and neglect confer a vulnerability to depression, anxiety, alcoholism, obesity, and even heart disease, while parental warmth and affection can lead to resistance and resilience (for review, see Meaney, 2001). In rodents and nonhuman primates, variation in early care regulates adult stress reactivity, exploratory behavior, learning and memory, and sensitivity to drugs of abuse (Francis et al., 1999; Liu et al., 2000; Levine, 2001; Meaney, 2001; Sanchez, 2006). Especially striking is the capacity of parenting to perpetuate particular behavioral profiles from one generation to the next, such as abuse behavior in macaques and licking and grooming behavior in rats (Francis et al., 1999; Meaney, 2001; Sanchez, 2006). At the heart of this connection between early life and adult behavior lies early social attachment (Bowlby, 1973, 1982; Sable, 2004). Situated within the theoretical framework of attachment theory, the use of animal models is beginning to provide neurobiological insights into how early social environment can introduce and perpetuate individual variation in a variety of behavioral phenotypes (Meaney, 2001; Sable, 2004). In this study, I was particularly interested in how early life attachment factors guide expectations and behaviors in later social relationships.

Human rearing conditions are remarkably diverse. One of the most distinct factors producing variation in early life social experience is family structure and composition. Prairie voles (*Microtus ochrogaster*) provide an excellent opportunity to experimentally examine the effects of early life social experience on adult sociality. Prairie voles are small, genetically diverse, microtine rodents that adapt easily to laboratory environments

and possess a rich repertoire of social behaviors (Carter et al., 1995; Young and Wang, 2004). Virgins of both sexes are highly social and exhibit spontaneous nurturing (or alloparental) care toward unrelated pups; adults form selective social attachments (or pair bonds) between mates; and breeding pairs usually display robust biparental care of offspring (Getz et al., 1981; Getz et al., 1993; Carter et al., 1995; Lonstein and De Vries, 2000; Young and Wang, 2004; Hammock and Young, 2005). In the wild, prairie voles maintain varied and often complex family structures. For most of the year, approximately one-third (37%) of family units are single-mothers, one-third (36%) are male/female breeder pairs, and one-third (27%) are small communal groups consisting of a breeder pair and several reproductively inactive alloparents (Getz and Carter, 1996). Thus, variation in family structure offers a superb ethologically relevant model in which to examine how differences in early social environment influence the development of adult social nurturing and bonding behaviors.

Another advantage of using prairie voles is the large body of knowledge regarding the neurobiological mechanisms that regulate sociality. For example, oxytocin (OT) modulates spontaneous alloparental behavior and plays a central role in the formation of partner preferences, the laboratory proxy of pair bonds, in female prairie voles (Williams et al., 1992a; Insel and Hulihan, 1995; Olazábal and Young, 2006b). Arginine vasopressin (AVP), acting through the vasopressin 1a receptor (V1aR), shows similar effects, predominately in males (Winslow et al., 1993; Wang et al., 1994). These two neuropeptide systems, as well as the corticotropin-releasing factor (CRF) and dopamine (DA) systems, have all been shown to play a role in complex prosocial behavior in prairie

voles (DeVries et al., 2002; Aragona et al., 2003; Young and Wang, 2004; Lim et al., 2007).

Interestingly, prairie voles display remarkable individual variation in social behaviors, which have in some cases been linked to variation in neuropeptide systems. For example, in nature, approximately 40% of males adopt a wandering, rather than a pair-bonded, mating strategy (Getz and Carter, 1996). Variation in V1aR expression patterns has been associated with pair bonding in the laboratory and linked to reproductive success in naturalistic enclosures (Hammock and Young, 2005; Ophir et al., 2008b; Ophir et al., 2008a). In addition, female prairie voles display remarkable individual variation in spontaneous alloparental behavior toward pups. Approximately 50-70% of virgin females respond favorably to unrelated pups by displaying alloparental nurturing behavior, while the remainder either ignore or attack them (Lonstein and De Vries, 2001; Bales et al., 2004; Olazábal and Young, 2005). Females that display alloparental behavior have higher densities of OT receptor (OTR) in the nucleus accumbens (NAcc), and blockade of those receptors inhibits alloparental behavior. The relative contribution of genetics and early family experience in producing this variation in social behavior is unknown.

In this study, I exploited some of the natural variation in prairie vole family structure to examine the influence of early life social experience on later-life social behavior. Specifically, I examined the adult social behavior of prairie voles raised in single mother (SM) or biparental (BP) rearing conditions. Our results suggest that early life rearing conditions can profoundly affect adult prosocial behavior.

MATERIALS AND METHODS

Animals

All animals used in this study were bred in our laboratory at Emory University. Our colony is derived from field-caught stock originating from Illinois and is consistently outbred to maintain genetic diversity. Breeder pairs were housed in large ventilated breeder cages (34 cm long x 30 cm wide x 18 cm high) lined with bedding (bed-o-cob, Maumee, OH) and a single nestlet. Pairs were maintained at 22°C on a 14:10 light-dark schedule with lights on at 06:00 h and had *ad libitum* access to food (LabDiet, rabbit chow) and water. Cages were changed weekly. At 18 days post-pairing, all males were handled and spot-shaved dorsally to distinguish male from female. Males that had been randomly assigned to the single-mother (SM) group were removed permanently to another room. Day 18 was used because earlier removals had been shown to influence *in utero* pup viability (McGuire et al., 1992). Males in the BP group were simply returned to the breeding home-cage after being handled and shaved. Pups were born between day 24-28 post-pairing. Prairie vole litter sizes are variable (ranging from 2 to 6), so litters were culled to provide approximately equal numbers of 2, 3, and 4 pup litters in each type of rearing condition. Pups were weaned at 21 d of age (PND22) and housed in same-sex, same-group pairs or trios. Same-group cage assignments were used to avoid having individuals from one particular group consistently be the smallest or least mature in a given cage.

All experimental offspring were derived from three strategically planned breeding cohorts (**Table 3.1**). Cohort 3 pups, which were derived from 24 family units (BP = 12, SM = 12), were weaned at PND22 and remained unmanipulated until adulthood when

they underwent open-field, elevated plus maze, and naïve spontaneous alloparental behavior testing, with a week interval between each test (PND65 to PND90). Cohort 4 pups, which were derived from another 24 family units (BP = 12, SM = 12), were weaned at PND22 and remained behaviorally unmanipulated until adulthood (PND90) when they were paired and tested for social attachment behavior in the partner preference test. Cohort 5 pups were derived from 36 (BP = 18, SM = 18) breeder pairs. At PND22 they were weaned and then left unmanipulated until approximately 90 d of age. From these unmanipulated cohort 3 adults, I created thirty-one male-female in large breeder cages. Each breeder pair consisted of one BP-reared male and one BP-reared female (BP/BP pairs = 14) *or* one SM-reared male and one SM-reared female (SM/SM pairs = 17). No pair consisted of siblings; and, at maximum, only one male and one female were used from each cohort 3 litter. Approximately 18-20 d post-pairing, males were handled and shaved dorsally. All males were returned to their partners. Twenty-six of these pairs produced pups (BP/BP = 11, 79%; SM/SM = 15, 88%).

Offspring weights

All pups from cohort 3 were weighed on the day of birth (PND1), then weekly until weaning at PND22. Pups were gently removed from the nipples and placed in a plastic weigh container mounted on a scale sensitive to 0.01 grams. Animals were again weighed in adulthood (PND65 + PND90), using the same scale.

A 2 (sex) x 2 (group) x 2 (PND) ANOVA was used to analyze mass at weaning and in adulthood of cohort 3 offspring, with PND as a repeated measure. *Post hoc* pair-

wise comparisons within each time point were used to examine group differences. $P < 0.05$ was considered statistically significant.

Open-field

To examine adult emotional and spontaneous nurturing behavior, cohort 3 animals were bred in the same manner as cohort I. At ~60 days of age, all cohort 3 (**Table 3.1**) offspring were moved to a behavior room, allowed to acclimate at least one hour, and then underwent open-field testing (Males: BP = 10, SM = 12; Females: BP = 12, SM = 10; one male and/or one female per litter). Between 09:00-11:00 h, rounds of four animals (2 males + 2 females) were run simultaneously for 20 min in 40 cm x 40 cm x 40 cm Plexiglas boxes separated by opaque white material and video-recorded from above; boxes were designated for male or female use only. Between each round, the open-field boxes were cleaned with a mild detergent (Alconox), wiped dry, cleaned with deionized water, and wiped dry again. The video-recordings were analyzed *post hoc* via an automated video-tracking program, EthoVision 3.0 (Noldus Information Technology, The Netherlands). Using features within the program, each open-field chamber was divided into the perimeter (wall + 10 cm) and the center (20 cm x 20 cm)—matching taped outlines applied to the underside of the boxes themselves. Behavior was processed in four consecutive 5 min time intervals. The first and last 5 min intervals were analyzed: the first represented responses to novelty and has been shown to be sensitive to early life experience (Meaney, 2001), while the last interval represented general activity. Behavioral measures included frequency (counts) in center, total time in center, and distance moved in the entire arena. The experimenter was blind to rearing condition.

Each measure was divided by group and sex and tested for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and for homogeneity of variance (Levene's test). Due to non-normality, all data from all groups and sexes were square-root transformed, tested for normality and homogeneity again, and then analyzed using a 2 (group) x 2 (sex) x 2 (interval) repeated measures ANOVA, with interval as the repeated measure. Each test was followed by appropriate *post hoc* tests and pair-wise comparisons.

Elevated plus maze

Approximately one week after open-field testing, the same cohort 3 animals were moved to a behavior room (Males: BP = 10, SM = 12; Females: BP = 12, SM = 10; again, only one male and one female from each litter), allowed to acclimate at least one hour, and then underwent elevated plus maze (EPM) testing between 09:00-11:00 h. The apparatus, which was raised 80 cm above the floor, measured 6 cm x 6 cm at the center platform, and the center had two open arms (50 cm x 6 cm x 0.6 cm) and two closed arms (50 cm x 6 cm x 15.5 cm) extending from it. Each animal was placed in the center and allowed to roam for 5 min. Behavior was video-recorded from above. As with the open-field, behaviors were analyzed by EthoVision 3.0. Raw measures included frequency (counts) and total time in each type of arm, and total distance moved in the entire arena. Behavioral parameters relevant to anxiety and exploration were then calculated: percent entries into the open arms (open arms count / [open arms count + closed arms count]), percent time in open arms (time in open arms / [time in open arms + time in closed arms]), and total distance moved. The EPM is often used as an anxiety assay in rodents and has also been used in prairie voles (Pitkow et al., 2001; Olazábal and Young, 2005; Stowe et

al., 2005). Each measure was divided by group and sex and tested for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and for homogeneity of variance (Levene's test). Due to non-normality, all data from all groups and sexes were square-root transformed, tested again for normality and homogeneity, and then analyzed using a 2 (group) x 2 (sex) ANOVA. Each test was followed by appropriate *post hoc* tests and pair-wise comparisons on the transformed data. Two animals (BP = 1 male, SM = 1 male) were excluded from the analysis because they fell from the apparatus.

Spontaneous alloparenting test

Approximately one week post-EPM testing, the same cohort 3 animals were moved to a behavior room, allowed to acclimate for at least one hour, and then examined for naïve spontaneous alloparental behavior between 0900-1100 hrs. Prior to testing, each animal was moved to a large polycarbonate cage (19 cm x 45 cm x 30 cm) lined with bedding (bed-o-cob) and allowed to acclimate for approximately 25 min. After acclimation, testing began when 2 pups (2-5 d of age) were placed in the cage opposite of the test animal. Animals were then observed by a trained experimenter blind to rearing condition and performance on previous tests. The following behavior categories were rated with the assistance of Stopwatch+ (available to download at http://www.cbn-atl.org/research/stopwatch.shtml): latency to approach pups, number of animals that attacked pups, time immobile over at least one pup, time licking and grooming at least one pup, and time away from pups (not in contact). A detailed posture analysis was not performed. If the test animal did attack the pups, the test was stopped immediately and pups were returned to their mother. While both males and females show spontaneous

alloparental behavior, I was not concerned with a direct comparison of sex differences within rearing groups. After excluding animals that attacked pups, the spontaneous alloparental behavior data was split by sex (Males: BP = 8, SM = 11; Females: BP = 7, SM = 9) and then each behavior was analyzed using a 2 (group) Independent samples Kruskal-Wallis H, Monte Carlo exact non-parametric test, due to the extreme non-normality of the data. Finally, all animals were categorized as either parental or non-parental based on previously established criteria: >5s LG and >30s huddling or crouching over at least one of the pups (Olazábal and Young, 2005). Chi-squared tests compared the proportion of parental animals across groups, within each sex.

Partner preference test

To examine the formation of partner preferences, cohort 4 BP- and SM-reared animals (90 d old) were paired at 08:00 hr with sexually- and behaviorally-naïve colony animals of the opposite sex in a clean cage with *ad libitum* access to food and water and allowed to cohabitate for 24 hrs (N = 12/sex/group; one male and/or one female per litter). The females were unprimed and therefore not immediately sexually receptive. The next morning, at 08:00 hr, all cohabitated animals were moved to a behavior testing room. Females were examined vaginally to determine if mating had likely occurred by rating the presence or absence of vaginal redness and the presence or absence of vaginal opening. Only animals that had both vaginal redness and vaginal opening were considered to have possibly mated. All test animals then underwent a 3-hr partner preference (PP) test.

Briefly, the PP test consists of a socially "neutral" chamber flanked by two stimulus chambers (Williams et al., 1992a; Young and Wang, 2004). The "partner" animal is tethered in one stimulus chamber, while a "stranger" animal of similar sociosexual valence is tethered in the other. The strangers underwent the same cohabitation and testing regimen as the test and partner animals. The experimental animal is placed in the center, neutral chamber and is allowed to roam freely for 3 hrs. Following the 3-hr test, all animals were returned to their cohabitation cages and allowed to cohabitate for 24 more hours (a total 48 cohabitation hours) and tested again in the same manner; the "partner" remained the same, but the "stranger" was new. Animals were again returned to their cohabitation cage after testing and remained together for another 5 days (a total of 1 week of cohabitation) and then tested again—for a total of 3 PP tests per experimental animal. Regardless of time point, all tests were video-recorded and analyzed *post hoc* for time spent huddling and number of center cage-crossings by an automated software system (SocialScan 2.0, Clever Sys Inc., Reston, VA, USA). Automated scoring for huddling with SocialScan has been validated for use with the partner preference test and demonstrates a remarkably high correlation with manual scoring ($R > 0.99$ compared to human raters, see **Appendix 1**; Ahern et al., 2009). Partner preference data was analyzed using a three-way ANOVA for group, sex, and stimulus animal as factors. Significant interactions were followed up with *a priori* tests to examine partner preference for each group at each time point.

Animals from cohort 5 also underwent partner preference testing in the same manner. 24 females (BP/BP = 12 and SM/SM = 12) were tested after 24 hrs of cohabitation. After testing, females and male partners were returned to their large breeder

cages, where they remained throughout gestation and the family observations (see below). As with the cohort 4 data, cohort 5 videos were processed for huddling and chamber crossings (a proxy of locomotor activity) using the SocialScan automated system. Here, separate t-tests were used to examine partner preference in each group.

Next-generation family unit observations

All twenty-six cohort 5 pairs that gave birth (BP/BP = 11, 79%; SM/SM = 15, 88%) were observed as distinct family units in a manner similar to that presented in Chapter 3, study 2. Observation sessions occurred three times per day for one-hour each.. Observation sessions occurred at lights ON (0600-0700 hrs), noon (1200-1300), and late afternoon (1600-1700). The observational period lasted for 7 PNDs.

Data were again collected by visual spot-checks and data were logged based on three basic categories outlined in Chapter 3. Of these, I focused exclusively on two social behaviors for our statistical analysis: huddling and LG. Since this experiment aimed to test social parameters rather than PND effects, the entire 7 PND observation period was averaged before statistical analysis. Frequency huddling was tested using a t-test; nest exposure was tested using a two-way mixed ANOVA, with group as one factor and observed-vs-expected as a repeated measure. Time-of-day was again found to have no significant effect and was dropped from all analyses.

RESULTS

Pup weights

There was a significant main effect of rearing condition on pup weight ($F_{1,51}$ = 15.151, P < 0.001), but no sex or rearing-condition by sex interaction effects. *Post hoc* tests demonstrated that SM-reared animals weighed less at weaning than BP-reared animals, regardless of sex (P < 0.01; **Figure 4.1**). By adulthood (PND65), however, SM-reared animals from both sexes had achieved mass weights statistically indistinguishable from BP-reared animals (P = 0.469).

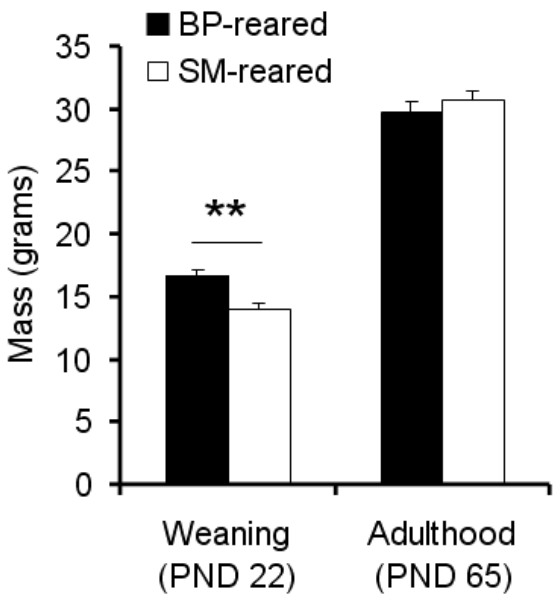


Figure 4.1 - The effect of rearing condition on pup weights
SM- and BP-reared pups were weighed to obtain maturational measurements. SM-reared pups weighed less at weaning than BP-reared offspring, but were statistically indistinguishable in adulthood. Bar graphs represent Means + SEM. ■ = BP-reared pups, □ = SM-reared pups. ** P < 0.01.

Adult offspring spontaneous alloparental care

Latency to approach

Mann-Whitney non-parametric analysis revealed no group differences between BP- and SM-reared females (U = 38, P = 0.825; **Figure 4.2A**) or males (U = 58, P = 0.897; **Figure 4.2B**) in their latency to approach the 2-5 day old, unrelated pups.

Attacks

Chi-squared analysis revealed that there was also no group difference in the number of animals that attacked for either sex: females (BP-reared: 4/12 vs SM-reared: 1/10: $X^2(1) = 1.69$, P = 0.193) or males (BP: 2/10 vs SM: 1/12: $X^2(1) = 0.630$, P = 0.427; data not shown).

Pup-directed behavior

Mann-Whitney analyses did reveal significant group differences in pup-directed care in females. SM-reared females exhibited significantly less licking and grooming of pups (U = 8, P = 0.005; **Figure 4.2A**) and less time immobile (huddling) over pups (U = 9, P = 0.004). Males showed equal amounts of both licking and grooming (U = 45, P = 0.357; **Figure 4.2B**) and huddling over pups (U = 58, P = 0.925) across rearing conditions.

Mann-Whitney analysis also revealed a significant effect of rearing group on the amount of time test animals spent away from pups. SM-reared females spent more time away from the stimulus pups than BP-reared females (U = 17, P = 0.04; **Figure 4.2A**); males showed no group difference (U = 59, P = 0.975; **Figure 4.2B**).

Parental versus non-parental

Many studies of prairie voles categorize individuals as either spontaneously parental or non-parental. Using standard criteria (>5s LG and >30s huddling or crouching over at least one of the pups; Olazábal and Young, 2005), I categorized each of our test subjects. A significantly smaller percentage of SM-reared females were spontaneously maternal compared to BP-reared females: 17% versus 60%, respectively ($X^2(1) = 4.43$, P = 0.035; **Figure 4.2C**). SM- and BP-reared males did not differ, with 83% and 70% categorized as spontaneously paternal, respectively ($X^2(1) = 0.55$, P = 0.457; **Figure 4.2D**).

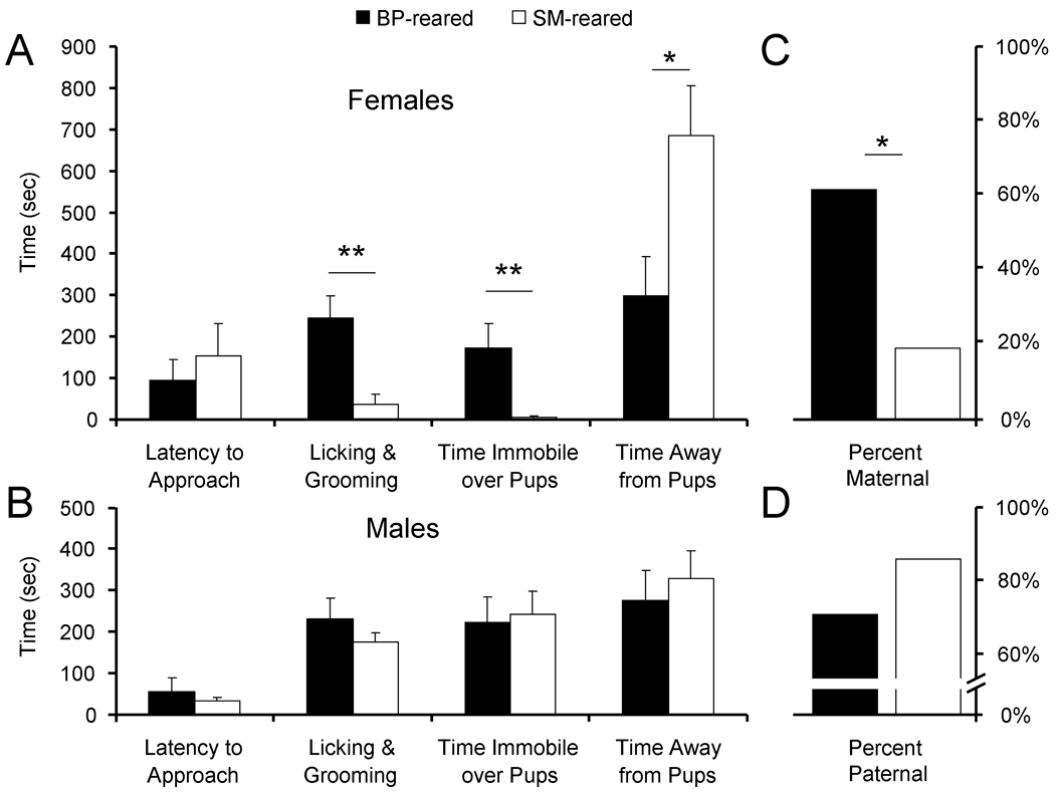


Figure 4.2 - Rearing condition influences adult alloparental behavior
SM- and BP-reared adults were tested for spontaneous alloparental nurturing behavior. SM-reared adult female offspring showed significantly lower levels of pup-directed alloparental care (time licking & grooming and time immobile over pups) and spent most of their time away from the pups, in comparison to BP-reared adult females **(A)**. SM- and BP-reared males showed no differences in key measures of alloparental behavior **(B)**. Based on previously published criteria (Olazábal and Young, 2005), individual animals from each sex and group were categorized as spontaneously maternal **(C)** or spontaneously paternal **(D)**. Bar graphs represent Means + SEM **(A + B)** or Mean percentages **(C + D)**. ■ = BP-reared adult offspring, □ = SM-reared adult offspring. * $P < 0.05$, ** $P < 0.01$.

Adult offspring partner preference formation

Cohabitation mating

Mating facilitates PP formation in prairie voles, but is not necessary given a 24 hour cohabitation period, although this varies across laboratories (Carter et al., 1995; Young and Wang, 2004). After 24 hours of cohabitation, our crude method of visual analysis which has correlated highly with actual matings observed on video showed that experimental groups from cohort 4 did not differ in the number of animals that showed indications of mating after 24 hrs (mated/total: BP-reared females (1/12) versus SM-reared females (3/12): $X^2(1) = 1.2$, $P = 0.273$; BP-reared males (2/12) versus SM-reared males (3/12): $X^2(1) = 0.253$, $P = 0.615$) and after 48 hrs of cohabitation (BP-reared females (8/12) versus SM-reared females (8/12): $X^2(1) = 0$, $P = 1$; BP-reared males (10/12) versus SM-reared males (7/12): $X^2(1) = 1.815$, $P = 0.178$). It is important to note that similar non-differences were observed if different criteria were used (e.g. only redness, only vaginal opening).

Partner preference testing

Cohort 4 BP- and SM-reared animals were assessed in the PP test at 3 time points: after 24 hrs, after 48 hrs, and after 1 week of total cohabitation time. A 2 (stimulus animal) x 2 (group) x 2 (sex) x 3 (time point) ANOVA, with time huddling as the dependent measure and with cohabitation period as a repeated measure, revealed a main effect of stimulus animal (partner vs. stranger; $F_{1,264} = 60$, $P < 0.001$), a main effect of sex ($F_{1,264} = 4.2$, $P < 0.04$), and a group by stimulus interaction ($F_{1,262}$, $P < 0.02$).

After splitting by sex and group, planned *post hoc* t-tests were used to compare time spent huddling with partner versus stranger in order to assess PP formation. A significant PP was observed after 24 hrs, 48 hrs, and at 1 week of cohabitation for both

BP-reared females (Partner > Stranger: 24hr: P = 0.014; 48hr: P = 0.015; 1wk: P < 0.001; **Figure 4.3A**) and BP-reared males (Partner > Stranger: 24hr: P = 0.04; 48hr: P = 0.001; 1wk: P = 0.002; **Figure 4.3B**). SM-reared animals, on the other hand, did not show this same PP formation profile: Both SM-reared females (**Figure 4.3A**) and males (**Figure 4.3B**) failed to show a significant PP at 24 hrs and 48 hrs, but did reach significance by 1 week (SM-reared females: Partner > Stranger: 24hr: P = 0.250; 48hr: P = 0.186; 1wk: P = 0.006; SM-reared males: Partner > Stranger: 24hr: P = 0.414; 48hr: P = 0.450; 1wk: P = 0.044).

Cohort 5 female offspring were also examined in the partner preference test. After 24 hrs of cohabitation, BP-reared females showed a significant group partner preference whereas SM-reared females did not (**Figure 4.3A**; P < 0.001 and P = 0.107, respectively). There were no differences in center chamber entries, which serves as a measure of locomotor activity (Mean entries ± SEM: BP-reared female: 133 ± 19, SM-reared female = 143 ± 28; T-test: P = 0.773).

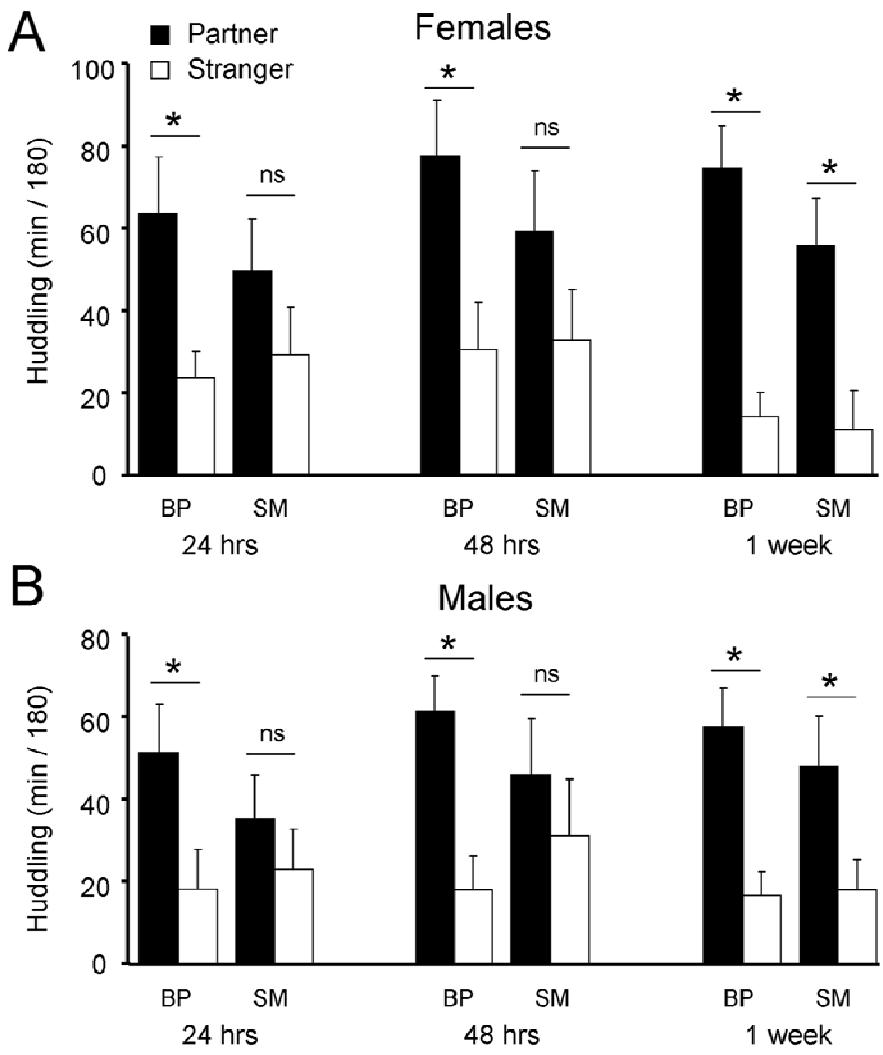


Figure 4.3 - Rearing condition influences partner preference formation
SM- and BP-reared adults were tested for social bonding behavior in the partner preference test at three time-points throughout cohabitation: 24 hrs, 48 hrs, and 1 week post-pairing. SM-reared adult females exhibited a slower onset of partner preference formation compared to BP-reared adult females **(A)**, with BP-reared females demonstrating significant group partner preferences at all three time-points and SM-reared females only during the 1 week test. SM-reared adult males also exhibited a slower onset of partner preference formation compared BP-reared adult males **(B),** again, with BP-reared males showing significant group partner preferences during all three time points, while SM-reared males demonstrated a significant partner preference only at 1 week. Bar graphs represent Means + SEM. ■ = BP-reared adult offspring; □ = SM-reared adult offspring. * $P < 0.05$, ns = not significant.

Adult offspring open-field testing

Cohort 3 animals underwent open-field testing for 20 min. To account for longitudinal changes in behavior over time and to allow comparison with typical 5 min open-field test studies, the total time was divided into 5-min intervals. The first (0-5 min) and last (15-20 min) 5-min intervals were used for statistical analysis: the initial period represents a locomotor response to novelty, while the last approximates general activity. Time spent in the inner 10 cm x 10 cm zone and total distance moved were scored to analyze anxiety and locomotor activity, respectively.

Time in center

Due to non-normality of the time spent in the center, all subsequent analyses were performed after a square-root transformation. A 2 (time interval: 0-5 min vs 15-20 min) x 2 (group) x 2 (sex) ANOVA, where interval was a repeated measure and time in the center was the dependent variable, revealed a main effect of time interval ($F_{1,40} = 29.43$, $P < 0.001$) and a sex difference ($F_{1,40} = 4.24$, $p = 0.045$), but no group effect ($F_{1,38} = 0.137$, $P = 0.713$), nor group by sex interaction ($F_{1,40} = 0.521$, $P = 0.474$). There were no interactions with time interval. *Post hoc* pair-wise comparisons revealed a significant sex difference during the first 5 min interval ($P = 0.004$; **Figure 4.4A**), but not during the fourth interval ($P = 0.751$; data not shown).

Total distance moved in the open-field

Again, due to non-normality, all subsequent locomotor activity analyses were performed after a square-root transformation. A 2 (time interval: 0-5 min vs 15-20 min) x 2 (group) x 2 (sex) ANOVA, with time interval as a repeated measure and distance moved as the dependent variable, revealed a main effect of group ($F_{1,40}$ = 4.949, P = 0.031; **Figure 4.4C**), but no effect of sex nor interval. There were no interactions. *Post hoc* tests revealed that both male and female SM-reared offspring moved a greater distance during the first 5 min interval than their BP-reared counterparts (P = 0.038 and P = 0.056, respectively), although the effect was only a trend in females. The difference disappeared in both sexes by the 15-20 min interval (P = 0.141 and P = 0.831, respectively; data not shown).

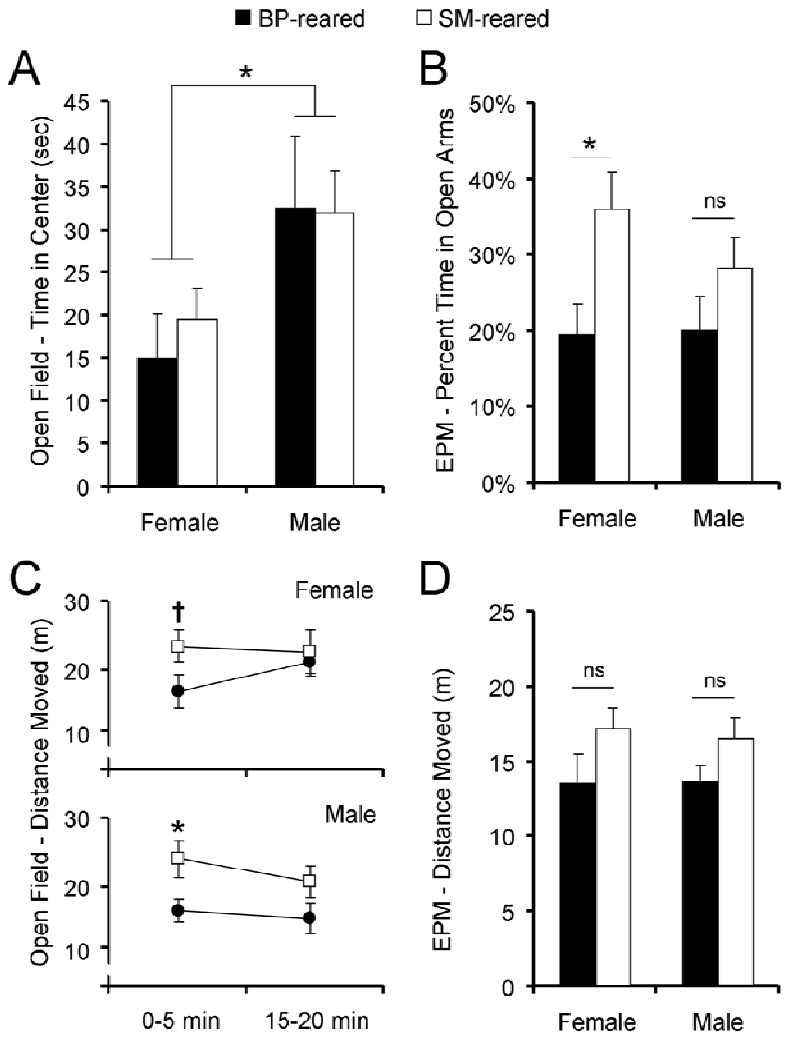


Figure 4.4 - The effect of rearing condition on anxiety and locomotor activity
SM- and BP-reared adults were tested for anxiety and exploratory/locomotor activity in the open-field and elevated plus maze (EPM). SM- and BP-reared adult animals showed no differences in time spent exploring the anxiety-inducing center zone of the open-field **(A)**, but SM-reared animals did spend a greater percentage of time in the anxiety-inducing open-arms of the EPM **(B)**, compared to BP-reared adult animals. In terms of locomotor activity, SM-reared adult animals of both sexes showed transient hyperactivity in both the first 5 min of the open-field test **(C)** and the 5 min EPM test **(D)**, compared to BP-reared counterparts. Bar graphs represent Means + SEM. ■ = BP-reared adult offspring; □ = SM-reared adult offspring. * $P < 0.05$, ns = not significant.

Adult offspring elevated plus maze testing

Animals underwent elevated plus maze testing for 5 min. Raw time in each zone (open-arms, closed-arms, and center) and total distance moved was scored. Anxiety-related measures were then calculated.

Percentage time in open arms

The percentage of time spent in the open arms was calculated (open arm time / [open arm time + closed arm time]), tested for normality and homogeneity of variance. Raw scores were non-normal, so the data were square-root transformed. A two-factor ANOVA revealed that there was a main effect of group ($F_{1,38}$ = 7.13, P = 0.011; **Figure 4.4B**), but no effect of sex, nor was there a group by sex interaction. *Post hoc* pair-wise comparisons revealed that SM-reared females, but not males, spent a greater percentage of time in the open arms compared to BP-reared controls (P = 0.015 and P = 0.202, respectively).

Total distance moved in EPM

Raw total locomotor activity data were square-root transformed to achieve normality and then analyzed. A two-factor ANOVA revealed a main effect of group ($F_{1,38}$ = 5.89, P = 0.019; **Figure 4.4D**), but no effect of sex, nor was there a group by sex interaction. Planned p*ost hoc* testing revealed that SM-reared offspring moved more throughout the entire EPM BP-reared animals (P = 0.012), but effects did not remain significant when each sex was analyzed separately: females P = 0.112; males P = 0.088.

Percentage of open arm entries

The percentage of open arm entries was also calculated (open arm entries / [open arm entries + closed arm entries]), tested for normality and homogeneity of variance, square-root transformed, and then analyzed using a two-factor ANOVA. The ANOVA revealed no difference in group ($F_{1,38}$ = 0.157, P = 0.694) nor sex ($F_{1,38}$ = 0.610, P = 0.439), nor was there a group by sex interaction ($F_{1,38}$ = 0.544, P = 0.465); data not shown.

Adult offspring gestational measures

Cohort 5 BP/BP and SM/SM pairs showed no differences in the time that elapsed between pairing and the birth of offspring (Mean ± SEM; BP/BP = 24.45 ± 0.37 days, SM/SM = 24.87 ± 0.49; T-test: P = 0.532). There were also no differences in the percentage of pairs that gave birth within a week of the first litter (BP/BP = 11/14, 79%; SM/SM = 15/17, 88%; Two-tailed Fisher's Exact Test: P = 0.636), nor were there differences in litter size (BP/BP = 3.91 ± 0.31 pups; SM/SM = 3.86 ± 0.38 pups; Mann-Whitney U = 79, P = 0.850).

Adult offspring parenting of the next generation

Family unit observations of cohort 5 BP/BP and SM/SM pairs revealed no differences in huddling frequency (**Fig 4.5B**; Two-way ANOVA; PND: F(1,1) = 2.693, P = 0.113, Group: F(1,25) = 0.35, P = 0.853, PND x Group: F(1,1) = 0.798, P = 0.380), nor were there differences in the frequency of nest exposure—either observed or how the

observed differed from expected, suggesting that both groups coordinated nest occupancy (**Fig 4C**; Two-way ANOVA; Exp-vs-Obs: $F(1,1) = 33.650$, $P < 0.001$, Group: $F(1,25) = 0.633$, $P = 0.434$, Exp-vs-Obs x Group Interaction: $F(1,1) = 0.125$, $P = 0.726$).

An analysis of LG behavior revealed that there was an overall group effect, with BP/BP parents engaging in a higher frequency of pup-directed LG than SM/SM parents. The decrease was found in both SM parents (**Fig 4D**; Three-way ANOVA; Object-of-LG: $F(1,2) = 122.705$, $P < 0.001$, Group: $F(1.53) = 8.126$, $P = 0.006$, Sex: $F(1,1) = 13.357$, $P = 0.001$, Object-of-LG x Group Interaction: $F(1,2) = 9.545$, $P < 0.001$, Object-of-LG x Sex Interaction: $F(1,2) = 31.942$, $P < 0.001$), Group x Sex Interaction: $F(1,53) = 0.051$, $P = 0.822$, Object-of-LG x Group x Sex Interaction: $F(1,2) = 0.039$, $P = 0.962$). Fathers again engaged in self-LG more than mothers, but there were no significant group differences in either self-LG or partner-LG.

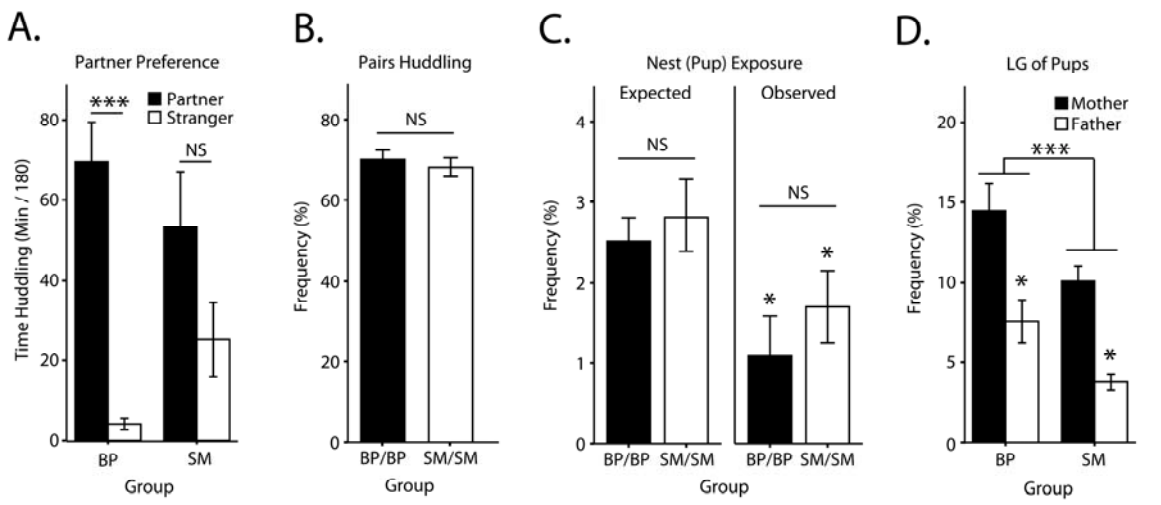


Figure 4.5 - Rearing condition influences parenting of the next generationFigure 4.6 **A**) BP-reared females showed a very clear partner preference after 24 hrs of cohabitation; SM-females did not. (**B**) BP/BP and SM/SM parents huddled at equal rates postnatally. (**C**) There were no differences in nest (pup) exposure and both groups showed nest coordination (* observed < expected, $P < 0.05$). (**D**) As a group and individually, SM-reared parents exhibited lower frequencies of LG than BP-reared parents, despite both groups showing a sex difference (* sex difference, $P < 0.05$). Bars represent Mean ± SEM. *** $P < 0.001$, NS = not significant.

DISCUSSION

In humans and other mammalian species, early life experience can profoundly influence long-term behavior, leading to marked individual variation (Meaney, 2001). There is also a vast psychological literature describing the effects of early parent-offspring interactions and the quality of adult relationships (Bowlby, 1973, 1982; Sable, 2004). On average, prairie voles are highly affiliative, but there is remarkable diversity in social behavior across individuals, and this behavioral diversity has been associated with variation in neurochemistry (Phelps and Young, 2003; Young and Wang, 2004; Hammock and Young, 2005; Ophir et al., 2008b; Ophir et al., 2008a). In this study, I exploited natural variations in family structure (Getz and Carter, 1996) to examine the

connection between early social environment and the expression of species-typical social behaviors, including nurturing and social attachment. I compared animals that had been reared by single-mothers (SM) and animals that had been reared biparentally (BP). Our findings demonstrate that, as predicted, SM- and BP-reared animals experienced different levels of care during the neonatal period and that these differences significantly influenced alloparental and bonding social behaviors in adulthood.

Most prairie vole offspring receive substantial care from both the mother and the father, with males exhibiting many of the same nurturing behaviors as females (Lonstein and De Vries, 2000). This paternal investment sets voles apart from standard laboratory rodents, such as rats and mice, but is comparable in many respects to other socially monogamous species such as California mice (Dudley, 1974; Bester-Meredith and Marler, 2003; Bredy et al., 2004; Frazier et al., 2006), titi monkeys (Kleiman, 1977; Anzenberger, 1988), and humans (Kleiman, 1977; Fuentes, 1998; Low, 2007). In previous work (see **Chapter 3**), I hypothesized that removing the male would have significant consequences on the quantity and quality of care received by offspring. Detailed observational comparisons of SM and BP family units revealed that SM-reared pups experienced significantly less care, including greater exposure and less LG in comparison to BP-reared counterparts.

Importantly, these differences were not a function of blatant neglect on the part of SMs. On average, SM-reared pups were left unattended only 10% of the time, and rarely were SM litters exposed for more than three consecutive observations (15-18 min), paralleling exposure intervals experienced regularly by other SM-reared rodents such as rats (Caldji et al., 1998; Meaney, 2001). In terms of LG, mothers neither lowered nor

increased their maternal investment in response to the presence or absence of the father; SM and BP mothers provided LG at essentially equal rates. Group differences in pup-care instead arose as a function of the father's presence in the BP group. With the assistance of the father, BP-reared pups were unattended less than 1% of the time. This remarkable rarity in pup exposure is consistent with prairie vole observations over PND1-3 in seminatural environments (McGuire et al., 2007) and suggests that, even if SMs only left the nest to eat and drink, they would be unable to compensate completely for the father's absence.

The effects of rearing on social behavior were dramatic. Compared to BP-reared animals, SM-reared adult females exhibited remarkably low spontaneous alloparental behavior (**Figure 4.2**) and both males and females exhibited a delayed onset of a stable group partner preference (**Figure 4.3**).

Spontaneous alloparental behavior is variable across mammalian species and sexes. For example, among adult rats, both males and females are typically infanticidal, whereas in mice, males are often infanticidal but females of most strains are spontaneously maternal (Lonstein and De Vries, 2000). In prairie voles, the majority of adults from both sexes are alloparental. There is, however, substantial individual variation within colonies and variation across laboratories (Roberts et al., 1998b; Lonstein and Fleming, 2002; Olazábal and Young, 2005; Olazábal and Young, 2006b, 2006a). Here I extend work showing that the absence of the father can significantly decrease spontaneous allo-maternal behavior. Wang & Novak (1992) had found an effect of the father's absence on juvenile alloparental behavior, but this was confounded by the presence of the father during test observations in the BP-reared group (Wang and Novak,

1992). Likewise, Roberts et al. (1998) extended this work to adult weanlings, but only found a non-significant trend based on categorical definitions of responsive versus non-responsive. Our data demonstrate that, in comparison to BP-reared adults, SM-reared females responded to unrelated pups with equivalent approach latencies, but ultimately engaged in strikingly little pup-directed behavior such as LG and huddling (**Figure 4.2**).

These data corroborate studies looking at more artificial early life manipulations. Neonatal infusions of OT, OTR antagonist, and steroids, all alter adult spontaneous alloparental behavior, as does early handling (Roberts et al., 1996; Bales and Carter, 2003a, 2003b; Bales et al., 2004; Bales et al., 2007d; Bales et al., 2007c). In fact, I note that, technically, our BP-reared animals are not identical to standard colony prairie voles. Throughout this study, all pups were handled weekly to obtain weight measurements. Based on previous work, I cannot rule out the possibility that the behavior of BP-reared animals was influenced by this handling. Despite this caveat, adult BP-reared offspring of both sexes showed historically normal levels of spontaneous alloparental behavior from our laboratory, with 60% of BP-reared females and 70% of BP-reared males considered spontaneously parental (**Figure 4.2C-D**; Lonstein and De Vries, 2000; Olazábal and Young, 2005). In comparison, only 17% of SM-reared females were spontaneously maternal (**Figure 4.2C**).

Our data show for the first time that natural variation in family structure can significantly influence adult bonding behaviors. In the partner preference test (a well-established laboratory proxy for pair-bonding), SM-reared offspring of both sexes exhibited a delayed onset of partner preference formation compared to BP-reared animals (**Figure 4.3**). Notably, SM-rearing did not obliterate partner preference formation; it

simply increased the likelihood that partner preferences would not be formed during the initial cohabitation periods. There was no indication that this difference was due to an effect on mating, a factor that can influence partner preference formation (Williams et al., 1992a; Young and Wang, 2004). Like alloparental behavior, partner preference formation is sensitive to a wide range of manipulations, both during the neonatal period and in adulthood (Carter et al., 1995; Insel and Hulihan, 1995; Bales and Carter, 2003b; Bales et al., 2004; Young and Wang, 2004), including handling (Bales et al., 2007b). Therefore, it is worth mentioning that, despite pre-weaning weight measurements, BP-reared animals demonstrated historically normal partner preference formation after a 24-hr cohabitation period (**Figure 4.3**; Carter et al., 1995; Young and Wang, 2004).

This study was also designed to test the hypothesis that manipulations of family social dynamics in one generation can affect family social dynamics in the next in a biparental prairie vole. In particular, I tested whether pairs derived from litters reared under SM conditions would coordinate nest occupancy at the same rate as pairs derived from litters reared under BP conditions, as well as whether there would be significant differences in LG dynamics. There were no statistically significant differences in the rate of nest occupancy or coordination (**Figure 4.5B-C**), but I do show for the first time that SM-reared animals exhibit pup-directed LG behavior toward their own pups that is significantly different animals reared under BP conditions. Interestingly, both sexes showed this decrease. Importantly, despite differences in partner preference formation, the groups showed no differences in gestational duration, reproductive success, or litter size that might have influenced an interpretation of the parenting results.

Our data provide ethologically-relevant support for the hypothesis that early life manipulations can alter species-typical adult nurturing and bonding behaviors in monogamous animals and demonstrate in another model species that less extreme early life manipulations—in comparison to extended maternal separation or active abuse, for example—can still have profound behavioral consequences. Finally, these findings may provide at least a partial explanation for some of the behavioral diversity observed in both field and laboratory studies of prairie voles and other highly affiliative species, which parallel phenomena reported in humans (Low, 2007).

A crucial question remains, however: what is the mechanism by which early life social behavior is transduced into variation in adult social behavior? Did family structure have a direct effect on the neural mechanisms regulating adult social behavior or was altered social behavior simply a secondary effect downstream of peripheral physiological consequences?

The consequences of family structure were apparent even before weaning. Throughout this study, pups were reared in 30 cm x 34 cm breeding cages, in which parents had easy access to food and water. Yet SM-reared offspring exhibited developmental delays that mirror findings obtained from father-absent offspring reared in substantially larger (1300 cm^2) semi-natural environments with seemingly greater foraging demands (Wang and Novak, 1992). I therefore wondered if SM-reared pups were less mature because they were malnourished. Malnutrition would of course impact any interpretation of adult social behavior. A more detailed analysis of the observational data, however, shows that SM-reared pups were attached to their mother and able to nurse at equal—if not higher—rates compared to BP-reared pups, and SM and BP

mothers both ate and drank at approximately equal frequencies (**Chapter 3, Figure 3.5**). While I have no data regarding milk quality or quantity, both findings suggest that malnutrition was not the primary cause of the maturational differences.

Another explanation could be social enrichment. In mice, pups reared by single-mothers tend to weigh less than mice reared in communal groups (Sayler and Salmon, 1969). Likewise, rat pups briefly separated from their mother show retarded growth and suppressed responses to growth hormones—a phenomena more associated with "nurturing touch" than food or temperature changes (Kuhn et al., 1978; Kuhn and Schanberg, 1998; Levine, 2001). In our sample, there was a non-significant correlation between cumulative LG and weaning body mass ($P = 0.085$; data not shown), whereas there was no correlation between body mass and cumulative pup exposure ($P = 0.495$; data not shown). This indicates that maturational differences may be more the effect of "nurturing touch" than exposure-induced thermoregulatory load.

Ruling out nutrition and thermoregulation does not mean that early social environment had a direct effect on the neurobiological mechanisms regulating adult social behavior. Evidence from both clinical and basic research indicates that early life experience can "program" stress axes, resulting in altered anxiety and exploratory behavior (Heim et al., 1997; Caldji et al., 1998; Francis et al., 2002; Branchi and Alleva, 2006; Sanchez, 2006). Behaviorally, increased stress responsivity or exploratory behavior could lead to decreased sociality. I tested SM- and BP-reared pups as adults in two standard, laboratory tests of anxiety and locomotor activity, the open-field test and elevated plus maze, which have been used previously with prairie voles (Pitkow et al., 2001; Olazábal and Young, 2005). Contrary to our predictions based on the rat literature

(Caldji et al., 1998; Francis et al., 1999; Meaney, 2001), SM-reared animals, particularly females, were generally less anxious, but marginally more active, than BP-reared animals (**Figure 4.4**). The hyperactivity, however, was very short-lived and only tended toward significance in each sex (**Figure 4.4C-D**). Moreover, in females, it is typically increased stress inhibits sociality (DeVries et al., 1996). Therefore, while I cannot rule out a specific social-anxiety, general emotional and locomotor profiles seem unable to account fully for the observed differences in social behavior between SM- and BP-reared animals.

Conclusions

I show here for the first time that ethologically relevant variations in prairie vole family structure have several important consequences for the development of offspring. In comparing SM- and BP-reared prairie voles, I find that both exhibit species typical behaviors, such as alloparental care, partner preference formation, and coordinated biparental care of offspring. But they appear to be at very different points along the behavioral spectrum. Very few SM-reared females were spontaneously maternal (**Figure 4.2**), both SM-reared sexes exhibited a delayed onset of partner preference formation (**Figure 4.3**), and both sexes interacted with their own offspring with lower rates of LG (**Figure 4.4**). Normal BP-reared colony animals, occasionally show these types of sociobehavioral profiles. SM-rearing made them occur significantly more often. Together, these data demonstrate that differences in life early family structure is a powerful means of introducing behavioral variation into the population of this biparental species.

Disclosure / Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 5

The impact of early life family structure on adult neuropeptide systems in monogamous prairie voles

Adapted from:

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ABSTRACT

Prairie voles (*Microtus ochrogaster*) exhibit remarkable variation in both adult social behavior, such as alloparenting and partner preference formation, and in neuropeptide receptor expression. Despite strong correlational and causal links between receptor variation and sociobehavioral variation, currently little is know about the primary factors that drive this individual variation receptor expression. Previous work (Chapters 3) demonstrated that prairie voles have a highly dynamic family life that is significantly altered by changes in family structure. Moreover, differences in family structure, such as biparental (BP) and single-mother (SM) rearing, lead to changes in the adult social behavior of the offspring. I exploited this ethologically-relevant difference in family structure to examine how structural differences in family environment can affect the development of the prairie vole oxytocin (OT), vasopressin, and CRF neuropeptide systems. Contrary to our hypotheses, early life family structure did not significantly account for neuropeptide receptor variation in the ventral forebrain ($P > 0.05$ for all). It did account for variation in hypothalamic OT mRNA expression ($P < 0.01$), OT receptor (OTR) in the lateral bed nucleus of the stria terminalis (lBNST; $P < 0.05$), and CRF2 in the dorsal raphe ($P < 0.05$). OT mRNA content and dorsal raphe CRF2 content significantly correlated with early life LG received. These data demonstrate for the first time that natural variation in family structure can influence the development of socially-relevant neuropeptide systems in a biparental species.

INTRODUCTION

Prairie voles (*Microtus ochrogaster*) are an excellent animal model to study the neurobiology of social bonding and social cognition. More recently, they have been used to study the impact of differences and shifts in biparental family dynamics (Chapters 3 and 4). Ethologically relevant variations in family structure, such as biparental (BP) and single-mother (SM) rearing, result in robust differences in early pup care (Chapter 3) that lead to significant differences in the expression of adult social behaviors in offspring, including alloparenting, partner preference formation, and post-partum parenting (Chapter 4). The neurobiological mechanisms by which differences in early life experience are transduced into differences adult social behavior in prairie voles are not yet known.

In natural and laboratory environments, prairie voles exhibit extraordinary diversity in the expression of several neuropeptide receptors, such as the oxytocin receptor (OTR), vasopressin 1a receptor (V1aR), and the two corticotropin-releasing factor (CRF) receptors (CRF1 and CRF2, respectively). Within prairie voles, individuals differ dramatically in the expression density of each of these receptors (Insel and Hulihan, 1995; Phelps and Young, 2003; Young and Wang, 2004; Hammock and Young, 2005; Lim et al., 2005; Ophir et al., 2008a). Interestingly, natural variations and artificial manipulations in the expression densities of these receptors in regions such ventral forebrain significantly influence adult species-typical social behaviors (Lim et al., 2004; Hammock and Young, 2005; Olazábal and Young, 2006a; Ross et al., 2009a). Despite some work associating diversity in the length and composition of a microsatellite upstream of the *avpr1a* gene with variation in the expression of V1aR (Phelps and Young,

2003; Young and Wang, 2004; Hammock and Young, 2005; Donaldson and Young, 2008; Ophir et al., 2008b; Solomon et al., 2009), overall, the factors that drive natural variations in neuropeptide receptor expression are not well-understood.

One possible contributor is early life experience. In rats, mothers that LG their pups at a high frequency also have increased OTR binding in several brain regions (Francis et al., 2000). Likewise, offspring that received high-LG had increases in OTR and V1aR binding densities in a region and sex specific manner (Francis et al., 2002). In voles, natural variations in early life experience have not been examined, but artificial neonatal manipulations have. Peripheral injections of OTR agonists and antagonists into prairie vole neonates resulted in altered neuropeptide receptor densities in adulthood (Bales et al., 2007d). Interestingly, some early manipulations alter the expression of the ligand for these receptors, not just receptors (Yamamoto et al., 2004)—an effect that also occurs in humans who experienced early child abuse (Heim et al., 2009).

Together, these data suggest that early life experience, such as differences in family structure, might drive differences in neuropeptide receptor densities that in turn could help explain the sociobehavioral differences between BP and SM rearing. This study aimed to test the hypothesis that differences in early life family structure can significantly affect neuropeptide and neuropeptide receptor expression. In particular, I examined whether OT expression might be decreased in SM-reared animals and whether they also have decreased OTR densities in the nucleus accumbens (NAcc) shell, decreased V1aR in the ventral pallidum, decreased CRF2 in the NAcc septal pole, and increased CRF1 in the NAcc shell. I initially focused on the ventral forebrain because expression densities in this region are highly variable, closely linked with social behavior

in prairie voles, and are regions that do not express appreciable densities of neuropeptide receptors in non-monogamous rodents, such as rats, mice, and promiscuous voles (Van Pett et al., 2000; Young and Wang, 2004; Lim et al., 2005). I also examined more canonical brain regions that are relevant to social behavior and are also susceptible to early life experience (e.g., the bed nucleus of the stria terminalis [BNST]).

MATERIALS AND METHODS

Animals

All animals were derived from the litters produced by cohort 2. As noted in Chapter 3, pups were reared either biparentally (BP) or by a single-mother (SM). Family units were observed from PND1-15. The offspring were weighed at birth (PND1) and then every 7 days until weaning. All offspring were weaned at PND22 into same-sex, same-group pairs or trios and housed in standard, ventilated, polycarbonate laboratory caging located in an independent cubicle. Animals were maintained at 22°C on a 14:10 light-dark schedule with lights on at 06:00 h and had *ad libitum* access to food (LabDiet, rabbit chow) and water.

Family unit pup care

For cohort 2 (see **Table 3.1**), parental behavior had been recorded and analyzed in great detail for PND1-15 in order to study the parents (see **Chapter 3**). Here, however, I interested primarily in the pups and how differences in early life experience may have altered brain development. To investigate this connection, I first calculated the accumulated sum total of observations that each litter was alone on the nest and each

litter (at least one pup) was being licked and groomed (LG). To gain a sense of how differences in parenting frequencies would affect divergence in what litters from each rearing condition experienced, I calculated the accumulated number of observations for PND 2, 5, 10, and 15. After testing for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and for homogeneity of variance (Levene's test), t-tests were performed to identify at which point, from the perspective of the pups, the two rearing conditions diverged. $P < 0.05$ was considered statistically significant. Cumulative LG and pup-exposure observations over PND1-10 were used in our correlations with neuropeptide receptor densities and oxytocin content.

Adult offspring brain and blood analysis

At ~60 days of age, a total of 36 subjects from cohort 2 litters (n = 6 males + 6 females / rearing group; only 1 male and/or 1 female per litter) were anesthetized with vaporized isoflurane and rapidly decapitated. Approximately 200uL of trunk blood were collected in 1.5mL EDTA-coated tubes (Brinkman Instruments Inc., 022379224) treated with 10uL of aprotinin (Trysol; Fisher Scientific: BP250310). Brains were then rapidly removed from the skull and snap frozen in powdered dry-ice. Brains were stored at -80°C until sectioned. Trunk blood was maintained on ice, centrifuged (4000 rpm, 4°C, 5 min), and plasma was aliquoted (10uL) and stored at -20°C for corticosterone assays. All brains were cryostat sectioned from the olfactory bulbs through the locus coeruleus in a 1:6 series at 20μM, thaw-mounted on super-frost plus slides (Fisher Scientific, 12-550-15), and maintained at -80°C until assayed. Each of the 5 series was treated for one of the following: ^{125}I autoradiographic binding for vasopressin 1a receptor (V1aR), oxytocin

receptor (OTR), corticotropin-releasing factor receptor 1 (CRF1), and CRF2, as well as *in situ* hybridization for oxytocin (OT).

Plasma corticosterone

A commercially available radioimmunoassay kit was used to quantify plasma corticosterone concentrations (MP Biomedicals, Irvine, CA). The kit has been validated previously for prairie voles (Taymans et al., 1997; Grippo et al., 2007c). Prairie voles are glucocorticoid resistant leading to high basal concentrations of corticosterone, so aliquots were diluted 1:1000, instead of the standard 1:200, to give results within the linear portion of the standard curve. The lowest detectable dose was 7.7 ng/mL, and the inter-assay variation coefficient was less than 7%. Cross-reactivity with other steroids was less than 1%. Values are given in ng/mL.

Oxytocin mRNA *in situ* hybridization

In situ hybridization for OT was performed using a well-established protocol in our laboratory (Wang et al., 2000; Patisaul et al., 2003). OT mRNA was probed using a single, 41-base, ^{35}S-labeled oligonucleotide probe (GGG CTC AGC GCT CGG AGA AGG CAG ACT CAG GGT CGC AGG CG) complementary to nucleotides 906-946 of the rat oxytocin mRNA (GenBank Accession Number K01701). Following *in situ* hybridization, slices were rinsed, washed, and dried according to the protocol and exposed to Kodak BioMax MR film (Eastman Kodak Co., NY) for 3 hours. After confirming OT mRNA detection using film, slides were dipped in photographic emulsion (Kodak NTB-2), exposed for 3.5 hours, and then developed.

Oxytocin mRNA *in situ* quantification and analysis

Quantification of *in situ* hybridization OT mRNA silver grains in the PVN was performed using AIS 6.0 (MCID, Canada) after digitization (MTI CCD72 camera). Both the number of OT-labeled clusters and the level of OT expression per neuron were estimated as follows. Under dark-field conditions, a 4X objective was used to identify and count silver-grain clusters (Young et al., 1994) throughout five adjacent sections of the paraventricular nucleus of the hypothalamus (PVN). Since adjacent sections were 120 uM apart, it is not possible that the same neuron could appear in adjacent sections. Grain clusters in all five sections from both left and right hemispheres were summed to represent OT cell count and used for analysis.

In situ slides were then nuclear-stained using cresyl violet, cover-slipped, and examined under bright-field conditions, using a 40X objective. The number of grains in approximately 20 clusters situated above large (and therefore neuron) nuclei were counted using a 50 pixel diameter circular cursor. Bilateral grain counts from 5 adjacent sections were quantified and averaged, and background readings from the lateral hypothalamus were subtracted before statistical analysis.

Both number of clusters and number of grains/cluster were analyzed using two separate two-way ANOVAs. *Post hoc* pair-wise comparisons followed significant effects. $P < 0.05$ was considered statistically significant.

Neuropeptide autoradiographic receptor binding

Autoradiography for V1aR, OTR, CRF1, and CRF2 each followed similar procedures and have been previously published (Phelps and Young, 2003; Lim et al., 2005; Olazábal and Young, 2006a). Briefly, slides were thawed at room temperature until dry, then fixed in 0.1% paraformaldehyde-PBS (pH 7.4) for 2 min, washed twice in 50mM Tris base (pH 7.4) for 10 min each. Slides were then incubated in tracer buffer (pH 7.4) for either 1 hour (V1aR and OTR) or 2 hours (CRF1 or CRF2). The tracer consisted of 50mM Tris base, 10mM MgCl, 0.1% bovine serum albumin, plus one of the following radioligands: for V1aR, 0.05nM ^{125}I-d$(CH_2)_5$(Tyr[Me])-AVP (PerkinElmer); for OTR, 0.05nM ^{125}I-d$(CH_2)_5$[Tyr$(Me)_2$-Tyr-$NH_2]_9$-OVT (PerkinElmer); for CRF1 and CRF2 0.2nM ^{125}I-sauvagine (PerkinElmer). ^{125}I-sauvagine binds to both CRF1 and CRF2 with high affinity (Primus et al., 1997; Lim et al., 2005). To achieve CRF1 and CRF2 selective binding, a cold antagonist was also added to the tracer: 500nM CP-154,526, a selective CRF1 antagonist (a generous gift from Michael J. Owens, Emory University), was used to reveal CRF2 binding; 500nM Astressin-2B, a selective CRF2 antagonist (Sigma-Aldrich, A5227), was used to reveal CRF1 binding. These antagonists show selective binding properties in prairie voles (**Chapter 2**; Lim et al., 2005). Regardless of the tracer cocktail, slides were then washed in 50mM Tris base plus 10mM MgCl (pH 7.4) 4 x 5 minutes, plus 30 minutes with slow stirring on a magnetic stir plate. Finally, slides were dipped briefly in deionized water, dried under a stream of cool air, and apposed to Kodak MR film for 72 hours (OTR and V1aR) or 85 hours (CRF1 and CRF2). ^{125}I-microscale standards (Amersham Biosciences / GE Healthcare) were applied to each film.

Autoradiographic receptor binding quantification and analysis

Quantification of autoradiograms followed previously published procedures (Phelps and Young, 2003; Lim et al., 2005). Films and microscale standards were digitized (MTI CCD72 camera) and quantified using AIS 6.0 (MCID, Canada). Once digitized, optical density measurements were taken bilaterally and averaged for each brain region across two to seven sections, based on the size of the region of interest. Optical density was converted automatically to decompositions per minute per milligram tissue (DPM) based on the microscale standards from each film. To control for non-specific background binding, DPM values from the corpus callosum were subtracted from the raw value for each region prior to statistical analysis, a procedure that has been used previously (Hammock and Young, 2005).

Due to the large number of brain regions that express each of the four neuropeptide receptor types, I analyzed the autoradiographic data in two tiers. Based on previous studies in prairie vole social behavior, our *a priori* objective was to test the hypothesis that early social experience could alter neuropeptide receptor expression in the ventral forebrain: in particular, OTR and CRF1 in the nucleus accumbens shell (NAcc-Shell), CRF2 in the NAcc septal pole, and V1aR in the ventral pallidum. Manipulations of all of these receptor populations have been shown to alter adult social behavior (Young and Wang, 2004; Lim et al., 2007). Therefore, in the first tier analysis, I conducted a 2 (group) x 2 (sex) x 4 (receptor-type/specific-region) multiple ANOVA (MANOVA) to examine the effect of rearing condition on neuropeptide receptor expression in the ventral striatum.

The ventral forebrain is not the only area that regulates social and emotional behaviors, nor is it the region most often associated with early life experience, since rats and mice express negligible levels of receptors there. In our second tier analysis, I chose 4-5 other brain regions for each receptor type based on their role in social and emotional behaviors and/or their sensitivity to early experience, and conducted a separate 2 (group) x 2 (sex) MANOVA for each one. *Post hoc* tests followed significant effects. Again, a $P < 0.05$ was considered statistically significant. To reiterate, all neuropeptide system analyses above were performed using adult, behaviorally naïve, brains obtained from cohort I adult offspring, for which I had early life experience data.

RESULTS

Family unit pup care

During the family unit observations of cohort 2 (**Table 3.1**), pups were considered exposed on the nest when neither the mother nor the father was on the nest during a spot-check, and considered to be receiving LG if at least one pup received it. I examined the cumulative effect of the frequency differences between BP and SM family units in **Chapter 3**. A total of fifteen family units (BP = 8, SM = 7) were fully observed from PND1-15. T-test comparisons at PND2, 5, 10, and 15 revealed that SM-reared offspring had experienced a cumulative number of exposures already by PND2 in comparison to BP-reared offspring: PND2 ($P = 0.01$; **Figure 5.1A**), PND5 ($P < 0.001$), PND10 ($P < 0.001$), and PND15 ($P < 0.001$).

A similar finding was observed for LG. Planned t-tests revealed that, by PND2, SM-reared offspring had already experienced a cumulatively lower number of LG bouts

than than BP-reared counterparts: PND2 ($P < 0.01$; **Figure 5.1B**), PND5 ($P < 0.01$), PND10 ($P < 0.01$), and PND15 ($P < 0.01$).

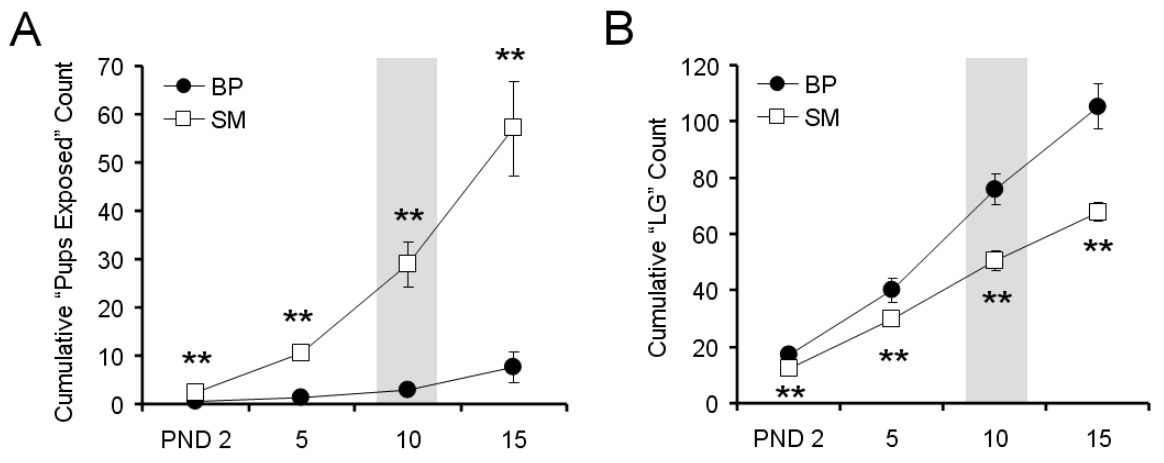

Figure 5.1 - The cumulative effects of rearing condition on pup care
Cohort 2 single-mother (SM) and biparental (BP) family units were observed to examine pup care (see **Chapter 3**). To get a sense of the pups' cumulative experience, the number of observations that pups from each litter were exposed **(A)** and licked and groomed (LG) **(B)** were summed over the first two weeks of life. Gray bands mark post-natal day 10 (PND10). Cumulative values at PND10 for each measure were is used in the correlational analyses with receptor densities and oxytocin mRNA content. Graphs represent Means ± SEM. ● = BP family units, □ = SM family units. ** $P < 0.01$.

Basal plasma corticosterone

I extracted and examined basal corticosterone concentrations from adult cohort 2 offspring reared under SM or BP conditions. Due to non-normality of the raw corticosterone plasma concentrations (ng/mL), animals were segregated by sex first and then group differences were analyzed using two separate non-parametric tests. Mann-Whitney U analysis revealed no group differences in baseline plasma corticosterone concentrations for females ($U = 11$, $P = 0.262$: BP = 910 ± 250 [mean ± SEM] ng/mL;

SM = 492 ± 106 ng/mL) and males (U = 17, P = 0.873: BP = 599 ± 202 ng/mL; SM = 418 ± 106 ng/mL.

Oxytocin mRNA expression

Number of OT mRNA clusters

OT mRNA was detected using *in situ* hybridization and photographic emulsion dipping. Dense silver-grain clusters were counted in five adjacent sections throughout the PVN using dark-field microscopy (Young et al., 1994). A 2 (group) by 2 (sex) ANOVA, with PVN OT mRNA clusters as the dependent measure, revealed a main effect of group ($F_{1,20}$ = 4.72, P = 0.042; **Figure 5.2A**), but no effect of sex ($F_{1,20}$ = 0.28, P = 0.60) nor group by sex interaction ($F_{1,20}$ = 0.19, P = 0.67). Planned *post hoc* comparisons within each sex demonstrated that SM-reared females had significantly greater numbers of OT mRNA clusters in the PVN than BP-reared females (t(10) = -3.1, P = 0.01), whereas males showed no statistical difference (t(10) = -0.96, P = 0.361).

For a more detailed analysis, I subdivided the PVN and found that the anterior portion demonstrated the most striking group difference, again with SM-reared females having substantially more OT mRNA clusters than BP-reared counterparts (t(10) = -3.62, P = 0.005; **Figures 5.2A-I**).

OT mRNA grains per cluster

After dark-field cluster counts had been performed, individual grains/cluster were counted over large neuron nuclei. This quantification estimates a measure of mRNA production on a cell by cell basis. A 2 (group) by 2 (sex) ANOVA, with PVN OT mRNA

silver-grains per cluster as the dependent measure, revealed no significant main effects or interactions (**Figure 5.2J**): group ($F_{1,20}$ = 0.544, P = 0.469), sex ($F_{1,20}$ = 0.662, P = 0.426), group x sex interaction ($F_{1,20}$ = 0.017, P = 0.897).

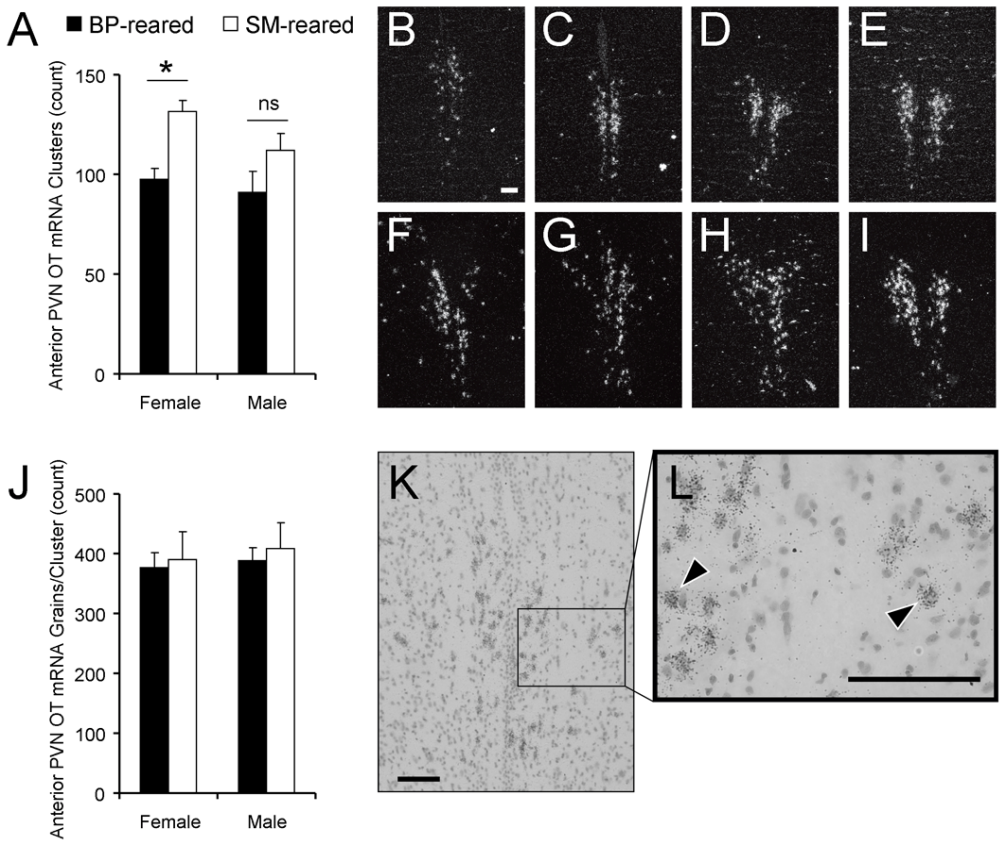

Figure 5.2 - Rearing affects oxytocin mRNA content in the PVN
Oxytocin content in the paraventricular nucleus of the hypothalamus (PVN). Brains were extracted from behaviorally-naïve SM- and BP-reared adults and analyzed for oxytocin (OT) mRNA expression using *in situ* hybridization and emulsion dipping. SM-reared animals had a significantly greater number of OT mRNA grain clusters throughout the PVN, particularly in the anterior portion, compared to BP-reared animals **(A)**. Representative, anterior to posterior, coronal, 10X images of the PVN of SM- **(B-E)** and BP- **(F-I)** reared females, under dark-field conditions, show grain clusters. OT expression was also analyzed by counting the number of grains per cluster over large nuclei. There were no group differences in grains per cluster **(J)**. **(K)** and **(L)** are representative images of PVN clusters over large nuclei at 20X and 40X, respectively. Arrow-heads **(L)** identify clustered grains and scale bars represent 100μM **(B, K, L)**. ■ = BP-reared adult offspring; □ = SM-reared adult offspring. * P < 0.05, ns = not significant.

Neuropeptide receptor densities in the ventral forebrain

A 2 (group) x 2 (sex) x 4 (receptor type) ANOVA, with receptor type as a repeated measure, and binding density for OTR in the shell of the nucleus accumbens (NAcc-Shell), V1aR in the ventral pallidum, CRF1 in the NAcc-shell, and CRF2 in the NAcc septal pole as the dependent measures. The analysis revealed a significant effect of receptor type ($F_{1,20} = 81.378$, $P < 0.001$), but no effect of sex ($F_{1,20} = 0.097$, $P = 0.759$) nor group ($F_{1,20} = 1.091$, $P = 0.309$); there was also no group by sex interaction ($F_{1,20} = 0.542$, $P = 0.470$). BP- and SM-reared adults did not significantly differ in any of the four neuropeptide receptor type in any of ventral forebrain regions examined (**Figure 5.3A**).

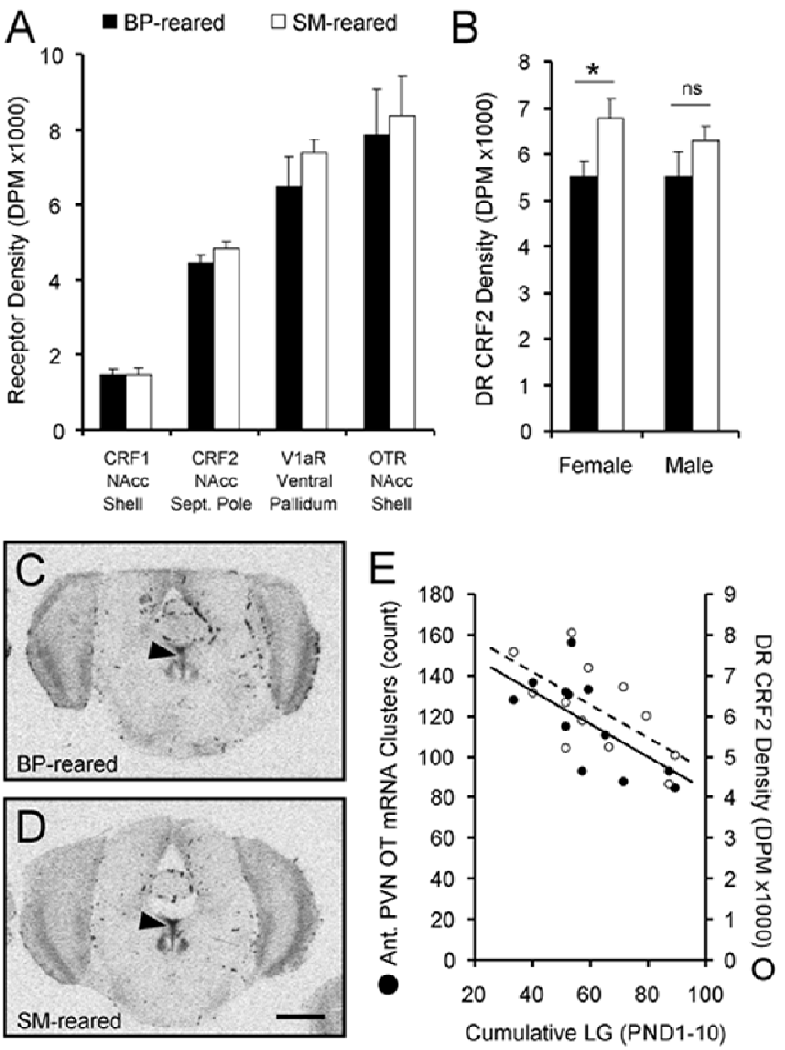

Figure 5.3 - The effect of rearing on neuropeptide receptor densities
Neuropeptide receptor densities. Brains from behaviorally-naïve SM- and BP-reared adults were assayed for oxytocin receptor (OTR), vasopressin 1a receptor (V1aR), corticotropin-releasing factor receptor 1 (CRF1) and CRF2 using radioligand receptor binding. None of the four neuropeptide receptors showed an effect of rearing condition in socially-relevant ventral forebrain regions **(A)**; NAcc = nucleus accumbens. SM-reared adult animals, particularly females, revealed a higher density of CRF2 in the dorsal raphe (DR), compared to BP-reared animals **(B)**. **(C)** and **(D)** show representative coronal sections of dorsal raphe CRF2 from BP- and SM-reared females, respectively. Arrow-heads identify the DR and scale bar represents 1mM. **(E)** graphically depicts the correlational relationship between early life licking and grooming (LG) and two neuropeptide system markers: the number OT mRNA clusters in the anterior PVN and the density of CRF2 in the DR. ■ = BP-reared adult offspring; □ = SM-reared adult offspring. ● = LG/OT correlation (R = -0.611), ○ = LG/CRF2 correlation (R = -0.652). * $P < 0.05$, ns = not significant.

Neuropeptide receptor densities in other regions of interest

I performed a second tier analysis that investigated the effect of rearing condition on four to five other brain regions that express high (and often variable) densities of neuropeptide receptors and are known to either underlie certain social behaviors or be affected by early life experience. Each set of regions for each receptor type was analyzed using a separate multiple ANOVA (MANOVA).

Oxytocin Receptor (OTR)

A 2 (group) x 2 (sex) MANOVA for OTR binding density in the lateral bed nucleus of the stria terminalis (lBNST), lateral septum (LS), medial preoptic area (MPOA), central amygdala (cAmyg), and basolateral amygdala (BLA) revealed no group effect for any of the brain regions (**Table 5.1**). The lBNST, however, showed a near-significant trend ($F_{1,24} = 4.281$, $P = 0.052$). The MANOVA also revealed a sex effect in the cAmyg ($F_{1,24} = 7.970$, $P = 0.011$) and BLA ($F_{1,24} = 6.054$, $P = 0.023$), but no group by sex interaction. *Post hoc* pair-wise comparisons revealed that, regardless of group, females expressed a greater density of OTR than males in the both the cAmyg ($P = 0.018$) and BLA ($P = 0.008$); data not shown. An exploratory *post hoc* t-test for lBNST revealed that SM-reared offspring had a lower density of OTR than BP-reared animals ($P = 0.049$).

Vasopressin 1a receptor (V1aR)

A 2 (group) x 2 (sex) MANOVA for V1aR binding density in LS, BNST, mediodorsal thalamus (MDThal), cAmyg, and posterior cingulate revealed no main effects of group or sex, nor a group by sex interaction for any of the brain regions (**Table 5.1**).

Corticotropin-releasing factor receptor 1 (CRF1)

A 2 (group) x 2 (sex) MANOVA for CRF1 binding density in the medial prefrontal cortex (mPFC), LS, BLA, and cAmgy revealed no group effect for any of the brain regions, but there was a main effect of sex in the mPFC ($F_{1,24} = 5.224$, $P = 0.033$). There were no interactions (**Table 5.1**).

Corticotropin-releasing factor receptor 2 (CRF2)

Lastly, a 2 (group) x 2 (sex) MANOVA for CRF2 receptor binding density in the LS, BNST, dorsal raphe (DR), and ventral tegmental area (VTA) revealed a main effect of group for the DR ($F_{1,24} = 6.089$, $P = 0.032$) and a main effect of sex for the BNST ($F_{1,24} = 31.560$, $P < 0.001$). *Post hoc* testing revealed that, regardless of group, males expressed a greater density of CRF2 in the BNST than females ($P < 0.001$), which replicates a sex difference previously described in prairie voles. *Post hoc* testing also demonstrated that SM-reared offspring, regardless of sex, expressed a greater density of CRF2 in the dorsal raphe than BP-reared offspring ($P = 0.019$; **Table 5.1**, **Figure 5.3B**). Representative sections of BP- and SM-reared females are presented in **Figure 5.3C** and **Figure 5.3D**, respectively.

Table 5.1 - Autoradiographic receptor binding densities by sex, receptor type, and brain region

		Male		Female	
Type	**ROI**	**BP-reared**	**SM-reared**	**BP-reared**	**SM-reared**
OTR	lBNST *	3687 ± 677	2397 ± 597	4260 ± 658	2882 ± 760
	LS	3497 ± 432	2995 ± 541	4198 ± 503	4034 ± 541
	MPOA	505 ± 275	522 ± 127	502 ± 235	314 ± 91
	cAmyg †	4993 ± 631	4475 ± 617	6002 ± 551	6995 ± 692
	BLA †	5851 ± 432	5669 ± 629	6697 ± 322	7076 ± 390
V1aR	LS	6448 ± 917	6920 ± 517	5785 ± 1071	6756 ± 854
	BNST	3178 ± 421	3810 ± 236	2954 ± 502	3669 ± 244
	MDThal	7945 ± 1233	7069 ± 721	6421 ± 1086	7239 ± 857
	cAmyg	4763 ± 620	5957 ± 233	4895 ± 890	5948 ± 345
	pCing	4321 ± 710	4341 ± 999	4530 ± 858	5160 ± 1267
CRF1	mPFC †	6143 ± 603	6550 ± 1024	4438 ± 548	5088 ± 456
	LS	3588 ± 501	3957 ± 506	3420 ± 451	3427 ± 775
	BLA	674 ± 61	930 ± 75	829 ± 158	649 ± 187
	cAmyg	528 ± 71	722 ± 197	552 ± 139	278 ± 95
CRF2	LS	2284 ± 218	2777 ± 259	3147 ± 521	2628 ± 333
	BNST †	2358 ± 586	2655 ± 290	646 ± 198	474 ± 114
	DR *	5505 ± 547	6282 ± 313	5514 ± 338	6827 ± 502
	VTA	4179 ± 413	4511 ± 586	4050 ± 588	3992 ± 397

Data are expressed as Means ± SEM

* $P < 0.05$ group difference; † $P < 0.05$ sex difference

Early life experience and neuropeptide system development

All of these neuropeptide system analyses occurred using brains extracted from adult animals for which I had extensive early life experience data (see **Figure 5.1**). This allowed us to investigate if particular early life metrics could predict a certain degree of variation in brain expression of OT and CRF2.

First, a bivariate Pearson's correlation revealed that, in females, the number of OT mRNA clusters in the anterior PVN positively correlates with the density of CRF2 in the dorsal raphe (R = 0.626, P = 0.039), while in males there was no relationship (R = -0.020, P = 0.952).

Second, I investigated the relationship between early life variables and adult neuropeptide system expression. During early life, individual pups were not marked, so our licking and grooming data represented how often at least of the pups within the litter was being licked and groomed. While not a perfect early life metric, I calculated the accumulated number of licking and grooming observations from PND1-10 (LG_{PND10}) for each litter and allotted that value to the test subject(s) that had been drawn from that litter. I then correlated that value with OT mRNA and CRF2 receptor data from those same individuals. Bivariate correlational analyses revealed that LG_{PND10} significantly correlated with both the number of OT mRNA clusters (R = -0.611, P = 0.035; **Figure 5.3E**) and DR CRF2 density (R = -0.652, P = 0.030; **Figure 5.3E**) in females.

DISCUSSION

Prairie voles exhibit remarkable individual variability in adult social behavior and neuropeptide receptor expression (Carter et al., 1995; Insel and Hulihan, 1995; Young and Wang, 2004; Lim et al., 2005; Donaldson and Young, 2008; Ophir et al., 2008b; Ophir et al., 2008a), with many studies suggesting or demonstrating a direct relationship (Bales and Carter, 2003b; Bales et al., 2004; Lim et al., 2004; Hammock and Young, 2005; Olazábal and Young, 2006a; Bales et al., 2007d; Lim et al., 2007; Ophir et al., 2008b; Ophir et al., 2008a; Ross et al., 2009a). The primary factors that drive this

variation in receptor densities, however, is not as well known. Moreover, it is unknown whether the same type of sociobehavioral variation might arise through variations in less studied neuropeptide systems or brain regions.

Work in **Chapter 3** clearly demonstrated that biparental prairie vole parents coordinate a rich, highly dynamic and social family environment and that this dynamic is altered by the removal of the father. Work in **Chapter 4** clearly showed that ethologically relevant variations in family structure—such as BP- and SM-rearing—resulted in significantly different sociobehavioral profiles in adult offspring. Here I find that differences in family structure and early life care can significantly influence neuropeptide systems that are known to regulate adult social behavior, although the connection between early life variation and differences in adult social behavior is not quite as clear as I had predicted. Our data demonstrate that, while neuropeptide receptor densities within the ventral forebrain were not significantly affected by prairie vole early life family structure, oxytocin content in the PVN and the expression densities of at least two neuropeptide receptors were. These findings are interesting for a number of reasons.

OTR, V1aR, CRF1, and CRF2 europeptide receptor density variation in the ventral forebrain, especially within the nucleus accumbens and ventral pallidum, has been directly related to variations in adult social behaviors, such as alloparental care (Olazábal and Young, 2006a) and partner preference (Lim et al., 2004; Hammock and Young, 2005; Lim et al., 2007; Ross et al., 2009a). Moreover, manipulations of these and other systems in the forebrain produce significant changes in adult social behavior (Young and Wang, 2004; Lim et al., 2007; Donaldson and Young, 2008). Most promiscuous rodents, including traditional animal models such as rats and mice, do not express appreciable

densities of these receptors in these reward and reinforcing brain regions (Young and Wang, 2004; Lim et al., 2005). Therefore, investigations using traditional animal models have not been able examine the relationship between early life experience, expression of neuropeptide receptors in the ventral forebrain, adult bonding behaviors.

Based on the sociobehavioral differences between BP- and SM-reared adult offspring observed in Chapter 4, I hypothesized that BP-reared offspring would have higher densities of OTR in the shell of the nucleus accumbens, higher densities of V1aR in the ventral pallidum, higher densities of CRF2 in the septal pole of the nucleus accumbens, and lower densities of CRF1. Contrary to these predictions, I found no group differences in the density of OTR, V1aR, CRF1, or CRF2 in any of these ventral forebrain regions (**Figure 5.2A**), nor did they significantly correlate with early life (PND1-10) LG or pup exposure parameters (data not shown). These findings are corroborated by work using artificial early life manipulations such as peripherally injected OTR agonists and antagonists. These treatments also did not affect ventral forebrain neuropeptide receptor densities, despite finding changes in other regions (Bales et al., 2007d). Given the outbred nature of our prairie vole population, our sample size (N = 6/sex/group) is likely too small to show definitively that early life family structure has no effect on ventral forebrain neuropeptide receptor densities, but it is unlikely to be the primary driver of individual variation—suggesting some other mechanism or mechanisms (such as the *avpr1a* microsatellite for V1aR).

While variation in the ventral forebrain has been the primary target of most neuropeptide research in prairie voles, it is not the only region of interest, nor is it the only region that regulates adult social behavior. Moreover, the forebrain is not the typical

area of focus for most studies examining the effects of early life experience on neuropeptide system development (Francis et al., 2000; Meaney, 2001; Francis et al., 2002; Plotsky et al., 2005; Branchi and Alleva, 2006; Bales et al., 2007d; Champagne and Curley, 2008). Our second-tier analysis focused on more canonical brain regions and revealed that the OT and CRF2 neuropeptide systems in other areas are sensitive to natural variations in early care. Compared to BP-reared animals, SM-reared adults had increased OT mRNA clusters in the PVN, a higher density of OTR in the lateral BNST, and a higher density of CRF2 in the caudal dorsal raphe.

Greater sociality is typically associated with a more robust OT system activation (Williams et al., 1992a; Carter et al., 1995; Insel and Hulihan, 1995; Young and Wang, 2004). Thus, finding greater PVN OT mRNA content in the seemingly less social SM-reared animals was initially surprising and paradoxical, but it is consistent with other literature. For example, one study found that even seemingly opposite neonatal manipulations of the OT system both increase the number of OT neurons in the PVN, particularly in females (Yamamoto et al., 2004). Interestingly, OT can be anxiolytic (Yoshida et al., 2009) and artificial increases in OT have been found to lead to transient hyper-exploration in prairie voles (Dharmadhikari et al., 1997). Thus increased OT in SM-reared animals may partially explain the transient hyperactivity and decreased anxiety I observed in this group, particularly in females where the effects were strongest. Increased OT mRNA content is also consistent with recent findings showing that post-weaning isolation can ramp up PVN OT expression. Pan et al. (2009) took weanlings and either pair-housed (as is usual) or isolated them for 6 weeks (Pan et al., 2009). In adulthood, the isolated weanlings had significantly higher expression of OT mRNA in the

isolated animals, suggesting that decreased social interaction in early life may result in some sort of compensatory overactivation of the PVN OT system—such as in SM-rearing conditions (**Figure 5.3A**). Whether the effect of early social experience on OT content helps explain differences in adult nurturing and bonding is still unknown and will require further investigation.

The receptor density differences also did not map clearly onto the behavioral differences I observed, but they too fit with the literature. In two studies comparing high- and low-LG rats, Francis and colleagues found their most striking OTR density differences in the lateral BNST, with high-LG subjects expressing more OTR (Francis et al., 2000; Francis et al., 2002). In the current study, BP-reared animals, which had received greater LG early in life, had significantly more lBNST OTR than SM-reared adults. Since OTR in the lBNST has been associated with increased maternal behavior in the form of LG (Francis et al., 2000), lower lBNST OTR in SM-reared animals might have contributed to the observed alloparental and parental LG differences.

Likewise, activation of CRF2 in the dorsal raphe has been implicated in anxiety, fear and escape behaviors, partially through the modulation of serotonin release in the NAcc (Hammack et al., 2003a; Hammack et al., 2003b; Lukkes et al., 2008; Lukkes et al., 2009a; Lukkes et al., 2009b). Currently, however, virtually nothing is known about the role of dorsal raphe CRF2 receptors in mediating prosocial behaviors, such as pair bonding.

One intriguing finding is the relationship between the OT mRNA content in the PVN, CRF2 neuropeptide systems in the DR, and early life LG. As noted above, post-weaning isolation in prairie voles results in increased PVN OT content (Pan et al., 2009);

in rats a similar isolation-manipulation results in long-term upregulation of CRF2 in the dorsal raphe, even after a period of resocialization (Lukkes et al., 2009b). I show here that SM-reared animals, which received less social in early life, have higher PVN OT mRNA content and higher dorsal raphe CRF2, and both show a significant negative correlation with early LG (**Figure 5.3E**). The effects were most exaggerated in females. Coupled with the findings that females showed more robust behavioral differences throughout this study, our data suggest that females are especially sensitive to LG during early life, potentially through its tandem effects on the OT and CRF2 systems. Future research will aim to elaborate the relationship between OT and CRF2, as well as each of these with early life experience.

Conclusions

Overall, these findings demonstrate that differences in prairie vole early life family structure and dynamics can significantly influence the development of at least two neuropeptide systems that have been related to social behavior. In both cases, however, a direct link to the observed sociobehavioral differences between BP- and SM-reared animals is not entirely clear. Future research (see Chapter 7) should be aimed at addressing this disconnect either by examining how the observed changes can influence adult social behavior directly or by identifying other changes in the brain.

Disclosure / Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 6

Corticotropin-releasing factor receptor 2 (CRF2), oxytocin (OT), and their anatomical relationship in monogamous prairie voles

PREFACE

While all the work in prairie voles and the Coste et al. mice is my own, as was much of the research, interpretations, and conclusions, the initial findings relating CRF2-like and OT co-expression were made in rat by my collaborators on this project, Joanna Dubrowski, PhD, and Donald Rainnie, PhD, of Emory University.

ABSTRACT

Corticotropin-releasing factor receptor 2 (CRF2) is a g-protein coupled receptor with both central and peripheral actions. Recently, CRF2 has been implicated in modulating stress in response to social manipulations. Considering the importance of oxytocin (OT) for the regulation of social behavior, there may be an intimate anatomical and functional link between CRF2 and OT. In this study I used competitive receptor autoradiography, *in situ* hybridization, and immunoflourescent histochemistry to further define CRF2 expression in monogamous prairie voles, a species in which CRF2 has had an interesting role in relation to social context. I find, based on species and technique comparisons, that CRF2 is distinctly expressed in the septal pole of the nucleus accumbens (NAccSP) in monogamous prairie voles and not in nonmonogamous rodents. Furthermore, I find CRF2-like immunolabeling exclusively in OT-expressing hypothalamic neurons, as well as in far-reaching large-caliber fibers throughout the brain (including the NAccSP, lateral septum, dorsal raphe, and elsewhere). I examine the specificity of the commercial CRF2 antibody used and add to an understanding of CRF2 knockout mice. I end with a discussion of how this immunolabeling could be used in the future.

INTRODUCTION

Corticotropin-releasing factor receptor 2 (CRF2) is a g-protein coupled, 7-transmembrane receptor, that like CRF1 is expressed in the brain and the periphery and is binding site for CRF and members of the urocortin family of neuropeptides (Chalmers et al., 1995; Van Pett et al., 2000; Reul and Holsboer, 2002; Bale and Vale, 2004; Chen et al., 2005a). In the periphery, CRF2 activity regulates a variety of basic functions, such as blood pressure (e.g., Dieterle et al., 2009) and gastric emptying (e.g., Nozu et al., 1999). Centrally, the focus of CRF2 research has been on stress- and anxiety-related behaviors (Bale and Vale, 2004). Initially, it appeared that CRF2 might act to oppose the activity of CRF1, the mechanism by which CRF induces anxiety- and stress-related behavioral responses (Reul and Holsboer, 2002). As CRF2 has been studied in more detail, it now appears that CRF2 activation can be anxiolytic, anxiogenic, or have no effect at all, depending on the site of action and the type of manipulation (Bale and Vale, 2004).

More recently, CRF2 has been identified as an important mediator between stress and sociality. In Syrian hamsters, CRF2 receptors in the bed nucleus of the stria terminalis (BNST) and the dorsal raphe (DR) modulate social defeat (Cooper and Huhman, 2005; Cooper and Huhman, 2007). In rats, CRF2 densities in the DR change in response to social isolation and subsequently affects anxiety (Lukkes et al., 2009a; Lukkes et al., 2009b). Likewise, CRF2 has been tied to social behavior and social context in several studies in monogamous prairie voles. For example, CRF2 activation within the septal pole of the nucleus accumbens (NAccSP) is necessary for CRF-induced partner preference formation in males (Lim et al., 2007). In this dissertation, central CRF2

mediated responses to pair bond disruption (**Chapter 2**), and CRF2 expression densities in the DR were altered by early life family structure (**Chapter 5**).

Placed in the context of social buffering and stress (DeVries et al., 2003), these data suggest there might be a close anatomical and functional relationship between CRF2 activity and the brain systems that drive and regulate social behavior, such as oxytocin (OT). In fact, in **Chapter 5** I note a curious parallel response to early life licking and grooming (LG) by OT mRNA producing cells and CRF2 densities in the dorsal raphe.

Anatomically, CRF2 mRNA has been localized in rats and mice to a number of regions that have OT innervation, such as the amygdala (Van Pett et al., 2000). More recently, Lim et al. (2005) used receptor autoradiography to characterize CRF1 and CRF2 receptor distributions in monogamous (prairie and pine) and non-monogamous (meadow and montane) voles (Lim et al., 2005). While many of the subcortical regions, such as the lateral septum, showed consistent overlap across vole species and a strong similarity with the rat and mouse CRF2 mRNA maps, other regions, such as the septal pole of the nucleus accumbens (NAccSP), did not. High densities of CRF2 were found in the NAccSP and medial striatum of monogamous vole species, but not in nonmonogamous voles. In promiscuous rats and mice, Van Pett et al. (2000) found no CRF2 mRNA in these regions, whereas Primus et al. (1997) using receptor autoradiography notes densities nearly equivalent to CRF1 (Primus et al., 1997; Van Pett et al., 2000). Interestingly, in prairie voles, the NAccSP, NAcc shell, and the medial striatum are areas where OT is released (Ross et al., 2009b) onto high densities of OT receptor (OTR)—OTR densities that are conspicuously decreased or absent in nonmonogamous species (Young and Wang, 2004).

Here, I aimed to extend our understanding of the anatomical localization of CRF2 in prairie voles, with a special focus on points of contact between CRF2 and OT systems. First, I made a direct comparison between rats, mice, meadow voles, and prairie voles using competitive receptor autoradiography with a special focus on CRF2 expression densities within the NAccSP. Because receptor autoradiography depends on the selectivity of the ligand and the sensitivity of the receptor (or receptors), competitive receptor autoradiography can only identify CRF2-like binding patterns. To clarify that prairie vole CRF2-like binding in the NAccSP (and other regions) is in fact the result of distinct patterns of CRF2 gene expression, rather than an indication of an altered selectivity for the radioligand for instance, I used *in situ* hybridization to localize CRF2 mRNA on adjacent sections. Finally, in an attempt to indentify which neurons and processes express CRF2 receptors, and how they relate to the OT system, I conducted immunofluorescence histochemistry using a relatively new antibody from Abcam that targets the extracellular N-terminus of CRF2. Based on the binding and *in situ* hybridization assays, I expected to identify a cluster of NAccSP cells that express CRF2 in prairie voles, but not rats, mice, and nonmonogamous voles, and that these cells would be intimately connected to the OT system.

MATERIALS AND METHODS

Animals

For CRF2 receptor autoradiography and *in situ* hybridization, brains were collected from 3 adult, males from each species: prairie voles, meadow voles, mice, and rats. (Prairie voles, *Microtus ochrogaster*, were from our colony at Emory University;

meadow voles, *Microtus pennsylvanicus*, were also from our colony at Emory University; C57BL/6J mouse brains were a generous gift of K Ressler at Emory University; and Sprague-Dawley rat brains were a generous gift of M Davis at Emory University). For immunofluorescence histochemistry, brains were collected from 4 adult prairie vole males, as well as adult male CRF2 wild-type (WT = 2) and knockout (KO = 2) mice. The CRF2 mouse brains were collected by Allison Anacker and were a generous gift provided by Andrey Rabyinin (Oregon Health Sciences University, Portland, OR). For western blotting, subjects consisted of 9 adult male prairie voles.

All subjects had been group housed from weaning until brain or tissue extraction, and all had been cared for under procedures that were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and OHSU, and were in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Brain and tissue processing

For receptor binding and *in situ* hybridization assays, prairie voles, meadow voles, and mice had been sacrificed with carbon dioxide, while rats had been injected with chloral hydrate. Brains were extracted between 0800-1200hr and snap frozen in finely crushed dry-ice. Brains were then wrapped in labeled aluminum foil and stored at -80C until sectioning on the cryostat. Finally, snap-frozen brains were cryostat sectioned at 20µm (Leica, CM1800), thaw mounted on SuperFrost Plus slides (Fisher), and stored at -80C until receptor autoradiography or *in situ* hybridization. Brains were cut in a 1:6 series.

For immunofluorescence, over-anesthetized animals were transcardially perfused with ice-cold 1X PBS and the 4% paraformaldehyde. Brains were extracted from the skull and placed in 4% paraformaldehyde for a 2hr post-fixation. Brains were then moved to 30% sucrose in 1X PBS cryoprotectant, and stored at -20C. Following storage, brains were sectioned at 30μm on a freezing microtome and moved to a cryoprotective medium consisting of 25% glycerol and 30% ethylene glycol in 0.05 M phosphate buffer until needed.

For western blot, animals were sacrificed with carbon dioxide. Brains were extracted, placed in 1X PBS, and then moved to a mouse brain mold (dorsal side down). Two clean razor blades were used to section out a 1 or 2mm thick brain slices targeting the lateral septum and paraventricular nucleus of the hypothalamus (PVN), respectively. Sections were then chilled on a slide that was dry-ice-cold, and 2mm diameter punches were taken from PVN and LS; heart punches were also taken. Tissues were immediately placed in ice-cold homogenization buffer (0.32M sucrose, 5mM HEPES, pH 7.4, plus protease inhibitor [cOmplete, Mini, EDTA-free, Roche: 11 836 170 001]). Each homogenization sample consisted of 3 subjects. After tissue collection, all tissue samples were homogenized using a Sonic Dismembrator set to 2 (Fisher, Model 100), chilled, vortexed, and then quantified using a BCA assay (Pierce Biotechnology, Rockford, IL) and a microplate spectrophotometer (BioRad Benchmark Plus). After quantification, samples were stored at -20C until western blotting within a week.

Receptor autoradiography

One series of thaw mounted tissue from each animal underwent autoradiographic binding for CRF2 following previously described procedures (Lim et al., 2005). In short, 0.2nM 125I-sauvagine in the tracer binds both CRF1 and CRF2; 500nM of CP-154,526, a highly selective CRF1 antagonist (**Chapter 2, Figure 2.2**), was used to displace the sauvagine from CRF1 receptors, putatively leaving only CRF2 binding. This assay is presented in more detail in **Chapter 2** and the protocol is presented in full in **Appendix 2**. Visual, qualitative analysis of the anatomical distribution of CRF2-like binding was performed after digitization with a digital camera (SPOT RT Slider).

CRF2 *in situ* probe generation

A 531bps fragment of the 5' end of the prairie vole CRF2 cDNA sequence was amplified from a prairie vole total cDNA library using primers that were designed by me based on a rat (NM_022714) and mouse (AY445512) cDNA sequence alignment. The 5' end was targeted because CRF1 and CRF2 mRNA sequences are >70% homologous in the transmembrane and carboxy-terminal coding regions (Chalmers et al., 1995). Primers were 5'-CCACAAACATCCAGAAGAAGTGG (now labeled PTA059) and AGCGGCACCAGACCTCATTGC-3' (now labeled PTA047). The 531 bps fragment was inserted into a pCR4-TOPO plasmid (Invitrogen) with flanking T3 and T7 sites (plasmid now called PLTA020).

CRF2 *in situ* hybridization

After digestion the digestion of PLTA020 by either NotI (to make anti-sense template) or PmeI (to make sense template), the 531 bps prairie vole CRF2 cDNA insert was used as a template for riboprobe amplification using T3 (anti-sense) or T7 (sense) RNA polymerase (Riboprobe Combination System - T3/T7, Promega). Slide treatment and hybridization methods followed a standard protocol (Ressler et al., 2002) and is presented in full in **Appendix 3**. Visual, qualitative analysis of the anatomical distribution of CRF2 mRNA was performed after digitization with a digital camera (SPOT RT Slider).

CRF2 and OT immunoflourescent histochemistry

Fluorescent immunohistochemistry was performed using either a rabbit polyclonal antibody directed against the N-terminal (extracellular) domain of the CRF2 protein (1:1000, ab12964, Abcam, Cambridge, MA) or a mouse monoclonal antibody, clone 4G11, directed against OT (1:7500, MAB5296, Chemicon-Millipore, Billerica, MA), or both for dual-immunoflurescence.

To examine the anatomical expression profiles of the CRF2 and OT in prairie vole tissue, I performed immunofluorescence histochemistry on free-floating serial prairie vole brain sections, from the olfactory bulb to the dorsal raphe. Here, sections were rinsed 3x (10 min each) in 1X PBS, permeabilized with 0.5 % Triton-X 100 in 1X PBS, and incubated for 48 hrs at 4C with either primary antibody or both diluted in 0.5% Triton-X/1XPBS solution. Sections were rinsed 3x (10 min each) in 1X PBS and then incubated at room temperature for 2 hrs with specific Alexa-Fluor secondary antibodies:

Alexa-Fluor 488 goat anti-mouse IgG and/or Alexa-Fluor 568 goat anti-rabbit IgG (1:500, Molecular Probes, Invitrogen, Carlsbad, CA, USA). In cases of dual immunofluroescence, both secondaries were applied simultaneously for the 2 hr incubation. Following immunolabeling, sections were rinsed 3x (10 min each) in 1X PBS and 1x in 0.05 M phosphate buffer (PB), mounted on gelatin-coated glass slides and coverslipped using Vectashield fluorescence mounting medium (Vector Laboratories, Inc., Burlingame, CA). Confocal laser scanning microscopy with Z-stacking was used to visually analyze co-expression of the two immunolablels and to obtain high-resolution photomicrographs using an Orca R2 cooled CCD camera (Hammamatsu, Bridgewater, NJ) mounted on a Leica DM5500B microscope (Leica Mircosystems, Bannockburn, IL).

Specificity of the CRF2 antibody

Because the Abcam CRF2 antibody has not been validated very thoroughly previously, several approaches were used in an attempt to validate the specificity of the primary anti-CRF2 antibody. First, I pre-absorbed each primary antibody with either a control blocking peptide or OT salt to test for cross-reactivity. Second, I employed genetic controls (2 independent lines of CRF2-/- [knockout] mice). And third, I performed western blot analysis. Details of these procedures are presented below.

Immunofluorescence staining after the CRF2 antibody pre-absorption

Based on the remarkable overlap in expression of CRF2-like and OT staining, the specificity of the rabbit polyclonal primary antibody against CRF2 (dilution 1:1000) and mouse monoclonal primary antibody against OT (dilution 1:7500) were tested for cross-reactivity by pre-incubation in blocking solutions. In one solution, both were pre-

incubated with a 20x molar excess of the control CRF2 peptide (1:50, ab80010, Abcam). In a second solution, both were incubated with a high molar excess of OT salt (0.07mM oxytocin acetate salt hydrate in 0.5% Triton/PBS solution, Sigma, Sigma Aldrich, St. Louis, MO). A third solutionwas also prepared and contained only the two primaries; this served as a positive control. Each solution incubated for one hour at room temperature before being applied to free-floating, 30μm-thick prairie vole sections according to the procedure above.

CRF2 Knockout Mice

CRF2-/- and CRF2+/+ mice were obtained from two independent sources: Paul Sawchencko and Wylie Vale, generously provided 4 CRF2-/- and 2 CRF2+/+ mice derived from the Bale et al. (2000) knockout line (Bale et al., 2000); Andrey Rabyinin, Oregon Health and Sciences University, generously provided 2 CRF2-/- and 2 CRF2+/+ mice derived from the Coste et al. (2000) knockout line (Coste et al., 2000). These mice, which are described briefly below, were used to test the specificity of our CRF2 antibody.

The Bale et al. knockout line was generated by isolating the CRF2 locus from a 129 strain mouse genomic library and replacing exons 10-12 (transmembrane domains 5-7) with a phosphoglycerate kinase linked neomycin resistance (PGK-neo) cassette using homologous recombination. ES-cell cultures and chimaeric mice were then generated, followed by heterozygote mutant mouse breeding on a C57BL/6 and 129 genetic background. Non-sibling heterozygous matings were used to produce CRF2-/- homozygosity (Bale et al., 2000).

Likewise, Coste et al. knockout mice were generated by isolating the CRF2 locus in a λ phage clone from a 129/Sv mouse strain genomic library and replacing the genomic sequence that encodes TM domains 3 and 4 with a PGK-neo cassette. RW-4 ES-cells from a 129/SvJ embryo were electroporated and C57BL/6J blastocyst chimeras were produced. Chimaeric mice were bred to C57BL/6J mice to generate mutant offspring (Coste et al., 2000).

Mouse brain tissue preparation for the immunohistochemistry was performed according to the procedures outlined above, and immunofluorescence staining for CRF2 (1:1000 primary) was identical to the procedure described above.

Western Blot experiment

After removal from the -80C freezer, protein homogenates were vortexed and 25ug of protein per sample were loaded into polyacrylamide-SDS mini-gels (Invitrogen, NP0336BOX), separated electrophoretically, blotted onto nitrocellulose membranes (Invitrogen, LC2001), and blocked for 1hr in blocking buffer containing 2% nonfat dry milk, 0.1% Tween 20, 0.05 M NaCl, and 0.01M HEPES (pH 7.4). Subsequently, the membranes were incubated overnight at 4C with the Abcam primary anti-CRF2 N-terminal antibody (1:1000, Abcam) diluted in the blocking buffer. On the following day, the membrane was incubated with HRP-labeled specific secondary antibody (peroxidase conjugated anti-rabbit IgG antibody, Vector Labs, 1:2000) for 1 hr at room temperature. The CRF2 proteins in prairie vole (PVN, LS, and heart) and rat (rat BNST from Joanna Dubrowski), as well as CRF2+/+ and -/- Coste et al. mice (reproductive fat pads), were detected by SuperSignal West Chemiluminescence (Pierce Biotechnology) and visualized

with an Alpha Innotech Fluorochem imaging system (Alpha Innotech, San Leandro, CA). Approximate band sizes (in kDa) were estimated by comparison to a standard ladder (Invitrogen, LC5925) that was run simultaneously in the same gel. To ensure approximately equal amount of protein were added to each lane, membranes were stripped (10 min; Pierce Stripping Solution), washed in blocking buffer, and then re-incubated with a GAPDH primary antibody (1:7500) for 2 hrs diluted in blocking buffer. This was followed by a secondary antibody incubation and reexposure using the SuperSignal West Chemiluminescence and Alpha Innotech imaging system. See **Appendix 4** for complete protocol.

Specificity of the OT antibody

The anti-OT antibody is a monoclonal antibody raised in mouse and targeted against the OT. No cross-reactivity to various AVP analogues (AVP, vasotocin, and [Asu1,6,Arg8]-vasopressin) was shown in a competitive ELISA (Jia et al., 2009).

RESULTS

CRF2 receptor autoradiography across species

Qualitative comparisons of the anatomical distribution of CRF2-like autoradiographic binding revealed several similarities, as well as several differences, across mice, rats, and the two vole species (prairie and meadow; **Figure 6.1**). All four species showed moderate densities of CRF2-like binding in the internal plexus of the olfactory bulb, in the Ctx, BNST, DR, and cerebellum, as well as high densities in the LS and choroid plexus (choroid plexus CRF2-like binding likely corresponds to the CRF2β

isoform, rather than CRF2α brain isoform; **Figure 6.1B-E**). As noted in Lim et al. (2005), prairie and meadow voles differ in the expression of CRF2-like binding in the septal pole of the NAcc (NAccSP) and medial striatum (Lim et al., 2005), with prairie voles having high densities and meadows having low densities (**Figure 6.1B-C**). Here I show that rats and mice also appear to have very low CRF2-like binding in the NAccSP (**Figure 6.1D-E**). In the CA1 field of the hippocampus, both vole species have moderate to high CRF2-like binding (with meadow voles having more), whereas rats and mice have low to none (**Figure 6.1B-E**).

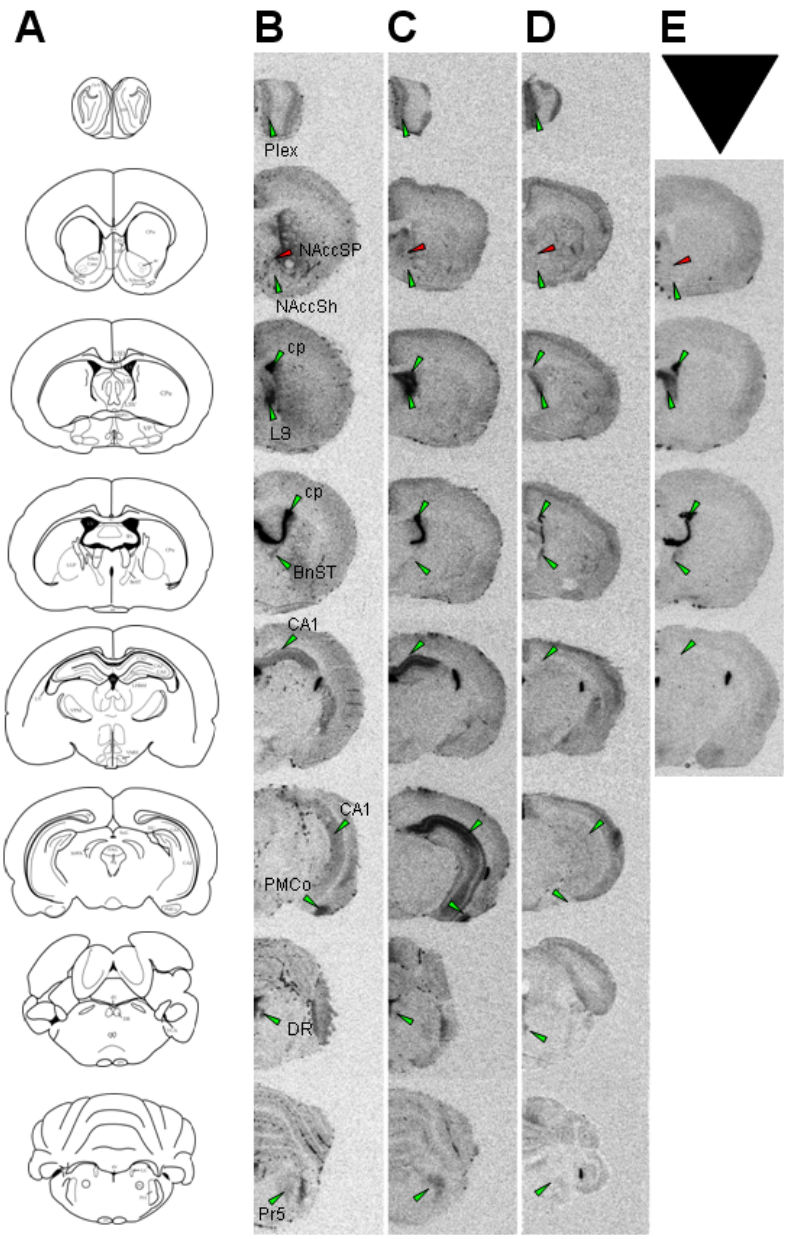


Figure 6.1 - CRF2-like receptor binding in four rodent species

See previous page for figure. **(A)** A series of anterior to posterior schematic representations of the rodent brain (modified from Paxinos and Watson, 1998), providing an anatomical reference for each of the CRF2-like receptor binding autoradiography panels: **(B)** prairie vole, **(C)** meadow vole, **(D)** mouse, **(E)** rat. Red arrows identify the septal pole of the nucleus accumbens (NAccSP); green arrows identify other regions of moderate to high CRF2-like binding density. Plex: internal plexus; NAccSh: shell of the NAcc; cp: choroid plexus; LS: lateral septum; BnST: bed nucleus of the stria terminalis; CA1: CA1 field of the hippocampus; PMCo: posterior-medial cortical nucleus of the amygdale; DR: dorsal raphe; Pr5: principle sensory area 5.

CRF2 *in situ* hybridization

Qualitative description of the anti-sense CRF2 mRNA *in situ* hybridization revealed strong overlap between CRF2-like receptor binding and CRF2 mRNA in the NAccSh, medial striatum, LS, and choroid plexus (**Figure 6.2B-C**). Because binding and *in situ* sections are adjacent, it appears that the localization of the CRF2-like binding is more diffuse than the mRNA, particularly within the LS. Qualitative analysis of the sense CRF2 mRNA *in situ* hybridization treatment revealed essentially no background or regions of non-specific labeling (**Figure 6.2D**).

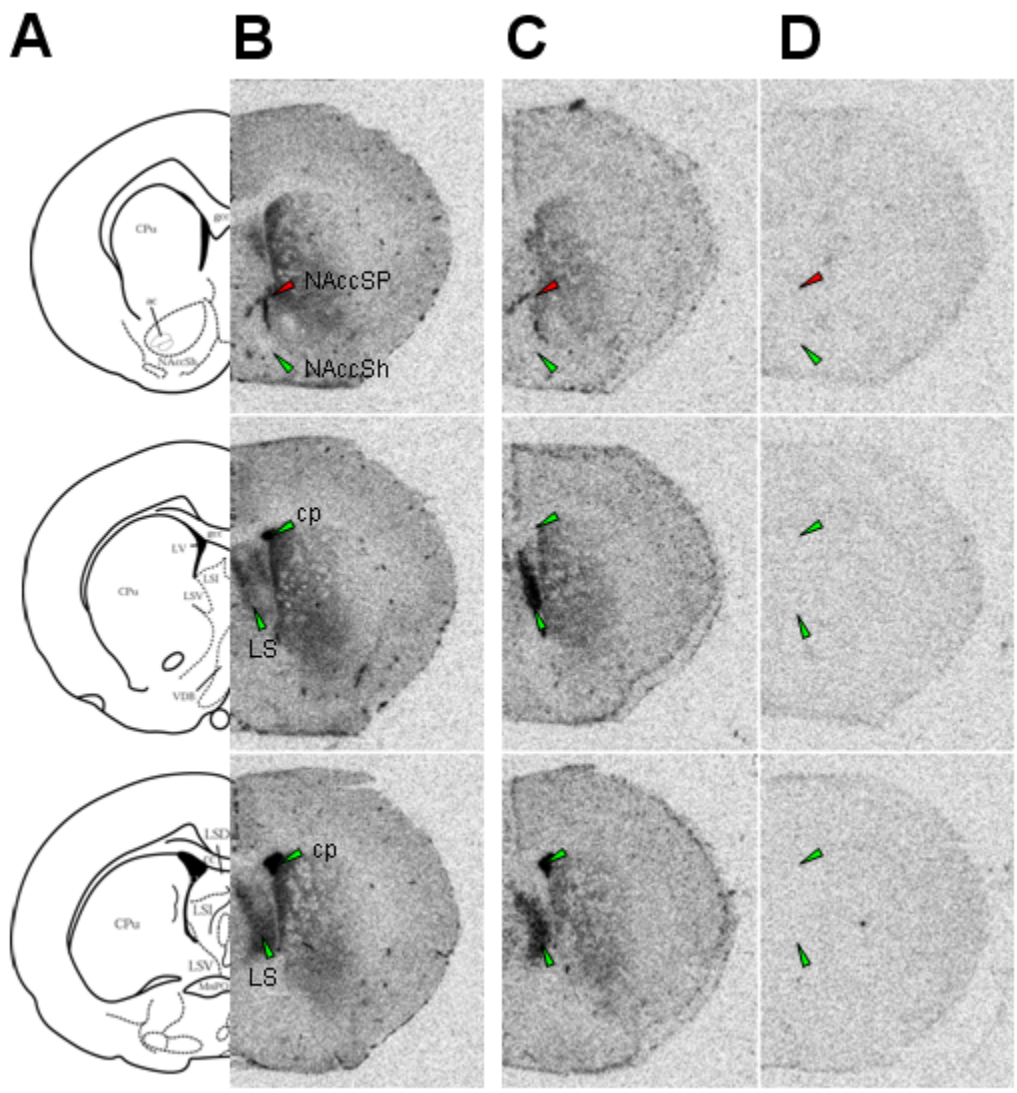


Figure 6.2 - CRF2-like receptor binding versus CRF2 mRNA comparison
(A) A series of anterior to posterior schematic representations of the rodent brain (modified from Paxinos and Watson, 1998), providing an anatomical reference for the CRF2-like receptor binding autoradiography and CRF2 mRNA panels. **(B-D)** are adjacent prairie vole brain sections with CRF2-like receptor binding **(B)**, anti-sense *in situ* hybridization for CRF2 mRNA **(C)**, and sense *in situ* hybridization for CRF2 mRNA **(D)**. Red arrows identify the septal pole of the nucleus accumbens (NAccSP); green arrows identify other regions of high CRF2-like binding density. NAccSh: shell of the NAcc; cp: choroid plexus; LS: lateral septum.

CRF2 and OT immunofluorescent histochemistry

CRF2-like and OT immunofluorescence both revealed high somatodentritic expression in neurons of the paraventricular nucleus (PVN; CRF2-like **Figure 6.3A+C**; OT **Figure 6.3B+D**) and supraoptic nucleus of the hypothalamus (SON; data not shown). The majority of the PVN and SON CRF2-like and OT immunopositive cells were large, magnocellular neurons. Dual-immunofluorescence revealed that nearly all CRF2-like immunopositive neurons in the PVN and SON also express OT, and vice versa (**Figure 6.3E**).

In addition to somatodendritic staining in the hypothalamus, CRF2-like immunoreactivity was found in large-caliber fibers that coursed throughout the brain, including the BNST, LS, NAcc (**Figure 6.3F**), and the DR. As seen in **Figure 6.3**, CRF2-like immunoreactive fibers are characterized by multiple, beaded varicosities and apparent pseudopoda. Significantly, nearly all CRF2-immunopositive fibers co-express OT (for example, **Figure 6.3G** in the NAcc).

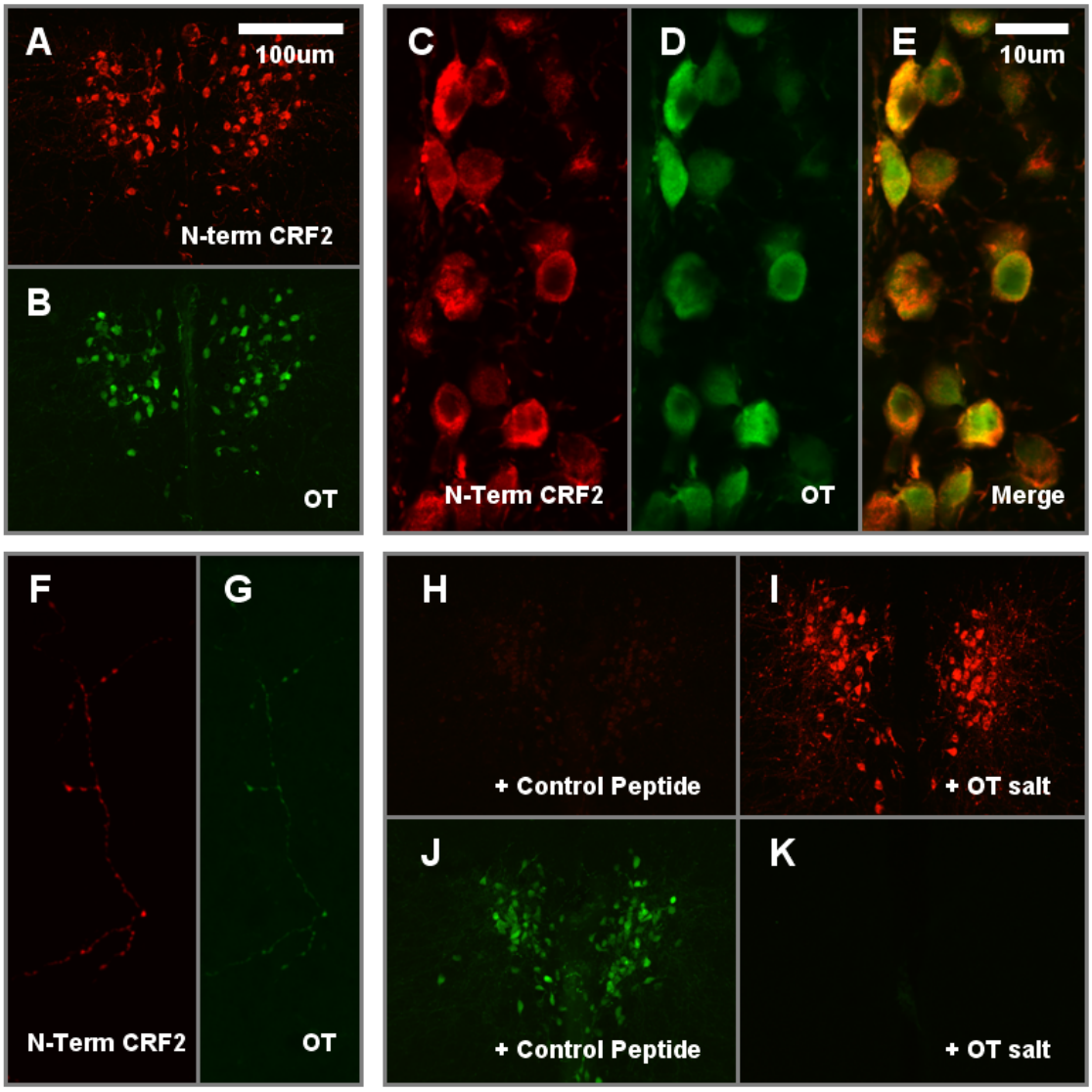


Figure 6.3 - CRF2-like and oxytocin immunoreactivity colocalization
(A) CRF2-like somatodendritic immunofluorescence in the paraventricular nucleus of the hypothalamus (PVN) at low magnification using an N-terminal CRF2 antibody from Abcam. **(B)** Oxytocin (OT) somatodendritic immunofluorescence in the PVN at low magnification. **(C-E)** High-magnification of CRF2-like **(C)**, OT **(D)**, and CRF2-like/OT colocalized immunoreactivity in PVN magnocellular neurons. **(F-G)** High-magnification of immunoreactive fibers in the NAccSP that are double-labeled with CRF2-like and OT staining. Note the beaded profile of this large co-labeled fiber. This type of colabeling in fibers is found in many parts of the brain, including the LS, BNST, DR and cortex (not shown). **(H-K)** Illustrate the selectivity (and non-cross-reactivity) of each primary antibody: the CRF2 antibody staining is abolished by pre-absorption with the Abcam CRF2 control peptide **(H)**, whereas the OT antibody is not **(J)**; likewise, the OT antibody staining is abolished by pre-incubation with OT salt **(K)**, whereas the CRF2 antibody is not **(K)**. Ruler bars represent 100um for **(A-B, H-K)** and 10um for **(C-G)**.

CRF2 antibody specificity

Pre-absorption

Due to the high-degree of overlap in the CRF2-like and OT immunofluorescence, cross-reactivity was tested using three separate series of floating brain sections. One series was pre-incubated with a 20x excess of a commercially sensitive CRF2 control peptide (generated for the CRF2 antibody by Abcam, ab08001); the other was treated with an excess of OT salt; the third had no blocking reagent. In the first series, the CRF2 control peptide essentially abolished somatodendritic and fiber CRF2-like staining in the PVN and throughout the brain (**Figure 6.3H**), whereas it had no effect on the anti-OT antibody (**Figure 6.3J**). The opposite results were found in the second series: 0.07mM OT salt completely abolished anti-OT antibody immunofluorescence (**Figure 6.3K**), but had no effect on CRF2-like staining (**Figure 6.3I**). Dual-labeling occurred normally in the third treatment (e.g., **Figure 6.3A-B**).

CRF2 knockout mice

To ensure specificity, I also examined CRF2-like immunoreactivity in brain sections derived from two independent lines of CRFR-/- (knockout) and CRF2+/+ (wild-type) mice (Bale et al., 2000; Coste et al., 2000). Like prairie voles, CRF2+/+ mice exhibited robust CRF2-like immunoreactivity in the PVN and in fibers throughout the brain (**Figure 6.4A**). Surprisingly, both lines of CRF2-/- mice had robust CRF2-like immunoreactivity in neurons throughout the PVN (**Figure 6.4B**, Coste et al. mice) and SON, as well as in fibers throughout the NAcc, LS, DR, BNST, and elsewhere. See the

discussion for a closer examination of the methodology used to generate the CRF2-/- mice and their appropriateness as genetic controls.

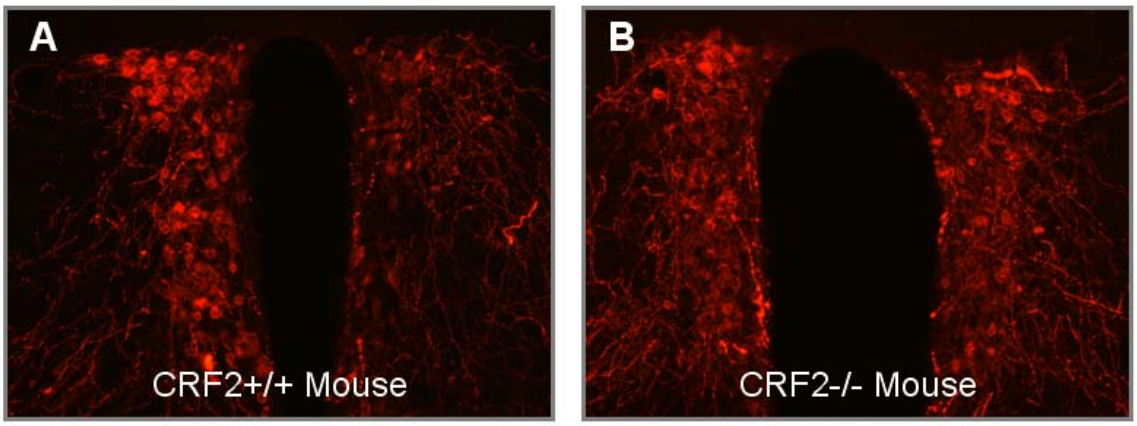


Figure 6.4 - CRF2-like immunoreactivity in Coste et al. (2000) CRF2 mice
(A) CRF2-like somatodendritic immunofluorescence in the PVN of CRF2+/+ mice using an N-terminal CRF2 antibody from Abcam. **(B)** CRF2-like somatodendritic immunofluorescence in the PVN of CRF2-/- mice using the same N-terminal CRF2 antibody from Abcam. Note how they are essentially indistinguishable.

Western blotting

The Abcam CRF2 antibody was used in western blot analysis. Expression of CRF2 protein was examined in a variety of tissues, including prairie vole PVN, LS, and heart tissue extracts, BNST from rats, and reproductive fat pads from CRF2+/+ and CRF-/- mice. As shown in **Figure 6.5A**, the Abcam N-terminal anti-CRF2 antibody binds several bands that differ in intensity across different tissues. Perhaps the four most prominent bands occurring across tissues and species are located at ~41 kDa, ~48 kDa, ~62 kDa, and ~75 kDa. CRF2+/+ and CRF2-/- mouse reproductive fat pad tissue was loaded in alternating lanes of the gel, but there was no alternation in the pattern of the bands (**Figure 6.5, "Mouse"**).

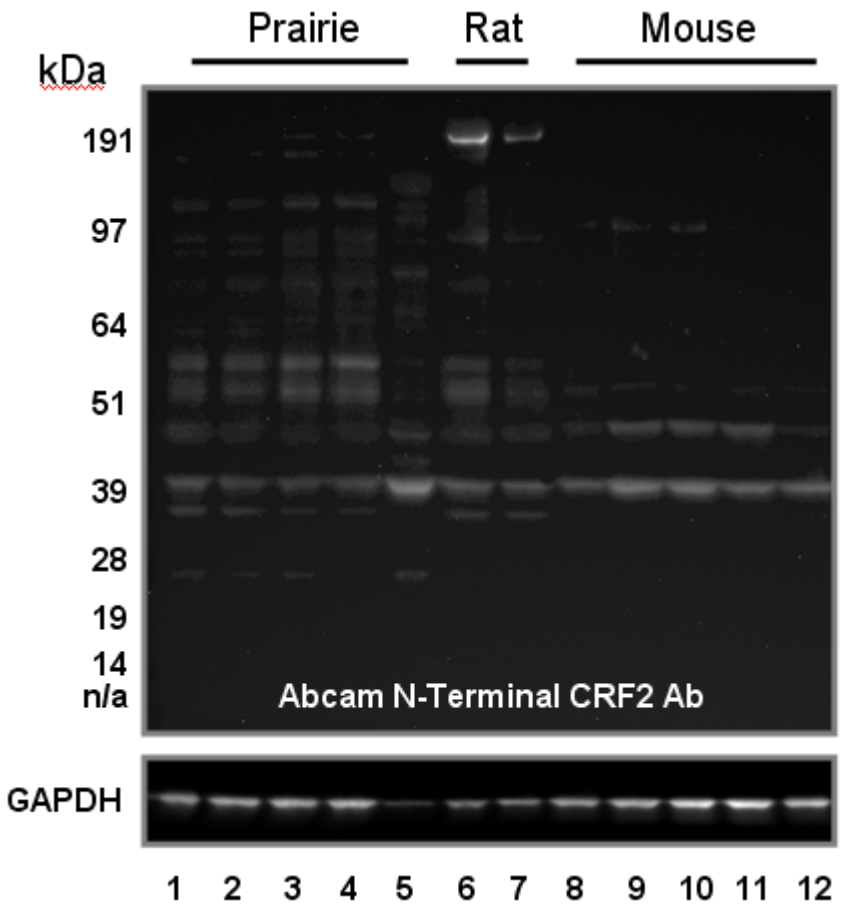


Figure 6.5 - Western blot with the N-terminal targeting CRF2 antibody from Abcam in a variety of tissues

Western blot for the N-terminal CRF2 antibody from Abcam in a variety of tissues. CRF2 immunoblotting using the N-terminal CRF2 antibody from Abcam (1:1000 dilution) revealed variety of bands that differed across tissues. Prairie tissue was derived from tissue punches from the PVN (prairie lanes 1-2), LS (prairie lanes 3-4), and the heart (prairie lane 5); rat tissue (provided by Joanna Dubrowski) was derived from dissected BNST (rat lanes 6-7); mouse lanes consisted of reproductive fat pad tissue from 2 CRF2+/+ and 3 CRF2-/- mice derived from the Bale et al. (2000) line: +/+ tissue was loaded in mouse lanes 8, 10, 12, -/- tissue was loaded in mouse lanes 9, 11. Note there is no alternation in the mouse bands as would be expected if the CRF2 antibody was detecting a band that was present in the +/+ mice, but absent in the -/- mice. After exposure, the blot was stripped and probed for GAPDH to serve as a loading control.

DISCUSSION

CRF2 localization

Despite the need to know anatomical location in order to understand function, the results presented here demonstrate the difficulty of attempting to localize CRF2 expression. The CRF2-like receptor binding presented above is wholly consistent with the binding profiles presented previously for voles (Lim et al., 2005) and similar to mRNA maps in mice and rats (Chalmers et al., 1995; Van Pett et al., 2000). It also clearly demonstrates that the monogamous prairie vole have a distinct expression pattern within the NAccSP that differs from nonmonogamous voles (here the meadow vole) and from promiscuous rats and mice (**Figure 6.1**). The question remained, however, whether this difference is due to species specific gene expression, receptor transport, or radioligand selectivity. To help distinguish between these possibilities, I performed *in situ* hybridization for CRF2 mRNA with a prairie vole specific probe targeting the 5' end of the mRNA. Qualtitative nalysis at the level of the NAccSP and LS revealed a striking similarity in the expression pattern and density of the CRF2-like receptor binding and CRF2 mRNA that was not the result of background or artifact in this region (**Figure 6.2B-D**). Despite indications that CRF2-like receptor binding might be slightly more diffuse than CRF2 mRNA (see the LS in **Figure 6.2**), the CRF2 mRNA profile confirms that the observed CRF2-like receptor binding is in fact CRF2.

But which neurons and neuronal processes actually express CRF2? Since neither receptor binding nor *in situ* hybridization for mRNA can fully address this question, I aimed to use a commercial antibody targeted against the extracellular N-terminal of the CRF2 protein. I expected to find clusters of CRF2-labeled cells within brain regions that

also had high levels of mRNA and CRF2 binding (e.g., NAccSP, LS, DR, BNST, etc.). Instead I found highly localized somatodendritic labeling only in the PVN and SON of the hypothalamus (similar to what was described in rats by Joanna Dubrowski, PhD, and Donald Rainnie, PhD, just prior to the work described here). The hypothalamic labeling was highly specific and appeared in large magnocellular neurons, with fibers projecting in each of the cardinal directions (**Figure 6.3A**). Most other regions of the brain exhibited CRF2-like immunoreactivity only in large-caliber fibers that were characterized by punctate beads (**Figure 6.3F**). Interestingly, the immunoreactivity was reminiscent of OT staining recently described in prairie voles by Ross et al. (2009).

When I performed CRF2-like and OT dual-immunofluorescent histochemistry and used confocal microscopy for analysis, I found a near perfect overlap in the expression of OT and CRF2-like staining, within magnocellular neurons of the PVN (**Figure 6.3E**) and SON, as well as in the large-caliber fibers that coursed throughout the brain, including the NAcc, LS, DR, BNST, and cortex (**Figure 6.3F-G**). This colocalization of OT and CRF2-like immunoreactivity appears to be a general rodent phenomenon, rather than a vole-specific curiosity, for a similar profile was found in mice (e.g., **Figure 6.4A**) and in rats (collaboration and communication with J Dubrowski and D Rainnie).

These findings, which demonstrate a close anatomical relationship between the OT and CRF2 systems, have several important implications for our understanding of how the CRF and OT systems might interact on mechanistic level. Such conclusions, however, depend on the accuracy of the evidence that CRF2 and OT do in fact colocalize; and the accuracy of that claim depends on the specificity of the commercial CRF2 antibody I

employed. While antibody specificity is a concern in every study, the issue is especially pronounced here due to the unexpected disconnect between CRF2 mRNA and binding maps and CRF2-like immunoreactivity, as well as the near perfect overlap between CRF2 and OT immunostaining.

With such high-degrees of overlap, there is an obvious concern over the possibility of antibody cross-reactivity. To address this, pre-absorption experiments were performed using Abcam's commercially available CRF2 control peptide and OT salt. Pre-absorption revealed no evidence of cross-reactivity, with the control peptide only abolishing CRF2-like immunoreactivity and OT salt only abolishing OT immunoreactivity (**Figure 6.3H-K**).

Preabsorption, however, only demonstrates that the synthetic CRF2 control peptide was the *best* epitope for the CRFR2 antibodies. In order to rule out any possibility of cross-reactivity as an explanation for the observed staining, I applied the Abcam CRF2 antibody to two lines of CRF2-/- (knockout) mice that were meant to serve as genetic controls. In 2000, Bale et al. reported CRF2-/- mice that showed no CRF2 receptor autoradiographic binding using radiolabeled sauvagine (Bale et al., 2000). Likewise, Coste et al. (2000) had also generated CRF2-/- mice and reported that there was no CRF2 mRNA (Coste et al., 2000). I therefore expected no CRF2-like immunoreactivity in either of these knockouts. What I found instead was robust magnocellular staining in the PVN and SON in the CRFR2-/- mice, as well as CRFR2-like immunoreactive fibers throughout the NAcc, BNST, LS, DR and cortex (**Figure 6.4B**). The immunoreactivity was essentially indistinguishable from the staining that occurred in CRFR2+/+ mice (**Figure 6.4A**).

Ordinarily, these data would have moved us to reject the specificity of the Abcam CRF2 antibody and conclude that the Abcam antibody is detecting some other protein. Closer examination of the knockouts, however, offered an alternative explanation: It is plausible that the antibody is detecting a truncated form of the normal CRF2 protein. The Abcam CRF2 antibody reportedly targets some (commercially sensitive) portion of the N-terminus. Even in wild-type animals, the CRF2 gene can be expressed in several different truncated and soluble forms (Miyata et al., 1999; Miyata et al., 2001; Chen et al., 2005b). These truncated and soluble forms contain the entirety of the N-terminal domain and would therefore be detected by the Abcam CRF2 antibody.

A similar phenomenon might be occurring in the CRR2-/- mice, in which only a part of the gene has been knocked out. Neither the Bale et al. group nor Coste et al. group produced CRF2-/- mice by the complete deletion of the CRF2 gene. CRF2-/- mice from Bale et al. (2000) were generated by deleting exons 10, 11, and 12, which code transmembrane (TM) domains 5-7 and CRF2-/- mice from Coste et al. (2000) were generated by deleting transmembrane (TM) domains 3 and 4, which are coded by exons 6-9. In both cases, the sequence that codes for the transcription start site and most of the N-terminus remains intact. Since the Abcam CRF2 antibody targets a portion of the N-terminal domain, such an artificially truncated CRFR2 protein, if it were produced, would also result in robust immunostaining even in CRF2-/- mice.

What about the nonsense-mediated RNA decay (NMD) pathway? The artificially truncated mRNA should be degraded by the NMD (Chang et al., 2007). Recent work by Evans & Seaholtz (2009), however, demonstrates that at least one of the endogenous truncated CRF2 mRNAs is able to escape the degradation process (Evans and Seasholtz,

2009). The same may be happening with the artificially truncated mRNAs in the CRF2-/- mice (see **Figure 6.6** for schematic representation).

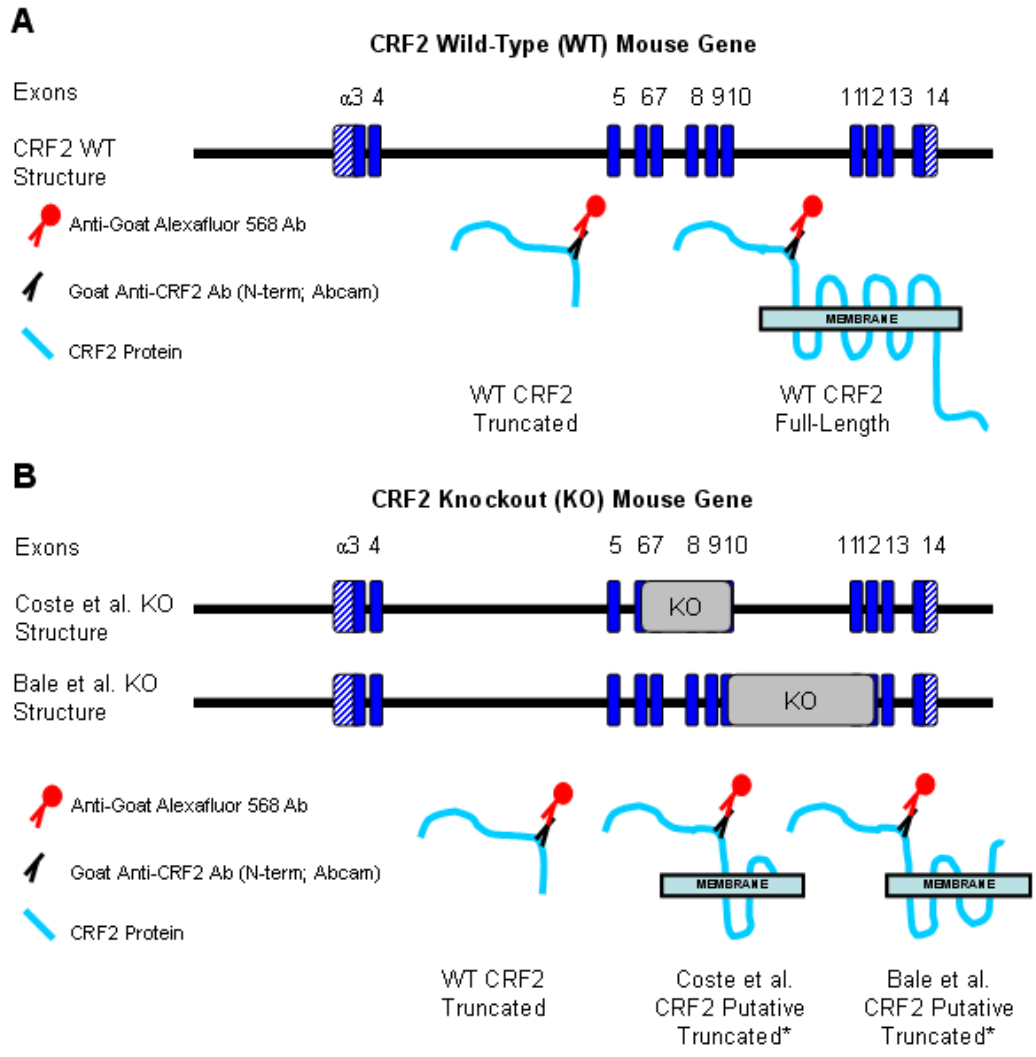


Figure 6.6 - Schematic representation of the Abcam CRF2 antibody in relation to various genes and gene products

(A) Schematic representation of the normal wild-type (WT) gene with the 12 exons that code for the brain isoform of CRF2α, as well as two normal gene products: a soluble truncated form and a full-lenth, 7 transmembrane (TM) form. **(B)** Schematic representation of the CRF2-/- (knockout) genes generated by Bale et al. (2000) and Coste et al. (2000) (Bale et al., 2000; Coste et al., 2000), as well as the various protein products in relation to the Abcam N-terminal antibody. Note that the Bale et al. gene could still have two transmembrane domains, the Coste et al. gene could still have four. * denote a hypothetical situation. Antibody target on the N-terminus is also hypothetical, since the true epitope is commercially sensitive.

Such an explanation might work for the Bale et al. (2000) mice, but the Coste et al. (2000) group demonstrated no CRF2 mRNA in their mice (Coste et al., 2000). Without any mRNA, there should be no protein. Here again, closer examination of the methodology proves informative. Coste et al. examined CRF2 mRNA transcript levels using an RNase protection assay in which the protective probe was aimed at only 239 nucleotides. These 239 nucleotides correspond exactly to the region encoding TM domains 3 and 4. Since TM domains 3 and 4 are the parts of the gene Coste et al. deleted, it is plausible that all the CRF2 mRNA that was produced in the CRF2-/- mice was left unprotected and RNase degraded it. It was thus undetected in their assay.

Therefore, in both lines of CRF2-/- mice, it is possible that the first series of CRF2 exons are transcribed and this truncated mRNA escapes degradation and is translated. Once translated, the N-terminus of the CRF2 protein would be available for detection. In support of this suggestion, McEuen and Bale recently demonstrated the presence of CRF2 mRNA in their CRFR2-/- mice (TL Bale *personal communication*).

In sum, it would appear that both CRF2-/- mice are not sufficiently characterized to disprove the specificity of the Abcam CRF2 antibody. These findings even suggest that a better characterization of these CRF-/- mice is necessary for interpreting the data they are used to produce. The soluble CRF2 variant described by Chen et al. (2005) is known to bind to CRF, but not sauvagine (Chen et al., 2005b). Sauvagine, however, is what Bale et al. (2000) used to demonstrate that their CRF2-/- mice were functional knockouts. If, however, a truncated form of the CRF2 protein is being produced, it may have same ligand binding profile as the endogenous soluble CRF2, making these mice in effect, soluble CRF2 over-expressers. Since soluble CRF2 is thought to sequester CRF (and

perhaps other ligands) to prevent it from acting at receptors, this could have important implications for our understanding of behavioral responses to stressors of CRF2-/- mice.

With CRF2-/- mice coming under suspicion as a negative genetic control, I also attempted western blotting to get a sense of the Abcam antibody specificity. Immunoblots revealed multiple bands with differing intensities depending on the source tissue (**Figure 6.5A**). Four bans appeared consistently across tissues and across species; the bands were approximately 75, 64, 48, and 40 kDa, respectively. The 48 kDa band may correspond to the full-length CRF2 protein calculated from the published sequence (Miyata et al., 2001). Others, however, have interpreted larger bands (between 60-80 kDa) as the full-length receptor, with the size shift due to N-glycosylation (Lee et al., 2004; Bishop et al., 2006; Tian et al., 2006). This banding pattern could be due to other factors, however, including non-specificity of the Abcam antibody. In support of this later interpretation are the bands from the CRF2-/- and CRF2+/+ mouse tissues (**Figure 6.5A**). Since these tissues were loaded in alternating fashion, one would expect an alternating banding pattern. In fact, even if the CRF2-/- mice still produce an artificially truncated form of CRF2, bands of at least one size (the true full-length) should also alternate. This was not observed, but reproductive fat pad (the only CRF2-/- tissue to which I had access) does not have many of the bigger bands. The data are therefore incomplete currently.

The significance of CRF2/OT colocalization

Based on these data, I cannot know with even reasonable certainty whether the CRF2-like immunoreactivity is in fact CRF2. If, however, I accept the specificity of the CRF2 antibodies, then the light microscopic immunofluorescence data showing

CRF2/OT colocalization is extraordinarily interesting. First, if the majority of CRF2 is expressed only in OT producing cells, then in order to justify it with the CRF2 mRNA map (**Figure 6.2C**), the mRNA would need to be trafficked to the CRF2 expressing terminals in the large-caliber fiber network. I might expect all three signals (binding, mRNA, and immunolabeling) to disappear if I lesion the PVN. Second, if CRF2 is expressed on magnocellular neurons and processes containing OT, activation of CRF2 by selective agonists (such as Urocortin 2) may provide a means of directly manipulating OT release. Indeed, hypothalamic CRF2 activation does increase OT release, at least into the neurohypophyseal system (Bruhn et al., 1986). Third, this colocalization could help explain the concominant increases in both OT mRNA clusters and CRF2 densities in the dorsal raphe described in **Chapter 5**. One might have expected increases in all CRF2 densities, but the region specific effect is still possible. Ross et al. (2009) found that specific regions of the OT system project to the NAcc (Ross et al., 2009b); it may be that the anterior region of the PVN that saw the most significant increase in OT mRNA clusters in single-mother reared females is the region that projects to the DR. Lastly, there is the intriguing possibility that much of the current CRF2 literature could be reinterpreted in light of its relationship with OT. Indeed both, CRF2 and OT seem to act to dampen HPA axis activity, but only in response to particular stressors (e.g., Mantella et al., 2005; Amico et al., 2008).

Conclusions

Here I aimed to extend our understanding of the CRF2 receptor by gaining a more complete picture of its anatomical expression and it potential relationship with the OT

system. While others have already shown CRF2-like receptor binding in the monogamous prairie vole (Lim et al., 2005), I show here for the first time that the CRF2-like autoradiographic binding distinctly overlaps with CRF2 mRNA expression, even in the NAccSP. As powerful as CRF2 autoradiography and *in situ* hybridization are for understanding the basic anatomical distribution, they cannot easily identify both the neurons and neuronal processes that express CRF2 and respond to its activation. In collaboration with Drs J Dubrowski and D Rainnie, I identified a commercial CRF2 antibody that provided very interesting CRF2-like immunoreactivity due to its colocalization with OT PVN magnocellular neurons and OT containing large-caliber fibers. Using many of the best techniques available, including pre-absorption, CRF2-/- mice, and western blotting, I can conclude that the CRF2-like immunoreactivity is selective in brain tissue, but I cannot state whether the target is CRF2 or not. In the process, this work also identified an important area of research relating to the current iterations of CRF2-/- mice.

In the end, if CRF2 is localized with OT, I propose using CRF2 activation as a means of selective regulating OT release and examining the effects of early life experience on both systems simultaneously. Instead, if the Abcam CRF2 antibody is selective for some other target, I propose identifying that target through immunoprecipitation and mass spectrometry, and then, depending on the protein's identity, using it as a selective target for manipulating OT neurons and fibers.

CHAPTER 7

General conclusions and future directions

Major findings

This thesis aimed to develop prairie voles (*Microtus ochrogaster*) as an animal model that can be used to study how differences and shifts in family dynamics affects behavior, mental health, and vulnerability to psychopathology. In **Chapter 1**, I briefly reviewed and synthesized the literatures from several research fields to illustrate how the "family" is an integrated, dynamic social system, in which each member and relationship has the ability to influence the development, behavior, and mental health of every other member. I also introduced current animal models that are used to study the neurobiological basis of this influence. In **Chapter 2**, I examine the impact of the male-female family bond on behavioral and neurobiological outcomes by helping to study the effects of pair bond disruption in prairie voles. We found increased passive-coping in males separated from their female partners and this behavioral response depended on the activation of both CRF1 and CRF2. In **Chapter 3**, I proposed to use prairie voles as an informative, nontraditional, animal model of family interactions that can be studied in a quantitative and repeatable manner, and that can be manipulated in ethologically and biomedically relevant ways. I found that prairie voles show a rich set of family dynamics, including maternal and paternal care and parental coordination on several direct parenting behaviors. I also found that parental care changes dramatically in single-mother (SM) rearing environments, despite normal maternal care. In **Chapter 4**, I examined how ethologically relevant manipulations of prairie vole family structure can influence the diversity of social and emotional behavioral profiles in adult offspring. I found striking differences in female alloparental care, marked differences in the time course of stable partner preference expression in both sexes, as well as different levels of pup care

between biparentally (BP) reared and SM-reared animals. In **Chapter 5**, I attempted to find a neurobiological link between ethologically relevant differences in family structure and the dramatic differences in behavioral development observed in Chapter 4 by focusing on variation in neuropeptides and neuropeptide receptors. I found altered OT, OTR, and CRF2 in key brain regions, but also found early life family structure to have no observable impact on the highly variable neuropeptide receptor expression in the ventral forebrain. Finally, in **Chapter 6**, I examined the anatomical relationship between CRF2 and OT, as a possible mechanistic link between stress and sociality. I found data consistent with differential CRF2 gene expression across species, and found colocalization of CRF2-like OT immunoreactivity using commercial antibodies in the NAcc and DR.

In sum, this thesis examines the contribution of family life and its dynamics to diversity in neurobiology and behavior in prairie voles, with the hope that findings made with this model system can inform developmental neuroscience, family and child psychology, and medicine in humans, as well as behavioral ecology and ethology.

Disruption of selective social bonds

In humans, the formation of selective social bonds, such as marriage, is a common occurrence; so is bond disruption. In both cases, we know relatively little about the neurobiological factors that drive and mediate the dramatic behavioral, emotional, and psychological changes that accompany these shifts in selective social (and family) environment. Loss of a spouse is an especially traumatic experience that is normally accompanied by grief. Unfortunately for some individuals, the response can turn

pathological (Biondi and Picardi, 1996; Zisook et al., 1997; Shear and Shair, 2005). Therefore, understanding the neurobiology that mediates normal responses to social loss may provide some insight into maladaptive human responses as well as treatments. In **Chapter 2** of this dissertation, I helped study the effects of pair bond disruption in monogamous prairie voles. Prior to my joining the Larry Young laboratory and the social loss project, OJ Bosch had started studying the effects of pair bond disruption in prairie voles. He had found increased passive-coping behavior in the forced swim and tail suspension tests (FST and TST, respectively), as well as increased hypothalamic-pituitary-adrenal (HPA) axis activity, in response to pair bond disruption. Since the FST and TST are typically used to screen antidepressants, OJ Bosch concluded that the passive-coping behavior and the increased HPA axis activity might represent a depressive-like state that is, in certain respects, similar to human grieving. Indeed, a similar "separation syndrome" had been described by JN Crawley in the mid-1980s in monogamous Siberian dwarf hamsters (Crawley, 1984b, 1984a, 1985), which also had increased HPA axis activity (Castro and Matt, 1997). In humans, pathological forms of grieving are often marked by abnormally severe and dysregulated bouts of depression and HPA axis activity (Biondi and Picardi, 1996; Shear and Shair, 2005; Hensley, 2006). Given the connetion between the stress of partner loss, increased HPA activity, and the known dysregulation of CRF activity in human depression, OJ Bosch then aimed to manipulate the CRF system by blocking receptor activation at the point of loss. By nonselectively blocking the activity of the two known CRF receptors (CRF1 and CRF2), he found that the depressive-like effect of pair bond disruption could be prevented. At this point, however, it was unclear which receptor mediated the effect. I used competitive

autoradiography to identify selective receptor antagonists that worked with appropriate specificity in voles and helped perform surgery, and score the behavioral tests. We found that blockade of either receptor type (CRF1 or CRF2) by selective blockade from the time of partner loss was sufficient to prevent the induction of the depressive-like passive coping. These findings were a step in the right direction, but there are still many unanswered questions for future investigation.

For prairie voles (as for many humans), the pair bond is a sociosexual bond. Therefore pair bond disruption results in the loss of both a social partner and future sexual opportunity. While in humans it is the loss of social attachment that is most painful, in prairie voles it may be different: the passive-coping may actually be a response to loss of sexual opportunity rather than loss of a partner. If so, the relevance of this model for human research may be markedly diminished. To tease these two possibilities apart, one could use the central infusion of a selective vasopressin 1a receptor (V1aR) antagonist within the ventral pallidum to selectively block the onset of the social partner preference during an extended cohabitation period (Young and Wang, 2004). Since the use of a V1aR antagonist has shown no effect on sexual behavior (Winslow et al., 1993), males could be tested for HPA axis dysregulation and passive-coping in response to the removal of sexual opportunity alone.

Another, and perhaps more important, avenue of research would be identifying which brain regions are necessary for the induction of the behavioral phenotype. CRF1 and CRF2 each have distinct distributions within the brain. As noted by Lim et al. (2005) and **Chapter 6**, the distribution within the shell and septal pole of the nucleus accumbens is dramatically different between monogamous and nonmonogamous species.

Interestingly, activation of both CRF1 and CRF2 within the NAcc is necessary for CRF-induced partner preference formation (Lim et al., 2007). Evolutionarily, these receptors, with their distinct patterns in monogamous species, may have arisen in order to induce proximity seeking in response to stressors or relatively long foraging bouts. This would have the practical consequence of maintaining the bond. In response to permanent partner loss, however, the activation of this system could result in maladaptive behavioral strategies such as passive-coping.

Situated at the nexus of many parts of the social bonding circuit, localized infusion into the NAcc of a selective CRF1 and/or CRF2 receptor blocker would be an excellent first target. From there, other parts of the circuit could then be tested. Another potential anatomical target is the dorsal raphe (DR). CRF2 within the DR is sensitive to social context (Cooper and Huhman, 2007; Lukkes et al., 2009b; **Chapter 5**) and both CRF1 and CRF2 regulate serotonin release (Lukkes et al., 2008), one the neurotransmitters that is often targeted in the treatment of depression. Since high and low doses of CRF have opposing effects on serotonin release into such regions as the NAcc, this could be regulating coping-strategies.

Finally, another avenue of research stemming from this work is identifying which factors make animals more or less vulnerable to passive-coping in response to pair bond disruption. In addition to identifying genetic factors, such as polymorphisms in the CRF receptor genes that might regulate affective responses (e.g., Bradley et al., 2008), it would be interesting to identify early life experience factors as well. In particular, the ethologically relevant manipulations of family structure presented in this thesis could be

used to test the the hypothesis that early life experience can affect responses to partner loss.

Prairie vole family dynamics

For many socially monogamous, biparental species, family life consists of a broader array of social interactions than for uniparental species, including mother-father, mother-infant, and father-infant interactions. The impact of differences, shifts, and changes in these relationships on the behavioral, emotional, and psychological outcomes of each family member has been the focus of a large number of studies in such fields as family psychology (Maccoby, 2000) and pediatric medicine (Schor, 2003). While traditional animal models (rats, mice, and rhesus macaques) have provided helpful insight into the endocrine and neurobiological systems that are sensitive to severe and subtle differences in mother-infant interactions, they do not recapitulate many of the other influential family relationships, including mother-father and father-infant relationships. I proposed to use monogamous, biparental prairie voles as a complementary alternative to traditional animal models, but it was unclear whether their family interactions would mimic human family interactions to a degree that would be relevant and amenable to translational biomedical research. In **Chapter 3**, I found that, in laboratory environments, prairie voles demonstrate a rich, dynamic family system, in which both parents contribute to the care of offspring. Moreover, I show that they exhibit parental coordination on several measures, including nest attendance and LG. In the second part of **Chapter 3**, I manipulated family structure in an ethologically manner by creating biparental (BP) and single-mother (SM) family units and found dramatic differences in pup care, despite

comparable levels of maternal investment. This work creates a novel approach to early life manipulations. For example, in rats, the traditional approach is to screen mothers for maternal behavior and take the extremes (Meaney, 2001). My approach in prairie voles offers greater ease of the manipulation of early life experience and avoids the genetic segregation that might occur in selection paradigms. It also allows manipulation in an outbred, naturally occurring species that demonstrate a rich set of social behaviors.

My findings in relation to prairie vole family dynamics also beg a number of follow up questions. Some of the most obvious relate to family structure and offspring development and are the focus of **Chapters 4** and **5**. But there are other important questions that could be profitably addressed. In humans, little is known about the neurobiological factors that help maintain long-term social bonds. Neuropeptide systems such as OT and AVP and their respective brain receptors (OTR and V1aR) have been implicated in partner preference formation in prairie voles, as have dopamine and dopamine receptors (Young and Wang, 2004). Less, however, is known about which brain systems drive the continued investment in the partner or how the stability of the male-female bond influences pup care.

Future research based on my findings could address these questions in several ways. For example, viral vector technologies could be used to permanently up-regulate (via adeno-associated viral [AAV] gene transfer; e.g., Lim et al., 2004) or down-regulate (via shRNAs; e.g., Garza et al., 2008) V1aR expression densities in the ventral pallidum, an area that has been implicated in modulating reward and reinforcement and facilitates partner preference formation in males (Lim et al., 2004; Young and Wang, 2004). Investigators could then examine whether manipulated males alter their interactions with

their partners, their offspring, or both. A similar experiment could be attempted with females. Viral vectors containing OTR shRNAs or an AAV containing the OTR gene (Ross et al., 2009a) could be injected bilaterally into the shell of the NAcc. Maternal behavior and partner-directed interactions could be quantified and examined for changes. Such targeted manipulations may reveal novel roles for well-studied brain regions and may highlight the need to examine other brain regions such as the OTR-rich prefrontal cortex during this phase of the pair bond.

Experimental manipulation of the family unit could also be used to address how mother-father interactions impact paternal care of offspring, an investigation that would be far less meaningful if conducted in rats or mice. Prairie vole mothers do not appear to need the father's presence to maintain normal maternal care (**Chapter 3**), but does the father need the maternal influence? A perforated acrylic barrier that divides the nest in half (allowing pups access to both sides but not the parents) could be used to test whether paternal care occurs normally in the absence of regular contact with female partner. In such an experiment, pups would likely favor maternal care for nutritional reasons, but when the mother is away from the nest, would the separated father still interact with them in a manner comparable to the cohabitating father when he is the lone nest attendant? My analysis of prairie vole family dynamics makes this suitable for investigation. Since cohabitating human fathers contribute more parental care than non-cohabitating fathers (Kentner et al., 2009) such an experiment may offer a new model for identifying interventions that may increase paternal investment in fractured families.

Family structure and its influences on the social behavior of adult offspring

In the human literature, variations in parental care, whether severe or subtle, have marked effects on the behavioral and emotional outcomes of children throughout development (Maccoby, 2000; Meaney, 2001; Schor, 2003; Sarkadi et al., 2008). In **Chapter 4**, I found that ethologically relevant manipulations of the biparental family structure had a profound impact on the social behavior of adult offspring. Compared to biparentally (BP) reared counterparts, single-mother (SM-) reared prairie vole females showed decreased alloparental care, delayed onset of a stable partner preference, and decreased direct maternal care (in the form of LG rates) toward her own offspring (**Chapter 4**). Likewise, SM-reared males demonstrated a delayed onset of stable partner preference formation and decreased LG toward their own pups. These findings illustrate the important contribution of early family environment to the diversity of adult social behavior in this typically biparental species. These findings may also have broader implications that could be the focus of future studies.

Ethologically, future studies could examine the effect of family environment in wild or large semi-natural populations. Despite the monogamous label, nearly 40% of all wild prairie vole males are wanderers, animals that do not appear to form stable selective social bonds with their mates (Getz and Carter, 1996). Tracking offspring through development, future work could test the hypothesis that more of these males originate in SM nests than in BP- or communal nests. A similar analysis could be conducted in relation to the 30% of wild single-mother family units, as well as female alloparental care. For the former, SM family units can arise due to the death of a male partner, but some SM family units may arise due to diversity in female social behavior. This difference in

female social behavior based on early life family structure may also affect alloparental care. While most alloparental care in communal groups is provided by older, reproductively inactive offspring of the central breeder pair, there are occasions when genetically unrelated females join communal groups (Getz and Carter, 1996) and provide alloparental care. It would be interesting to test the prediction that a greater percentage of these animals originated in BP- or communal nests. In both cases, questions of adaptive significance arise.

MJ Meaney in examining high- and low- LG-ABN rats emphasizes that one is not better than the other; they are simply different (Meaney, 2001). He then proposes that the rate of LG-ABN would like correspond to how stressful an environment is and therefore LG-ABN is "programming" the stress axis of the pups to effectively navigate an environment they have never experienced first-hand. The same type of "programming" may be happening in prairie voles. Social structure in voles, monogamous and nonmonogamous, appears to be, at least to some degree, related to habitat (Getz et al., 1992a; Getz et al., 1993; Getz and Carter, 1996). As discussed by Z Wang and M Novak (1992), promiscuous meadow voles appear r-selected—rapid breeders, low parental investment, and shorter life-spans—and are therefore adapted to the unstable habitats in which they live. Prairie voles, however, appear k-selected—show slower maturation and higher parental investment—which functions effectively in areas of stable, but limited habits. Within prairie voles, differential developmental responses to differences in family structure may allow the prairie vole species to play both ends of the selection spectrum.

Expanding on this perspective, it may be that single-mother nests may arise due instability (e.g., increased predation). In turn, single-mother reared animals (which

receive less total parental care) may then shift their social behaviors (e.g., pair bonding, alloparental care, and parenting) more toward the r-selection end of the spectrum (e.g., wandering and promiscuous). Alternatively, communal groups arise in areas in which territories are already well-established and stable and during the later part of the breeding season (Getz and Carter, 1996). These animals (along with BP-reared animals) receive extensive parental care and grow up to be more likely to form stable bonds and invest in parental activities (e.g., alloparenting or parenting), which are adaptive in a habitat that has k-selection pressures. While there will be extensive individual variation in responses to family structure, over large populations the effect would allow prairie voles to effectively inhabit a range of environmental conditions and switch quickly between the two. For example, in cases where prairie voles are colonizing new territories, single-mother nests may dominate at first due to an initially high predator-to-prairie vole ratio, but as prairie voles continue to breed faster than predation can deplete, prairie voles may settle into more and more communal nesting. This chain of events is speculative, but offers testable hypotheses that relate the laboratory work conducted in this dissertation to field studies.

The findings in **Chapter 4**, however, need not only be important for field work; it may also have implications for studying child development, human psychology and psychopathology. While speculative, it is worth noting that both the social and emotional data presented in **Chapter 4** have some parallels with human borderline personality disorder (BPD). BPD is typically characterized by poor behavioral regulation and impulsivity (one way of interpreting elevated plus maze and open field data), as well as insecure attachment tendencies, which together serve to disrupt and prevent the formation

of stable social bonds throughout life (Zanarini and Frankenburg, 1997; Skodol et al., 2002; Zanarini and Frankenburg, 2007; Livesley, 2008). Moreover, BPD has been tied to disrupted parental relationships, particularly neglectful fathers (Frank and Paris, 1981; Zanarini and Frankenburg, 2007), and is more prevalent in females (Ekselius et al., 1996). It is therefore an intriguing possibility that prairie voles reared under SM-conditions might serve as an informative animal model for certain aspects of this disorder, just as early life manipulations of rats and monkeys have informed our understanding of the etiology of depression and anxiety disorders.

In fact, the focus need not be BPD alone, but instead on how family environments influence the vulnerability to psychopathologies that also have social deficits. Since our prairie vole population is outbred, and therefore genetically diverse, larger studies could examine gene x environment interactions. By identifying which animals with which genotypes get pushed to the extremes of the behavioral spectrum by each type of family environment, future investigations may identify genes of interest that are relevant to both social behavior and mood. Such rodent-to-human translation is already happening. For example, the relationship between the *avpr1a* microsatellite and social behavior in voles (Young and Wang, 2004; Hammock and Young, 2005) has spurred human geneticists to examine the human *AVPR1A* microsatellites in relation to human social behavior (e.g., Walum et al., 2008). Likewise, the environmental influence of maternal LG on the methylation and expression of the rat glucocorticoid receptor gene recently helped identify a similar effect in humans (McGowan et al., 2009). Work examining how prairie vole family environment influences adult outcomes may identify similar sites that are modified by biparental rearing and translate to humans.

Family structure and its influences on neuropeptide systems in adult offspring

Chapter 5 aimed to start addressing the neurobiological reasons that BP- and SM-reared prairie vole offspring exhibit differences in adult social behaviors. Oxytocin receptor (OTR) has been identified as an important contributor to numerous social behaviors, including partner preference formation, alloparental behavior, and maternal behavior (Williams et al., 1992a; Insel and Hulihan, 1995; Young and Wang, 2004; Olazábal and Young, 2006b, 2006a; Ross et al., 2009a). Unlike most neurotransmitter receptors, OTR expression densities in specific brain regions, such as the NAcc, vary widely across individuals (Young and Wang, 2004), in a sense mirroring the behavioral diversity observed in the wild (Getz and Carter, 1996) and laboratory (Young and Wang, 2004). In some cases the diversity in OTR expression has been correlated to diversity in behavior (Olazábal and Young, 2006a; Ross et al., 2009a). Since early life family environment significantly influenced the behavioral diversity of adult offspring, it was plausible that a significant portion of the variation in OTR expression might be the result of differences in early life care, and a similar effect may apply to other socially-relevant and variable neuropeptide systems within the ventral forebrain, such as V1aR, CRF1, and CRF2 (Phelps and Young, 2003; Young and Wang, 2004; Lim et al., 2005; Ophir et al., 2008a). In turn, the dependence of the neuropeptide receptors on early life family structure might help explain the dependence of adult social behavior on early life family structure.

Contrary to our predictions, the neuropeptide receptor expression densities within the ventral forebrain were not significantly influenced by early life family structure.

Examining **Figure 5.3** more closely, it is possible that prairie vole family structure has some modulatory impact on neuropeptide receptor densities in the ventral forebrain, my sample size (N = 6/sex/rearing-condition) to detect an effect in this outbred, genetically diverse species. Whether larger sample sizes would reveal a significant contribution of family structure or not, it is clear that early life family structure was not *the* primary driver of the striking individual variations in neuropeptide receptor densities in the ventral forebrain.

The ventral forebrain, however, is not the only area of interest. Work in other species have identified a host of brain regions that are both sensitive to early life experience and contribute to species typical social interactions, such as mothering (Francis et al., 2000; Francis et al., 2002). Like DD Francis and colleagues in rats, I found differences in OTR densities within the lateral bed nucleus of the stria terminalis (lBNST; Francis et al., 2000; Francis et al., 2002); the direction was even the same: animals that received greater amounts of LG (BP-reared) had higher lBNST OTR than offspring that received less (SM-reared). In rats, this increased lBNST OTR has been linked to maternal behavior and therefore may play a role in the difference in maternal behavior between BP- and SM-reared animals. CRF2 in the dorsal raphe (DR) was also affected by rearing condition. Here the link to differences in adult social behavior is less clear, but could serve as an important area of future research.

CRF2 activation in the DR is known to modulate serotonin release, particularly into the NAcc (Lukkes et al., 2008). Moreover, in rats, CRF2 is sensitive to social context, and alterations of the expression of these receptors in response to isolation has a significant effect on anxiety-related behaviors (Lukkes et al., 2009a). Currently, there is

little understanding of the role of serotonin in the normal expression of prosocial behaviors such as pair bonding and alloparental care. To directly test whether the regulation of serotonin release by differing densities of CRF2 in the DR is important, future studies could use viral vectors (again, carrying either shRNA or extra copies of the gene) to selectively up- or down-regulate CRF2 expression. Use of the partner preference and alloparental care tests could be used to access the impact of these manipulations on social and spontaneous parental behaviors.

In addition to the family-structure dependent changes in the receptors, I also found a striking increase in OT mRNA content within the paraventricular nucleus of the hypothalamus (PVN) in SM-reared animals. As noted in the **Chapter 5** discussion, this was unexpected and appeared paradoxical, based on the general assumption that more OT increases sociality. The increased OT content may simply indicate an overcompensated or dysregulated OT system instead. For example, in human subjects with melancholic depression, which is often accompanied by the negative symptom of social withdrawal (reviewed in Winograd-Gurvich et al., 2006), the number of OT producing cells in the PVN is increased (Purba et al., 1996), not decreased, as is OT mRNA expression (Meynen et al., 2007). Others have even found higher plasma OT in more severe cases of major depression (Scantamburlo et al., 2007). Indeed, in prairie voles, early life manipulations of the OT system by either overstimulating or inactivating OTR both resulted in increased OT cells in the PVN (Yamamoto et al., 2004), particularly in females. Dysregulation of the OT system in either direction thus may result in a modified expression of social behavior. For BP- and SM-reared prairie voles, it would be interesting to test differences in plasma OT, as well as central OT release using

microdialysis, to identify whether OT release has also been altered. Another experimental approach would be to generate transgenic voles (Donaldson et al., 2009) that over-express OT throughout development and then test social behaviors in adulthood. This type of experiment could tease apart the causal relationship between greater OT mRNA content in the PVN of SM-reared animals (particularly females) and differences in social behaviors as adults.

Family structure and its global influence

Taking the last two sections together, it would appear that my findings may have some translational relevance. As noted in the introduction, caution must be taken: individual findings may not generalize. Shortly after I published the work described in **Chapters 4** and **5**, a couple of other groups published findings that mesh with mine and suggest a global influence of family structure and paternal care. For example, Jia et al. (2009) reared monogamous mandarin voles under BP- and SM-conditions and found significantly decreased social interactions in adult SM-reared animals (Jia et al., 2009) that were accompanied by changes to emotional behaviors. Likewise, VS Gromov, who has surveyed a variety of species from a zoological perspective, recently (2009) described decreased maternal and paternal attentiveness in monogamous Mongolian hamsters that were the offspring of multiple generations of single-mothers (Gromov, 2009). In the brain, a group headed by K Braun published two recent papers that demonstrated the importance of biparental rearing in dendritic spine maturation in the orbitofrontal and somatosensory cortices (Helmeke et al., 2009; Pinkernelle et al., 2009). This continued work published by Ovtscharoff and K Braun in 2006 (Ovtscharoff et al., 2006) that was discussed in **Chapter 5**. Coupled with work by Marler's group on AVP and findings by

Bredy et al. (2004) on cognitive development, both of which were discussed in previous chapters, there are now multiple lines of evidence suggesting that studying early life family structure and paternal care in animal models may provide important insights that translate to human research.

CRF2 and the potential link with the OT system

CRF2 is a g-protein coupled receptor that has been implicated in the regulation of emotionality (Reul and Holsboer, 2002; Bale and Vale, 2004). More recently, CRF2 and social behavior (Cooper and Huhman, 2005; Cooper and Huhman, 2007; Lim et al., 2007 and **Chapter 2**), and is sensitive to change in response to early life social context (Plotsky et al., 2005; Lukkes et al., 2009b; **Chapter 5**). In light of the importance of OT for regulating social behavior (Ross and Young, 2009), CRF2 and OT may share an important anatomical and functional relationship.

While CRF2 expression has been described in broad anatomical terms, such as regions that express CRF2 mRNA (Chalmers et al., 1995; Van Pett et al., 2000) or CRF2-like receptor binding (Primus et al., 1997; Lim et al., 2005), the anatomical localization of CRF2 to specific cell populations and cellular compartments has been more difficult. With prairie voles demonstrating a role for CRF2 in modulating responses to social situations (Lim et al., 2007; **Chapter 2**) and a now clearer understanding of the OT fiber network (Ross et al., 2009b; Ross and Young, 2009), I aimed to extend work in our laboratory that initially characterized the vole CRF2 distribution in the brain (Lim et al., 2005). Lim et al. (2005) had found high densities of CRF2 in the NAccSh of monogamous, but not nonmonogamous, voles—the same region in which low doses of

CRF can induce partner preference formation in prairie voles (Lim et al., 2007). While no CRF2 mRNA map has described CRF2 mRNA expression in the NAcc of more traditional animal models, such as rats and mice, there was one receptor autoradiography study that mentioned CRF2 binding in the NAcc (Primus et al., 1997). I therefore started my analysis of CRF2 distribution by making a direct comparison across monogamous prairie voles, nonmonogamous meadow voles, mice, and rats using autoradiographic receptor binding. Consistent with Lim et al. (2005), prairie voles were the only species with CRF2-like receptor binding in the NAccSh. As noted in the **Chapter 6** discussion, this binding could have been due to differences in gene expression, receptor transport, or radioligand selectivity. A follow up mRNA analysis confirmed that the difference was most likely due to differences in gene expression. I then attempted to identify the cell populations that would have a distinct expression pattern in prairie voles using a commercial CRF2 antibody. What I found was unexpected. Instead of distinct cell populations in the NAccSh, LS, DR, and elsewhere, I found a massive network of CRF2-like-labeled large-caliber fibers extending throughout the brain, as well as magnocellular neurons in the PVN and SON. In addition, I found that all of these processes and hypothalamic neurons also express OT. These results, which parallel findings in rats (J Dubrowski and D Rainnie) and show a direct anatomical connection between CRF2 and OT, are extraordinarily exciting, but they must also be interpreted with caution. The many controls used to test the specificity of the CRF2 antibody were unclear at best, contradictory at worst.

Despite near perfect overlap in immunofluorescent signals between CRF2-like and OT labeling, my pre-absorption tests clearly demonstrated that cross-reactivity

between the antibodies was not the cause. My use of CRF2-/- mice as genetic, negative controls seemed ideal in theory, especially with the lack of CRF2 mRNA in the Coste et al. (2000) line, but in practice proved complicated (see **Figure 6.6**). Likewise, the western blot analysis revealed several bands that are difficult to explain. With these data in hand, there are several important steps to take in the future.

In the next month, I aim to immunoprecipitate whatever protein(s) the Abcam CRF2 antibody is detecting. After immunoprecipitation and purification, I intend to use the highly sensitive technique of liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) provided by the Emory Proteomics Service Center to identify the enriched proteins. Due to the near perfect overlap with OT expression, whatever LC/MS/MS identities as the unknown protein, it could prove the basis of future research. If the protein is full-length CRF2, then microdialysis and electrophysiology can be used to investigate how activation of CRF2 affects OT release. Already, there is some indication that CRF2 activation can result in the peripheral release of OT (Bruhn et al., 1986); a similar effect may occur centrally through the selective activation of OT fibers in the NAcc. Alternatively, if some other protein is identified, it may be useful as a target for manipulating OT release or OT cell and fiber function. Both findings are likely to prove productive.

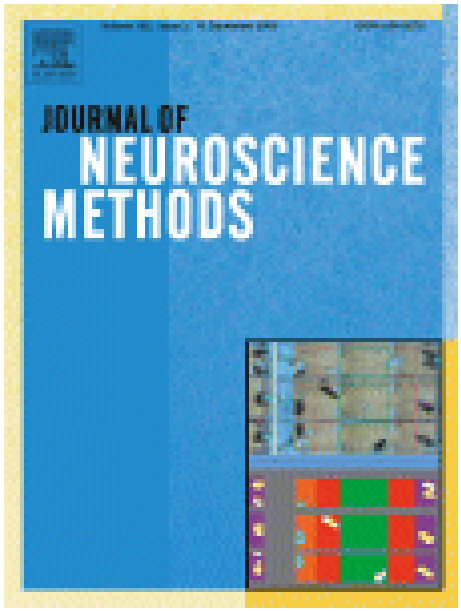
Final conclusions and comments

One of the strengths of this dissertation has been the use of a broad perspective. Anthropology, family and child psychology, pediatric medicine, behavioral ecology, and neuroscience have all had an interest in the phenomenon of family life. Each field has

focused on particular aspects of that phenomenon and made great advances in our understanding. But there have also been limitations. For good reasons, researchers in some fields have avoided using the term "family" and instead focused on mating strategy; in others there has has been a resistance to defining nonhuman social and reproductive networks as families; and in still others, there has been the entrenched belief that, even if rats and mice are not the best models, they are the most developed and therefore the most informative. I am sympathetic to all of these points, but I also propose a slight shift in perspective. Human families (however they are defined) have a profound impact on the behavioral, emotional, and psychological diversity and well-being of individual members. In some cases, human families can even exacerbate or protect against vulnerabilities to maladptive behaviors and psychopathologies that are debilitating to individuals and society (Maccoby, 2000). Given the complexity of human families, I see animal models as excellent source of complementary insight. The goal is not to deny the uniqueness of the human experience or to anthropomorphize monogamous rodents and nonhuman primates. I aim only to take an evolutionary perspective on mammalian brain development and suggest that the information can run both ways: monogamous and biparental species may inform human behavior and neurobiology, while at the same time human and family psychology can inform how we use these animal models. This perspective allows the separate fields to bleed into one another so that each research field gains life from each of the others. My work using prairie voles to study the effects of differences and shifts in family dynamics lends credence to this perspective.

APPENDIX 1

Development of an automated system for the analysis of prairie vole partner preference



Adapted from:

Ahern TH, Modi MM, Burkett JP, Young LJ (2009) Evaluation of two automated metrics for analyzing partner preference tests. *J Neurosci Methods* 182(2):180-188

ABSTRACT

The partner preference test (PPT) is commonly used to examine sexual and social preferences in rodents. The test offers experimental subjects a choice between two stimulus animals, and time spent with each is used to calculate a preference score. In monogamous prairie voles (*Microtus ochrogaster*), the PPT has been paramount to the study of pair bonding. Although powerful, use of the PPT in voles has depended primarily on human manual scoring. Manual scoring is time-consuming and is susceptible to bias and fatigue, limiting the use of the PPT in high-throughput studies. Here we compared manual scoring (real-time and 16x) and two automated scoring metrics: "social proximity" and "immobile social contact." We hypothesized that "immobile social contact" would provide data most comparable to manually scored "huddling", and thus be the most sensitive measure of partner preference in prairie voles. Each automated metric produced data that highly correlated with manual scoring ($R > 0.90$); however, "immobile social contact" more closely reflected manually scored huddling ($R = 0.99$; $P < 0.001$). "Social proximity" and "immobile social contact" were then used to detect group partner preferences in four data sets that varied by cohabitation length and sex. "Immobile social contact" revealed a significant partner preference in each data set; "social proximity" detected partner preferences in only three of the four. Our results demonstrate the utility of automated systems in high-throughput PPTs, and further confirm that automated systems capable of scoring "immobile social contact" yield results indistinguishable from manual scoring.

INTRODUCTION

The partner preference test (PPT) is a commonly used behavioral assay for the evaluation of social and sexual behavior. In the PPT, test subjects are offered a choice of two stimulus animals of differing social or sexual valence. Subjects are then observed and the number of approaches toward and time spent in close proximity to, or in contact with, each stimulus animal is used to calculate a preference score (Slob et al., 1981; Slob et al., 1987; Baum et al., 1990; Williams et al., 1992a; Crawley, 2004; Nadler et al., 2004). Use of the PPT has varied widely and has helped identify an array of biological factors regulating sexual preference (Johnson and Tiefer, 1972; Slob et al., 1987; Baum et al., 1990), social approach and investigation (Crawley, 2004; Nadler et al., 2004), and social attachment (Williams et al., 1992a; Williams et al., 1992b; Winslow et al., 1993; Carter et al., 1995; Insel and Hulihan, 1995; Nadler et al., 2004; Young and Wang, 2004). Each laboratory has modified the PPT to suit its own needs, but one commonality has been a dependence on human scoring for one or more of the behavioral metrics. Unfortunately, manual scoring is extraordinarily time and labor intensive and has prevented, in some cases, the use of the PPT in high-throughput studies. Manual scoring also suffers from questions of bias, fatigue, and inter-rater reliability (Noldus et al., 2001).

One superlative example illustrating the issue of time investment is the use of the PPT to study pair bonding in monogamous prairie voles (*Microtus ochrogaster*). Prairie voles are socially monogamous rodents that form selective, enduring attachments between mates after a period of cohabitation and/or mating. This emerging model organism has proven useful for investigating the neurobiological and genetic mechanisms underlying a variety of social phenomena, including bond formation (Carter et al., 1995;

Young and Wang, 2004), bond maintenance (Aragona et al., 2006), and the consequences of social loss and isolation (Grippo et al., 2007a). Central to nearly all of these investigations is the ability to measure a selective social bond using the PPT. Adoption of the PPT to study voles, however, has required several modifications. Before testing, prairie voles undergo a period of cohabitation with a conspecific. The "partner" is then loosely tethered in one stimulus chamber while a novel "stranger" is loosely tethered in another. The test animal is placed in the central, socially neutral chamber and allowed to roam for 3 hours. The amount of time spent "huddling" (which is variably denoted as contact, side-by-side contact, or affiliative contact) with each stimulus animal is then scored, since it is the most sensitive indicator of a selective partner preference in this species and its congeners (Williams et al., 1992a; Williams et al., 1992b; Winslow et al., 1993; Carter et al., 1995; Insel and Hulihan, 1995; Young and Wang, 2004). While the PPT has been highly effective in identifying socio-active manipulations in both male and female prairie voles, the near exclusive reliance on manual scoring has stalled the deployment of this animal model in large-scale drug discovery studies. This has remained true even with the use of time-lapse recording techniques.

In an attempt to increase the efficiency, scalability, and reliability of the PPT, we objectively compared two manual scoring methods (real-time and time-lapse) and two different automated metrics: "social proximity" and "immobile social contact." Early automated systems use center of gravity to track animal movement and can readily be adapted to quantify "social proximity." To process "social proximity," we used an early release of Noldus Ethovision, version 3.0 (release date November, 2002), which we already owned and had been using for elevated plus maze and open-field testing (see

Chapter 4). It is important to note that this older version of Ethovision 3.0 is just one example of automated systems that can easily and automatically process the location of a single animal over time within defined zones. A number of other commercial and custom-made video-tracking and beam-break systems have similar capabilities and can likely score ”social proximity.”

To process “immobile social contact,” we used Clever Sys Inc.’s newest version of SocialScan, 2.0 (release date January, 2007), with minor custom-modifications. We used this software because it is an example of software that can process whole-animal behaviors, distinguish three nearly identical-looking animals in a single arena, and could be adapted to provide a measure of “immobile social contact.” In our experience, SocialScan 2.0 was the first system on the market that could meet all of these specific needs simultaneously; however other software packages, such as newer versions of Noldus Ethovision (e.g., XT 6.0), report similar capabilities. It is likely that Ethovision XT 6.0 and other developing systems can or will be able to provide an “immobile social contact” metric under our testing conditions, but this was not tested here.

Ultimately, the goal of this study was to test the hypothesis that the automated metric of “immobile social contact” would better reflect “huddling” time as determined by a human rater than the metric of “social proximity,” where “huddling” is predominately immobile, affiliative social contact. After comparing the accuracy of each method in relation to manual scoring, we then tested the ability of the “social proximity” and “immobile social contact” metrics to detect significant group partner preferences in four data sets from subjects that differed by sex and cohabitation time. We again

hypothesized that the ability to detect “immobile social contact” would produce data more consistent with manual scoring methods than the “social proximity” metric.

Our results demonstrate that an automated approach to scoring the PPT yields data that are highly correlated with manual scoring methods. Systems that provide a “social proximity” metric provide good approximations in most situations and may be a cost-effective automated method, if used cautiously. Alternatively, software capable of detecting “immobile social contact,” appears to be a more viable option for high-throughput studies of social bond formation, since it more accurately approximates manual scoring and is able to detect partner preferences across several key variables.

Differences in scoring between the two automated methods were apparent, but not easily explained. The most obvious differences occurred when scoring male prairie voles that had cohabitated for a week. Males exhibit increasing levels of selective aggression toward strangers after extended periods of cohabitation with a partner (Aragona et al., 2006). Aggression therefore offered one plausible explanation for the discrepancy between the two metrics: aggression would likely increase stranger “social proximity” time without increasing stranger “immobile social contact” time. Aggression and other qualitative explanations are discussed.

Most importantly, both metrics reached a respectable level of accuracy and substantially reduced time and labor investments. Automation thus appears to be a highly efficient and generally accurate method of obtaining data from the PPT.

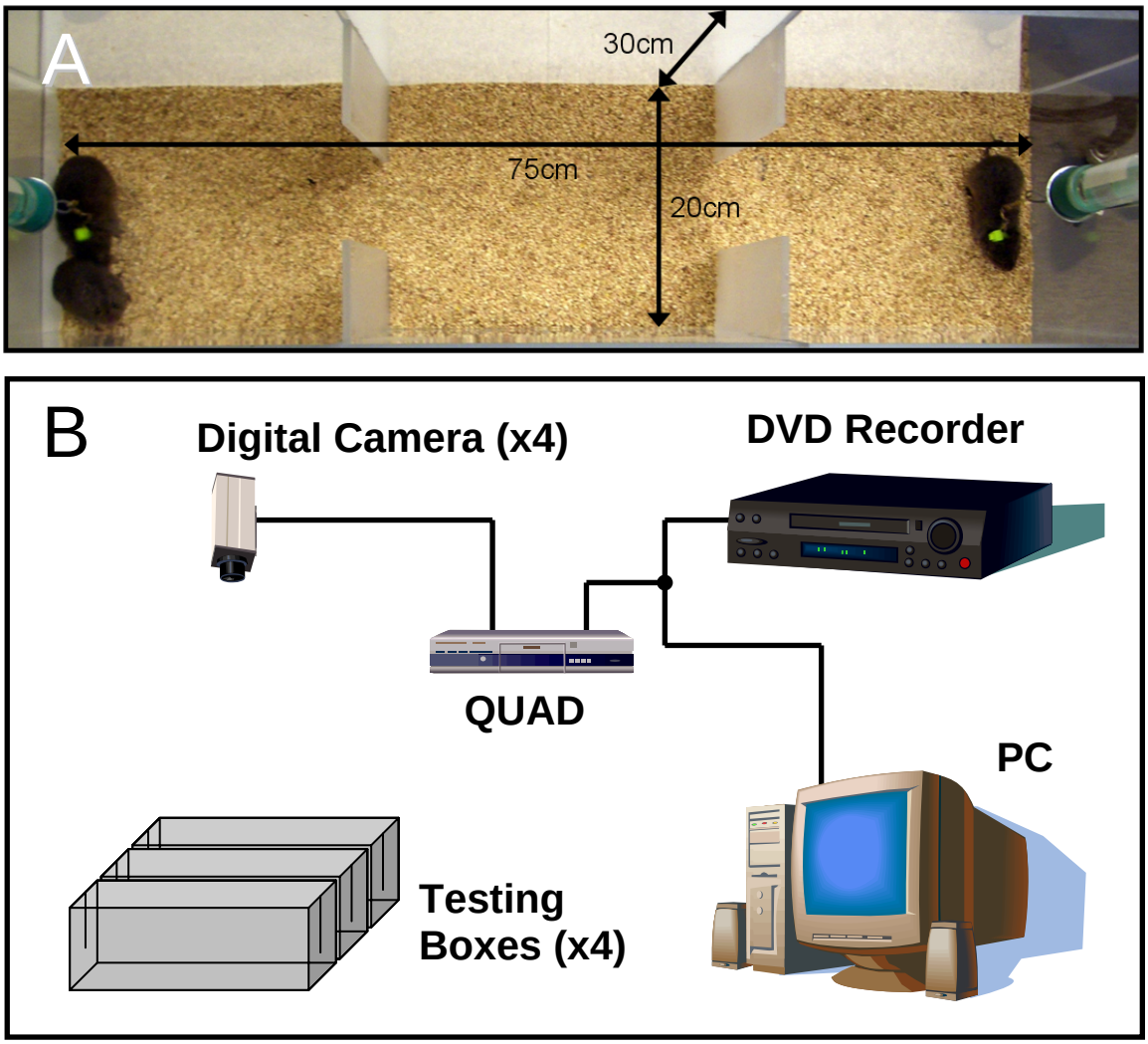
MATERIALS AND METHODS

The partner preference testing setup

Each PPT apparatus (**Figure 1.1a**) was constructed in-house out of 0.6 cm thick acrylic sheets and heat-resistant cement (Weld-On #16 Acrylic Solvent Cement, IPS Corporation, Gardena, CA). The apparatus consists of one long box 75 cm x 20 cm x 30 cm (length x width x height), divided into three chambers by placing two opaque slats, measuring 25 cm tall x 6 cm wide x 0.6 cm thick, opposite each other at 25 cm and 50 cm along the length of the walls. At each end of the box, a 20 cm deep x 1 cm wide slot was carved out to allow tethering anchors to slide in and out.

The anchoring apparatus was also made in-house. A small loop was made out of metal wire ($1/16^{th}$ inch thick) and then cinched (3/32 in. Aluminum Ferrule & Stop Set, The Lehigh Group, Macungie, PA). The free end was passed through three 1/4 in. nuts and two 3/8 by 1(1/2) in. zinc plated washers in alternation. The wire was then cinched again. To this loop-nut-washer combination, three interlinked Brass Snap Swivel fishing leaders (Size 10; Eagle Claw, Wright & McGill Co., Denver, CO) were attached. Through the most distal leader, an 8 in. cable tie (Commercial Electric, 826 843, USA) was looped to make a neck leash. The tethering anchor allows the researcher to tether the animal outside the apparatus then slide the anchor into the 20 cm deep slot, securing the tethered animal in the appropriate area.

Before each test day, the cages were steam washed and new bedding (Bed-O-Cob, Maumee, OH) was added to cover the floor. 50 mL conical tubes with water and sipper tubes were attached to aluminum frames and hung at each end over the anchoring slots; there was no water-bottle in the center chamber.



Appendix Figure 1.1 - Partner preference test apparatus and video recording setup
Partner preference tests (PPTs) were conducted in a three-chambered arena **(A)**. Stimulus animals (one partner and one stranger) were loosely tethered at each end; the test subject roamed freely for 3 hours. 12 PPT boxes were monitored by four cameras **(B)**. The four video streams are compressed to a single output by a QUAD box and sent to both a DVD recorder and PC.

Recording equipment

Our setup includes 12 PPT boxes. Video recording was performed with four digital video cameras (WV-CP284, Panasonic), each monitoring three adjacent testing boxes (**Figure 1.1b and 1.2a, c**). Cameras were situated vertically 91 cm from the floor

of the testing chambers, and all four cameras fed into a QUAD video box (4CQ, EverPlex); the QUAD collapsed the video streams into a single output. Exiting the QUAD, the video was simultaneously sent to a digital video disk (DVD) recorder (LQ-DRM200, Panasonic) and to a WinTV-PVR-350 video-card (model 990) installed in a PC (Dell Precision T3400) running Windows XP (**Figure 1.1b**). The DVD recorder outputs to a television for real-time viewing of the cameras' perspectives (not shown). By collapsing all four video streams into one output, there is some loss of resolution. The use of a vertical viewing perspective and our move to real-time rather than compressed video-recording, however, easily compensated for any resolution loss, while simultaneously allowing high-throughput testing.

Experimental animals

All subjects were either female or male prairie voles from our colony at Emory University (Atlanta, GA), which were weaned at 21 days post-birth, housed in same sex pairs or trios, and maintained on a 14:10 light:dark schedule (lights ON at 06:00 h) with *ad libitum* access to food and water. Animals were tested as adults (70-100 days old). All procedures were approved by Emory University's Institutional Animal Care and Use Committee and were in accordance with national guidelines.

Partner preference testing

Twelve sexually naïve, gonadally intact experimental females and twelve sexually naïve, gonadally intact experimental males were paired at 07:00 h with an equal number of sexually-naïve, gonadally-intact stimulus animals of the opposite sex and allowed to

cohabitate for 24 hours. At 07:00 h the following morning, all pairs were moved to a behavioral testing room and allowed to acclimate for >30 min. Each cohabitation "partner" was then tethered and anchored to one end of the PPT box, while a "stranger" was tethered and anchored at the other end. Water-bottles were placed at each end to cover the anchor slots. Finally, the experimental test animal was placed in the center, socially neutral chamber and allowed to roam freely for 3 hours.

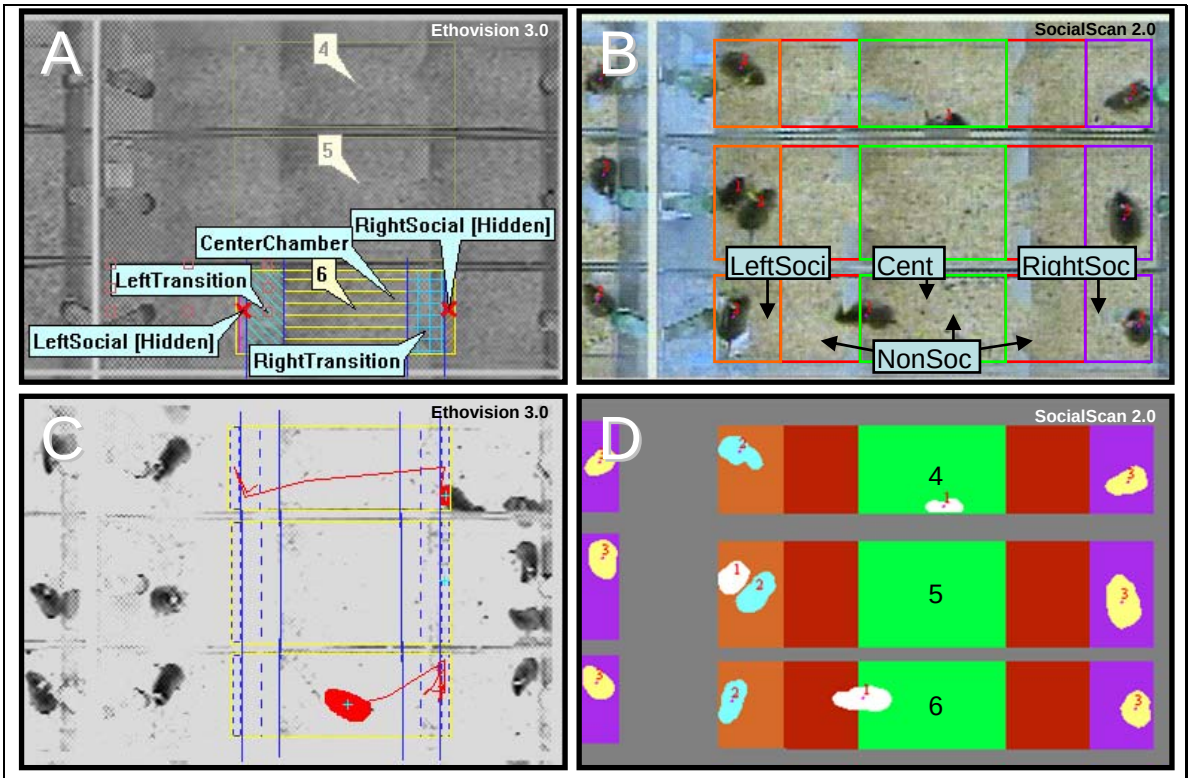
On each day of testing, a total of 12 experimental test animals were tested in the morning and 12 more were tested in the afternoon. The partners from the morning session served as strangers in the afternoon and vice versa. This procedure has been used previously and there are no measureable test order effects, which would confound the results (Lim et al., 2007). It also substantially reduces animal use. Our 24 experimental animals (12 males + 12 females) underwent PPT after 24 hours of cohabitation and again after 6 more days (a total 1 week of cohabitation). All 48 PPTs were video-recorded to DVD and compressed to MPEG files using the WinTV-PVR-350 video-card. All behavioral scoring occurred *post hoc*.

Automated video-tracking of "social proximity"

DVD recorded test sessions were analyzed for "social proximity" *post hoc* using an early version of Noldus Ethovision, version 3.0 (Noldus, The Netherlands). A DVD player (D-R410, Toshiba) fed video directly into a PC (Dell Dimension 8200), which contained a Picolo Frame grabber and video receiver. Ethovision processed all fourty-eight 3-h PPTs as they passed through the Picolo system.

For each DVD, at least 10 seconds of video containing clean PPT boxes with water-bottles had been recorded, providing a background image. This image served as a template for the virtual arenas (**Figure 1.2a**). A virtual arena defines where the program can track an animal; thus each PPT apparatus was defined by a separate arena. In each case, the virtual arena outlined the floor of the test box, but ended at a position in the stimulus chambers beyond which the tethered animals could not reach (**Figure 1.2a**). This prevented the presence of two animals in the arena simultaneously, which causes errors in the program.

Virtual zones define regions of interest within the virtual arena. Zone lines were therefore drawn across the base of each pair of slats and again a few centimeters from the ends of the arena definition. Six zones were defined: (1) LeftSocial, (2) LeftTransition, (3) CenterChamber, (4) RightTransition, (5) RightSocial, and (6) NonSocial (using the cumulative zone feature) (**Figure 1.2a**). LeftSocial and RightSocial were further defined as hidden zones. Hidden zones are typically used in assays where an animal can escape into an opaque house and thus out of sight of the camera. If an animal crosses the hidden zone border and disappears, Ethovision 3.0 allocates the interim time to the hidden zone. We modified the use of this feature by simply truncating the virtual arena. When the test animal crosses the marker for the left or right social zone and disappears out of the virtual



Appendix Figure 1.2 - Visual representations of two different approaches to automated video-tracking

An early version of Ethovision (version 3.0) and the newest version of SocialScan (version 2.0) were used to automatically process vole behavior in the partner preference test (PPT). Virtual arenas (area in which voles can be tracked) and zones (regions of interest) were outlined for Ethovision **(A)** and SocialScan **(B)**. For Ethovision 3.0, arenas were truncated at a point beyond which the tethered animals could not reach; zones included LeftSocial, RightSocial, two transitions zones, CenterChamber, and NonSocial (an accumulation of all non-hidden zones); the social zones were designated as hidden, allowing the program to track the amount of time the test subject spent in "social proximity" to the left and right tethered animals. For SocialScan 2.0, arenas included the entire test box floor; zones included LeftSocial, RightSocial, Center, and NonSocial; the NonSocial zone served to correct occasional ID swaps. During analysis, both Ethovision 3.0 and SocialScan 2.0 tracked the animals' location according to center-of-gravity. Ethovision 3.0 logged time in "social proximity" (**C**, see middle test box); SocialScan 2.0 logged time in "immobile social contact" (**D**, see middle test box).

arena, the program allocates time to the appropriate hidden social zone. Time in the hidden social zones thus served as a measure of time in "social proximity." With this software, "social proximity" is the best proxy of "huddling" without dyeing or marking the animals in some way—a potential confound for studies of social behavior. Regardless of zone, the test animal's location was measured by its center-of-gravity (**Figure 1.2c**).

After defining the arena, zones, and distance calibration, several other features were altered to optimize detection of the free-roaming, experimental animal. Trial protocol was set to 3:00 hours, with a sample rate of 15 samples/s. Hidden zones were set to 40 mm for entering and 0 mm for exiting. Detection was accomplished by background subtraction, but only when the object was darker than the background and larger than 40 pixels. To eliminate background noise, contrast and brightness were adjusted to +236 and -14, respectively (see **Figure 1.2c**). Each detectable object underwent a two-pixel erosion and then a three-pixel dilation. When the test animal was not found, the program used the last measured position. Detection thresholds were set to -255 to -25 and the reference image was updated before proceeding to the introduction of test animals.

With our setup, Ethovision 3.0 is able to process 12 PPT boxes simultaneously at a processor load of between 15 and 45, well below a load of 100, which can yield slow and inaccurate image processing. Ethovision processed the entire 3-hour test for each animal. Test animals were assessed for "social proximity" time in relation to the partner and stranger, entries into the center zone, and time spent in the center zone.

Automated video-tracking of "immobile social contact"

To process "immobile social contact," we used SocialScan 2.0, which is a relatively new video-tracking system that aims to process whole-animal behaviors of multiple, identical-looking animals in the same arena, without the need to uniquely dye or mark animals.

For our study, all fourty-eight 3-h PPTs were compressed to MPEG files and then batch processed *post hoc*. Before processing, arenas and zones were defined in a manner similar to Ethovision, except that the arena encompassed the entire floor of each PPT box and there were only four zones: (1) LeftSocial, (2) Center, (3) RightSocial, and (4) NonSocial (**Figure 1.2b**). The free- moving animal is assigned subject ID #1, the tethered animal in the "LeftSocial" zone is ID #2, and the "RightSocial" zone contains subject ID #3. The RightSocial and LeftSocial zones serve as markers for social proximity and allow for identification reassignment. Adjacent animals will occasionally switch IDs; to correct for this, Clever Sys Inc. has adapted the program such that only animal ID #1 (the test animal) can enter the NonSocial zone (**Figure 1.2b, red**). If IDs become swapped in one of the social zones, they immediately swap back once the test animal enters the NonSocial area. This process ensures that only one pair of IDs can be in social contact in the LeftSocial (#1 and #2) and RightSocial (#1 and #3) zones. Any social contact in either LeftSocial or RightSocial zones will therefore be logged as occurring between the test animal and the appropriate tethered animal (**Figure 1.2d**).

Animal detection was accomplished using the software's default parameters, and there was no need to change contrast or brightness.

SocialScan automatically assessed time in "immobile social contact" with each tethered animal, entries into the center zone, and time in the center. Since we are the first group to use this program to analyze partner preference, the ability of the program to simultaneously detect and log "immobile social contact" was added as a custom feature and we varied the "immobility value" from 0.01 - 1.00. The immobility value represents a percent movement criterion beyond which adjacent animals would be considered mobile and therefore not "huddling." For example, when animals are fighting one another, they are in contact, but not huddling or immobile, and consequently this time should not be counted as prosocial contact. Lower immobility values result in a more stringent definition of "immobile social contact;" higher values, a more liberal definition. As with Ethovision, SocialScan processed the entire 3-hour test for each animal. Data were exported and analyzed.

Verification of automated-scoring by hand-scoring

Fully scoring 48 PPTs in real-time (144 h) was prohibitive. Therefore, we used a series of 15-minute-long video segments to validate the accuracy of two manual scoring methods and two automated video-tracking systems. Segments were taken at 1 h and 2.5 h into the PPT. These time points were chosen based on the *a priori* experimental objective to capture data from test animals during periods with high levels of movement within the social zones (1 h) and huddling within the social zones (2.5 h). Tests of six males and six females were scored at each time period.

Each of the 15-minute video segments was scored under the following conditions: manually in real-time by two experimenters, manually at 16x fast-forward (time-lapse)

speed by the same two experimenters, once by Ethovision in real-time, and once by SocialScan in real-time.

Manual scoring was performed using PowerDVD 5 on a PC (Dell Dimension 4600). PowerDVD 5 allows a single jump from real-time (1x) speed to 16x speed at a bookmarked time point. With this setup, each 15-minute segment consistently played in 56.25 sec, exactly $1/16^{th}$ of 15 minutes. (We note, however, that several other setups resulted in highly variable 16x playback lengths, ranging from 50 to 60 sec. Because 16x behavioral measures must be converted back to real-time for analysis, having a consistent multiplier was paramount to obtaining reliable data.)

Manual raters scored: (1) left "huddling" time, (2) center chamber time, (3) center chamber entries, and (4) right "huddling" time, using Stopwatch+ (Center for Behavioral Neuroscience: http://www.cbn-atl.org/research/stopwatch.shtml). "Huddling" was characterized by close, physical, predominantly immobile or affiliative (e.g., grooming) contact. Center time and center chamber entries were characterized by the animal completely entering the center, socially neutral chamber (**Figure 1.1a**).

The old version of Ethovision and the new version of SocialScan each scored the same factors, but "huddling" was replaced by either "social proximity" or "immobile social contact," respectively. Each program had already analyzed the full 3-hour test for each animal, so a time-window function within each program was used to allow direct comparisons of the 15-minute segments with the two manual scoring methods.

Correlational analysis between automated-scoring and hand-scoring

The first set of analyses examined concordances between four different scoring methods: manual real-time, manual 16x speed, ”social proximity,” and ”immobile social contact.” By using time “huddling” (or the best proxy thereof), simple correlations were calculated to produce Pearson R-values. Because all the raters were blind to treatment, “huddling” time from both the left and right social zones were included (thus indiscriminately including both partners and strangers). Correlations were calculated using SPSS 15.0 (SPSS Inc., Chicago, IL). All dot plots represent individual data points and a linear regression line.

Data from the 15-minute video segments were compared across: (1) multiple raters in real-time, (2) multiple raters at 16x, (3) real-time scoring versus 16x scoring, within and across individual raters, (4) “social proximity” versus manual real-time and 16x “huddling,” and (5) “immobile social contact” versus manual real-time and 16x “huddling.”

Partner preference analysis

To examine whether differences in scoring between methods can positively or negatively affect our ability to detect a significant partner preference, a complete analysis of 48 PPTs were conducted using a 2 (sex: male vs female) x 2 (stimulus: partner vs stranger) x 2 (metric: “social proximity” vs “immobile social contact”) x 2 (cohabitation: 24 h vs 1 week) ANOVA, with cohabitation and metric as repeated measures and social time as the dependent measure. All statistical tests were performed in SPSS. All bar-graphs represent mean + SEM. A $P < 0.05$ was considered significant.

Analysis of aggression

Aggression is one important social behavior that could ostensibly increase "social proximity" time without also increasing "immobile social contact" time, particularly when males are the test subjects. Twenty-four different 15-minute video segments containing only male test animals were scored by two trained observers. "Aggression" was characterized by contact that resulted in a rapid withdrawal or sustained paw slapping, nipping, or flailing. Aggression included aggressive bouts initiated by either the test male or the tethered female. Aggression was only scored manually in attempt to explain the discrepancy between "social proximity" and "immobile social contact" PPT data in males after one week of cohabitation. All bar-graphs represent mean + SEM. A $P < 0.05$ was considered significant.

RESULTS

Comparison and verification of scoring methods

In an attempt to verify the accuracy of various manual and automated scoring methods, 15-minute video segments from 24 PPTs were scored six times for (1) left social time, (2) center chamber time, (3) center chamber entries, and (4) right social time: twice in real-time by two separate observers, twice at 16x speed by the same two observers, once by Noldus Ethovision 3.0, and once by SocialScan 2.0. Human observers measured time "huddling," Ethovision 3.0 scored time in "social proximity," and SocialScan 2.0 logged time in "immobile social contact."

A summary of the results can be found in **Table 1.1**. Overall, correlations were high between the different methods of scoring social time, center time, and center entries. Most importantly, there were high inter-rater correlations for "huddling" in real-time (R = 0.998; **Figure 1.3a**) and at 16x speed (R = 0.982), high intra-rater reliability for "huddling" between observation speeds (1x vs 16x for two different raters: R = 0.980-0.998;

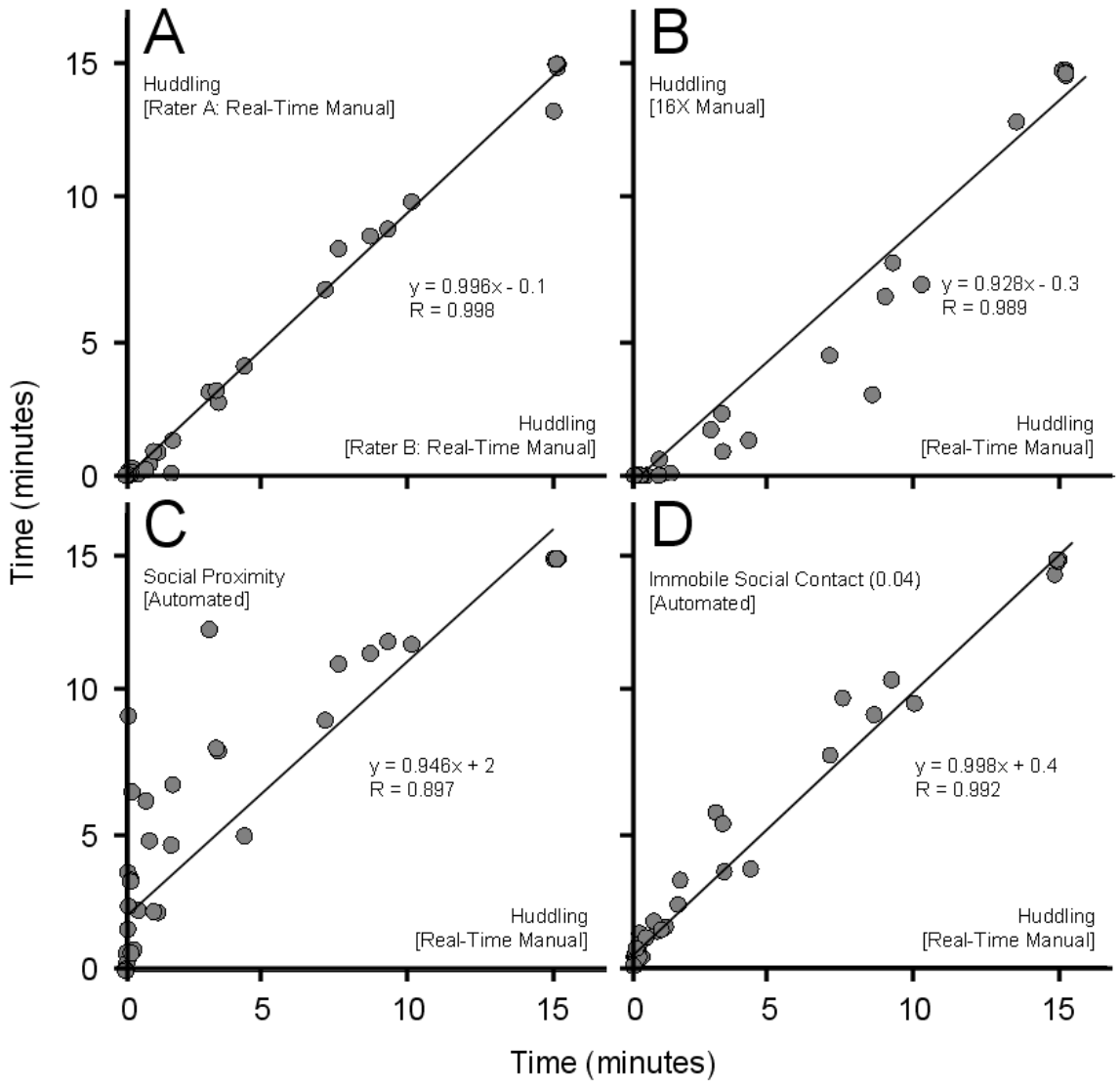
TABLE 1.1

Social time			**Pearson's R**
Real-time manual huddling	vs	16X manual huddling	**0.989**
		Social proximity (Ethovision 3.0)	0.897
		Social proximity (SocialScan 2.0)	0.904
		Social contact	**0.972**
		Immobile (0.05) social contact	**0.987**
		Immobile (0.04) social contact	**0.992**
		Immobile (0.03) social contact	**0.991**
		Immobile (0.02) social contact	**0.960**
16x manual huddling	vs	Social proximity	0.879
		Immobile (0.04) social contact	**0.980**

Center zone time			**Pearson's R**
Real-time manual	vs	16x manual	**0.996**
		Ethovision 3.0	**0.998**
		SocialScan 2.0	**0.999**
16x manual	vs	Ethovision 3.0	**0.993**
		SocialScan 2.0	**0.987**

Center zone entries			**Pearson's R**
Real-time manual	vs	16x manual	0.925
		Ethovision 3.0	0.814
		SocialScan 2.0	**0.992**
16x manual	vs	Ethovision 3.0	0.815
		SocialScan 2.0	0.950

Appendix Table 1.1 - Correlations of manual and automated scoring methods
Manual and automated scoring methods were used to analyze twenty-four 15-minute video segments for social behavior, center zone time, and center zone entries. Each scoring method was correlated to each of the others and a Pearson's R-value was calculated. **Bolded** R-values met an arbitrarily good correlation cutoff of 0.95. All social contact data were produced by SocialScan 2.0. The values in parentheses denote the "immobility" value used by SocialScan 2.0 to distinguish "immobile social contact" from mere "social contact." All correlations produced significant linear models ($P < 0.001$).



Appendix Figure 1.3 - Dot-plots of four key correlational comparisons
Dot-plots and linear correlations were generated for key scoring method comparisons to illustrate variation. Plots demonstrate that there is high inter-rater reliability in real-time **(A)** and intra-rater reliability across scoring speeds **(B)**. The metric of "immobile social contact" **(D)** approximated manually scored "huddling" better than the metric of "social proximity" **(B)**. Equations (y = mx + b) and Pearson's Rs illustrate how closely the linear models approximate a 1:1 ratio with no scale shift and the degree of concordance, respectively.

Figure 1.3b), and high concordance between real-time ratings of "huddling" and both automated scoring metrics: "social proximity" (R = 0.897; **Figure 1.3c**) and "immobile social contact," with the "immobility" criterion set to 0.04 (R = 0.992; **Figure 1.3d**). It is important to note that "immobile social contact" was examined using several different "immobility" values, ranging from 1 (equaling mere "social contact") to 0.01, but the peak concordance occurred around 0.04 and this value was used in all subsequent analyses.

Each bivariate comparison (e.g., real-time "huddling" vs 16x speed "huddling") produced a significant linear model ($P < 0.001$). To examine if they were equivalent, the residuals for each correlation were calculated (|predicted – observed|) and then averaged. An ANOVA and subsequent *post hoc* analyses revealed that "immobile social contact" was significantly more accurate, in terms of having smaller residuals, than "social proximity" (ANOVA, $F_{2,141} = 44.92$, $P < 0.001$: "social proximity"/real-time-"huddling" |residuals| > "immobile social contact"/real-time-"huddling" |residuals|, $P < 0.001$ [Tukey's]). Interestingly, "immobile social contact"/real-time-"huddling" and 16X-"huddling"/real-time-"huddling" correlation residuals did not differ significantly ($P = 0.643$ [Tukey's]).

Automated analysis of partner preference

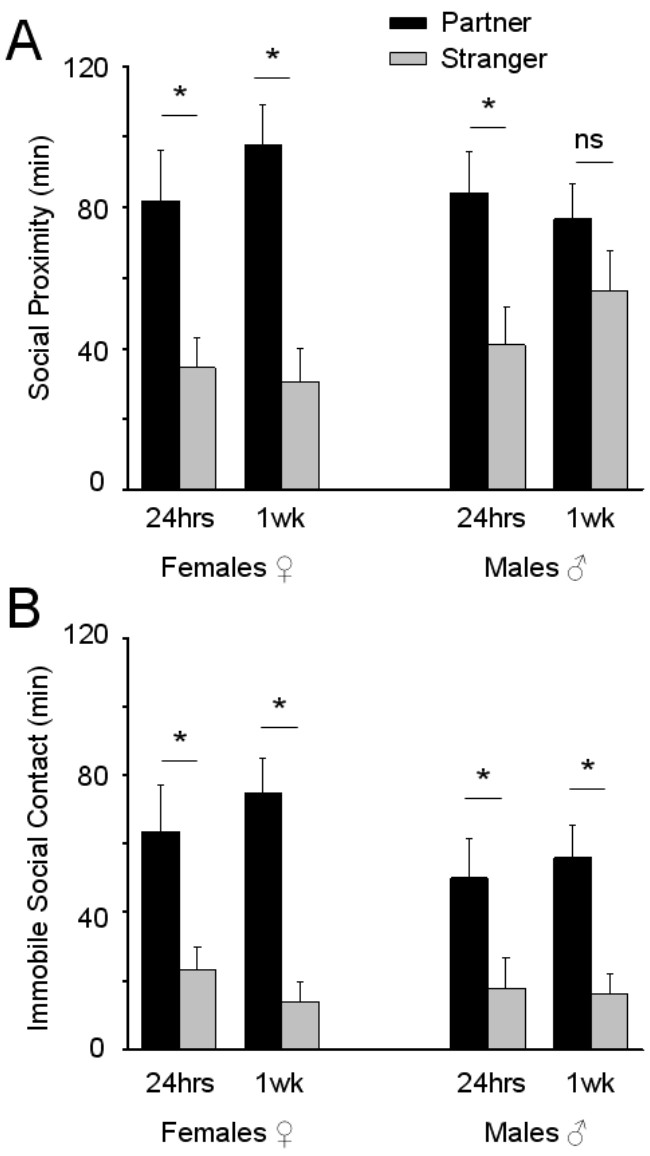
The essential test of an automated behavioral analysis system is whether it can detect a selective partner preference when one occurs. We compared two different automated video-tracking software packages for their ability to detect group partner preferences using four different data sets, varied by the sex of the experimental subject

and the duration of cohabitation. We hypothesized that these variables were capable of altering the types of social interactions that occur during the PPT (for example, time huddling, exploring, mating, and being aggressive) and that these alterations would influence our ability to detect significant partner preferences using "social proximity" or "immobile social contact" as the social metric. PPTs for 12 females and 12 males were conducted after 24 h and 1 week of cohabitation; all 48 recorded tests were scored *post hoc* for "social proximity" and "immobile social contact." Ethovision 3.0 measured "social proximity;" SocialScan 2.0 measured "immobile social contact" (immobility criterion = 0.04).

A repeated measures, 2 (sex: male vs female) x 2 (stimulus: partner vs stranger) x 2 (social measure: "social proximity" vs "immobile social contact") x 2 (cohabitation period [repeated measures]: 24 hrs vs 1 week) ANOVA revealed a between subjects effect of stimulus animal ($F_{1,44} = 28.7$, $P < 0.001$) and of social metric ($F_{1,44} = 97.3$, $P < 0.001$; **Figure 1.4**). There were also significant cohabitation period x social metric x sex ($F_{1,44} = 5.9$, $P = 0.020$) and cohabitation period x social metric x stimulus animal ($F_{1,44} = 5.5$, $P = 0.024$) interactions.

Planned *post hoc* Student's t-tests revealed that, in females, both "social proximity" and "immobile social contact" detected a significant partner preference during both the 24-h cohabitation PPT ("social proximity": $t(17.69) = 2.825$, $P = 0.011$; "immobile social contact": $t(15.69) = 2.672$, $P = 0.017$; **Figure 1.4**) and the 1 week cohabitation PPT ("social proximity": $t(21.15) = 4.575$, $P < 0.001$; "immobile social contact": $t(17.22) = 5.132$, $P < 0.001$).

In males, the data were similar, but ultimately disparate. Planned *post hoc* t-tests revealed that, in males, “immobile social contact” detected a significant partner



Appendix Figure 1.4 - Comparison of partner preference results obtained from two different automated metrics

Males and females underwent two separate 3-hour PPTs, once after 24-hours of cohabitation, and once after a full week of cohabitation. All tests were processed *post hoc* and assessed for "social proximity" time **(A)** and "immobile social contact" time **(B)**. The "immobile social contact" metric detected a significant partner preference in all four data sets; the "social proximity" failed to detect a partner preference in males after 1 week of cohabitation. Bar-graphs represent mean + SEM; (*) represents a statistically significant preference for the partner over the stranger, $P < 0.05$. "ns" = not significant.

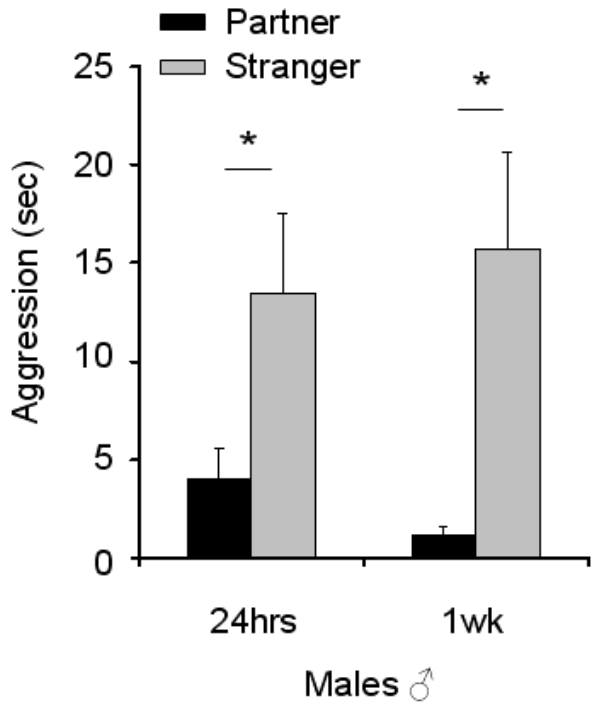
preference during both the 24-h and 1-week PPTs (24-hr: $t(21.20) = 2.207$, $P = 0.038$; 1 week: $t(18.59) = 3.588$, $P = 0.002$), while "social proximity" detected a significant partner preference after 24 h ($t(21.65) = 2.73$, $P = 0.012$), but not after 1 week ($t(21.63) = 1.670$, $P = 0.109$). This discrepancy left us with a question: what were the test males doing at the stranger end during the 1 week PPT, such that "social proximity" was increased, but "immobile social contact" was not?

Analysis of aggression in the partner preference test

We hypothesized that an increase in aggressive behavior may account for the increase in stranger "social proximity" seen in the males at 1 week, which prevented the detection of a significant partner preference (**Figure 1.4**).

Twenty-four 15-minute video segments (12 from the 24-hr PPT; 12 from the 1-week PPT) were scored manually by two observers in real-time for aggression and exploratory behavior within each social zone. A 2 (stimulus: partner vs stranger) x 2 (cohabitation period: 24 h vs 1 week) ANOVA, with cohabitation period as a repeated measure and aggression time as the dependent variable, revealed a between subject effect of stimulus animal ($F_{1,22} = 15.6$, $P = 0.001$; **Figure 1.5**), but no within subjects effects of cohabitation period ($F_{1,22} = 0.66$, $P = 0.426$), nor a stimulus x cohabitation interaction (24 h vs 1 week; $F_{1,22} = 0.04$, $P = 0.840$). Finally, correlations between aggression time and "social proximity" time were examined to determine if the increases in stranger "social proximity" time were accounted for by increases in aggression time. Increases in aggression did not significantly lead to or follow from increases in stranger "social proximity" time (Pearson's $R = 0.132$; $P = 0.540$; data not shown). Interestingly, we did

find that, at least during the 15-minute episodes we scored, males were involved in significantly more aggressive bouts at the stranger end than at the partner end, after both 24 h ($P < 0.05$; **Figure 1.5**) and 1 week of cohabitation ($P < 0.05$).



Appendix Figure 1.5 - Aggression analysis
Males were assessed for aggression in twenty-four 15-minute video segments, half during the post-24 hour cohabitation PPT test and half 1-week PPT. Males spent more time in aggressive scuttles with stranger females than with their female partners. Bar-graphs represent mean + SEM. (*) $P < 0.05$.

DISCUSSION

The partner preference test (PPT) has been and continues to be a powerful laboratory test for the study of social and sexual behavior in a wide range of species. Already shown to be sensitive to an array of treatments, the PPT promises to be a key assay in the identification of socially and sexually active manipulations in the future. Wide, extensive adoption of the PPT, however, will require a move away from the time

and labor intensive investment of human scoring toward more automated methods. Already, some groups have started using automated systems to assess social behavior (Crawley, 2004; Nadler et al., 2004; Moy et al., 2008; Scearce-Levie et al., 2008). These methods, however, are limited in scope and do not account for all the complexities of the PPT adapted for monogamous prairie voles.

We attempted to broaden the use of automation by analyzing two different approaches to automated behavioral PPT scoring in voles. One approach was to use a commercially available, center-of-gravity based software package that calculates a single animal's location and its distance moved. We used this type of system to automatically when the test animal was in "social proximity" to the tethered animals. Our second approach was to use a more sophisticated software package that calculates whole-animal behaviors and thus can track multiple, nearly identical-looking animals in a single arena. We used this software to automatically score when the test animal was in "immobile social contact" with the tethered animals. We then tested the hypothesis that "immobile social contact" would provide a more accurate approximation of manually scored "huddling" than a measure of "social proximity."

To verify that these automated metrics could provide behavioral scoring similar to human raters, we compared the results of twenty-four 15-minute video segments across four different rating methods. We confirmed that human raters generally have high inter- and intra-rater reliability and that the automated methods of scoring "social proximity" and "immobile social contact" correlate highly with manual methods of scoring "huddling" (see **Figure 1.4**; **Table 1.1**). Software capable of scoring "immobile social contact," however, approximated manual, real-time scoring of "huddling" to an accuracy

of 99%, which was significantly better than software providing "social proximity" data (only 90% accurate). With investigators attempting to achieve at least 95% inter-rater reliability (e.g., Cushing et al., 2001), the metric of "immobile social contact" appears to be the best automated measure we examined for our prairie vole partner preference testing.

The true test of an automated system, however, is whether it can consistently find group partner preferences when they in fact occur. Our results indicate that the two automated methods produce similar, but ultimately disparate, results (see males at 1 week; **Figure 1.4**). With different results of partner preference formation in hand, investigators are likely come to wholly different conclusions. For example, the automated "social proximity" data suggest that males rapidly form selective partner preferences but fail to express them during a PPT after 1 week. Alternatively, the automated "immobile social contact" data suggest that males display selective partner preferences over an extended period of time.

Since these data were obtained from the same set of video-recorded tests, the comparison clearly demonstrates that the move to automated systems cannot proceed indiscriminately. The assumption that "social proximity" is essentially equivalent to "huddling" as rated by humans could lead to erroneous conclusions.

"Social proximity," however, did provide comparable results for females at both time points and for males during the post-24-hr PPT. Thus, automated measures of "social proximity" in the form of center-of-gravity video-tracking may be a cost-effective and relatively accurate alternative in many circumstances. Likewise, infrared beam-breaks could also be used to detect "social proximity." With photobeams placed just

beyond the reach of the tethered stimulus animals, we found that Vole Tracker (R. Henderson, Florida State University), which has been used to measure locomotor activity in a number of studies (Curtis et al., 2001; Aragona et al., 2003; Curtis and Wang, 2005), provided nearly identical "social proximity" results when compared to our early version Noldus Ethovision, 3.0 (TH Ahern, unpublished data). This suggests that "social proximity" can be obtained through multiple methods, not just our 2002 version of Ethovision 3.0, and they are likely to have comparable accuracy.

As we predicted, "immobile social contact" provided a measure of "huddling" that was significantly more accurate than "social proximity." (**Table 1.1**). Furthermore, this automated metric successfully detected partner preferences after 24 hours and 1 week of cohabitation in both males and females. Both analyses confirmed our central hypothesis that automated "immobile social contact" would better reflect manually scored "huddling" than the measure of "social proximity."

Although we confirmed our hypothesis, we were intrigued by the factors that resulted in inaccuracies or discrepancies between scoring methods. As noted, males after 1 week of cohabitation demonstrated a significant partner preference with "immobile social contact" as the metric, but not with "social proximity"—primarily because of a relative increase in time spent in "social proximity" to the stranger (**Figure 1.4**). Based on known behavioral changes in males after extended cohabitation periods (Aragona et al., 2006), we examined whether an increase in stranger-directed aggressive bouts could explain the discrepancy. Our findings suggest it does not. Qualitative reanalysis indicated that different animals increased stranger "social proximity" due to a number of differing factors between animals. For some, it was aggression, for others it was chewing on the

stranger's tethering anchor, for others it was pushing the bedding around at the stranger end, etc. In sum, the test male behaviors that increased "social proximity" time in the social zone with stranger were many and varied.

A similar pattern of variation in behavior was seen in the 15-minute video segments used for our correlational analyses. We had initially thought that part of the reason our automated systems were not 100% accurate is that they might occasionally lose track of animals. However, this was rarely the case since the cages were well-lit, providing high-contrast between the dark animals and the light-colored bedding, and the cages were viewed from above.

Behaviorally, the sources of inaccuracy were more obvious, but difficult to quantify. During the measure of "social proximity," test animals would occasionally cross the boundary into one of the social zones and then proceed to sit, explore, dig, or attempt to climb the wall in a part of the zone removed from the tethered animal. When these behavioral bouts extended for long periods of time, they would substantially increase the amount of "social proximity" time while having no effect on "huddling" time. Disparities such as these ultimately had a deleterious effect on the overall accuracy of "social proximity" as a social metric.

For the measure of "immobile social contact," the reasons for inaccuracy were more subtle. One of the sources was the process of defining the outer edge of each animal. At times animals will sit near each other, but with a space between them. A human will see this space, whereas the software may overestimate the animal outlines enough to create a point of contact. The software would therefore count "immobile social contact," but the human rater would not score "huddling." Another source of inaccuracy likely

depends on how long "immobile social contact" bouts need to extend before a human will rate it as "huddling." Occasionally, animals investigate one another and pause briefly. The automated software is likely to add a second or two of "immobile social contact" time here, whereas a human would not. Both sources of inaccuracy could, in theory, be modified by changing specific parameters within the software's interface. At approximately 99% accuracy using SocialScan 2.0's default parameters and our testing conditions, minor adjustments to achieve slightly better accuracy seemed unnecessarily burdensome.

Achieving a respectable level of accuracy was not the only benefit to using these systems. Automated scoring also greatly decreased our time investment. Forty-eight PPTs would have taken 144 hours to score manually in real-time. Even using time-compression equipment, manual scoring would have taken 9-11 hours (requiring three to seven days to score fully, in order to avoid fatigue and drift). Alternatively, once standard virtual arenas were set, only 30-60 minutes of human time were required to obtain complete automated results by the morning following the final tests. This equals more than an 85% reduction in the expenditure of human time and a 67-86% reduction in the time delay to obtain complete data sets. This drastic reduction in time without a substantial reduction in accuracy suggests that the PPT in combination with the automated scoring of "immobile social contact" is ready for high-throughput discovery.

The success of automated video-tracking systems to analyze social behavior in prairie voles provides evidence that these systems could easily be extended to other rodent models, in which stimulus animals have restricted movement. While mating behavior may still have to be scored manually, many other social metrics could be

automated. For instance, SocialScan, Ethovision XT 6.0, and potentially others, are all capable of simultaneously measuring latency to approach, time in social contact, distance from a social object, stretch-attend postures, and even sniffing. In fact, it might be possible to couple these behavioral metrics with a measure of "immobile social contact" to provide a more complete picture of social behavior within the PPT, although we have not yet attempted this. In conclusion, video-based behavioral analysis software capable of tracking whole-animal behaviors of multiple animals may provide the most flexible, accurate, comprehensive, and time-efficient approach to studying social behavior on a large scale. Already, automated systems can be adapted to score important behavioral metrics and they appear ready to start replacing human scoring methods.

Disclosure / Conflict of interest

The authors declare that, after this research had been conducted absent of any conflicts-of-interest, Clever Sys Inc donated funds ($5000) along with the National Science Foundation and the Center for Behavioral Neuroscience to a pool of money that LJY used to organize and fund the 2009 Annual Vole Meeting (Atlanta/Decatur, GA). In return, Clever Sys Inc was permitted to bring a booth to the meeting.

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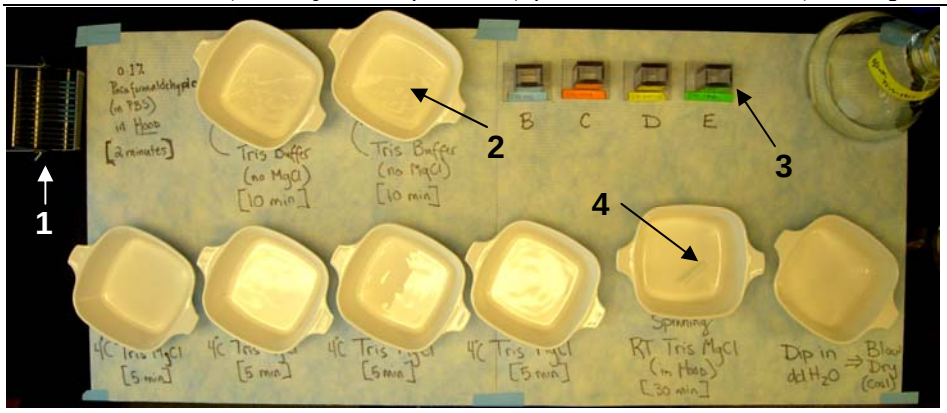
APPENDIX 2

CRF1 CRF2 Autoradiography Receptor Binding Protocol

Todd H. Ahern (Emory Univ | 7/2006) | Cf *Lim et al., 2005 (J Comp Neurol)*



1. Metal 30-Slide Rack *(Young Lab: drawer beneath the Radioactivity Bench)*
2. 450mLWhite Ceramic Wash Dishes *(Young Lab: cabinet beneath the Radioactivity Bench)*
3. 8mL 10-Slide Binding Chambers *(Young Lab: drawer beneath the Radioactivity Bench)*
4. White Teflon Stir-bar *(Young Lab: left-hand drawer beneath the microwave)*

Protocol:

1) Remove slides from -80ºC and put in Metal Slide Rack; thaw slides at Room Temperature (RT) and allow to dry, *~20 minutes*
   - While slides warm and dry, make or thaw all solutions.
2) Mix **8mL** of **1X Tracer Buffer + 0.2nM I-125 Sauvagine** in each Binding Chamber
   - For competitive binding add **500nM CP-154,526** (CRF1 antagonist) or **500nM Astressin-2B** (CRF2 antagonist): See Lim et al., 2005
3) Under hood, fix slides in 450mL of **0.1% paraformaldehyde** in White Ceramic Wash Dish, *2 minutes*
4) 2 washes in **1X Tris Buffer (No MgCl) pH 7.4** (change every 2 racks), *10 minutes each wash (~21 minutes total)*
5) Transfer slides from Metal Slide Rack to 10-Slide Binding Chambers containing I-125 Tracer Buffer: bind for *2 hours*
6) Transfer slides from Binding Chambers to Metal Slide Rack
7) 4 washes in **4ºC 1X Tris + MgCl Buffer**, *5 minutes each wash (~24 minutes total)*
8) 1 wash RT **1X Tris + MgCl Buffer**, spinning on stir-plate, *30 minutes*
9) Dip in **ddH2O** and dry completely with blow dryer (set to *cool*): *~15 minutes*
10) In dark room, appose slides to Film (dull-side faces slides); let expose for *72-90 hours*, then develop.

Solutions:

0.1% Buffered Paraformaldehyde (1L)*
800mL dH2O
1g paraformaldehyde
1-2 pellets NaOH
100mL 10X PBS†
Adjust pH to 7.4
Fill to 1L with ddH2O
* see Paraformaldehyde specs below

20X Tris - No MgCl *(1M Tris Buffer)* (500mL):
400mL ddH2O
60.3 g Tris (Trizma Base), add HCl to pH = 7.4
Fill to 500mL with ddH2O
(dilute this 1/20 to get 1X 50mM Tris+No MgCl)

1X Tris - No MgCl *(50mM Tris Buffe*r) (1L):
50mL 20X Tris - No MgCl *(see above)*
950mL ddH2O
(can pH again, but don't have to)

10X Tracer Buffer (50mL):
50mL 0.5M Tris (25mL 1M Tris, 25mlLddH2O)
1 g MgCl
0.5 g BSA *(powder at 4ºC in fridge)*

1X Tracer Buffer (8mL): *(50 mM [pH=7.4], 10mM MgCl, 0.1% BSA)*
7.2mL ddH2O
0.8mL 10X Tracer Buffer *(see above)*

20X Tris MgCl *(1M Tris + 4% MgCl)* (500mL):
400mL ddH2O
60.3 g Tris (Trizma Base), add HCl to pH = 7.4
20g MgCl
Fill to 500mL with ddH2O
(dilute this 1/20 to get 50mMTris+.25MgCl)

1X Tris MgCl *(50mM Tris + .2%MgCl)* (3L):
150mL 20X Tris MgCl *(see above)*
2850mL ddH2O
(can pH again, but don't have to)
(cool to 4ºC in fridge)

50%-EtOH/5mM-HCl (10mL):
5.00mL 200 proof EtOH
4.95mL ddH2O
50ul of 1M HCl

Trizma Base | Sigma-Aldrich, Cat # T-6066
Paraformaldehyde EM | Polyscience's Inc., Cat # 00380
BSA | Sigma-Aldrich, Cat # A2153-100g
*BioMax **MR** Film* | Kodak, Cat # 871-5187, 35x43cm

I-125 Sauvagine | PerkinElmer, Cat # NEX306010UC (10uCi, 370kBq) | On I-125 Sauvagine Shipping Chamber and Product Sheet, find the following:
Current uCi : ***x*** = ___ uCi *(e.g., 33.35uCi | see Lorra's radioactivity files to calculate current uCi value based on date)*
Specific Activity : ***y*** = ___ Ci / mmol *(e.g., 2000Ci/mmol)*
# of ul : ***z*** = ___ ul *(e.g., 250ul)*

Open the following file **<Autoradiography Isotope Conversion Sheet.xls>**. Now add ***x***, ***y***, and ***z*** from above to columns F, G, & H.
(For calculations, see page 2 of this protocol or page 76 of *Todd Notebook March 2006* for the original calculations)
To *8mL of 1X Tracer Buffer*, add *calculated amount (ul) of I-125 Sauvagine* to obtain *0.2nM I-125 Sauvagine*

500nM CP-154,526 (CRF1 Selective-Antagonist) | Generously provided by Michael Owens, PhD | Emory University
Add an equal number of mL of 50%-EtOH/5mM-HCl to "x"mg of CP-154,526 = 2.745mM CP-154,526 (e.g., 1.12mL 50%-EtOH/5mM-HCl to 1.12mg CP-154,526 = 2.745mM)
To *8mL of 1X Tracer Buffer*, add *1.807ul of 2.745mM CP-154,526* to obtain *500nM CP-154,526*

500nM Astressin-2B (CRF2 Selective-Antagonist) | Sigma-Aldrich, Cat # A5227-250ug, 250ug of Astressin2B trifluoroacetate salt, MW = 4041.69g/mol
Add 1mL of ddH2O to 250ug of Astressin-2B = 61.855uM Astressin-2B
To *8mL of 1X Tracer Buffer*, add *64.65ul of 61.855uM Astressin-2B* to obtain *500nM Astressin-2B*

I-125 Sauvagine *Example* Calculations: (Again, see **<Autoradiography Isotope Conversion Sheet.xls>** for fast calculations)

Current uCi : ***x*** = 33.35 uCi = 33.35E-6 Ci
Specific Activity : ***y*** = 2000 Ci / mmol = 2000 Ci / 1E-3 mol
# of ul : ***z*** = 250 ul = 250E-6 L

To calculate the Molarity of the I-125 Sauvagine that arrived from Amersham:

x / ***y*** = mols of I-125 : 33.35E-6 Ci / (2000 Ci / 1E-3 mol) = 1.6675E-11 mols of I-125

mols of I-125 / ***z*** = Molarity of I-125 in quantity ***z*** : 1.6675E-11 mol / 250E-6 = 6.67E-8 M = **66.7nM**

*To calculate the number of microliters (ul) of **66.7nM** I-125 Sauvagine to add to 8mL of 1X Tracer Buffer to have 0.2nM (0.2E-9M) of I-125 Sauvagine*

(Note: 8mL and 0.2nM are variable, depending on the purpose of the study. Simply adjust calculations accordingly.)

$[Volume]_1 * [Concentration]_1 = [Volume]_2 * [Concentration]_2$

$[Volume]_1$ * 6.67E-8 M = 8E-3 L * 0.2E-9 M

$[Volume]_1$ = (8E-3 L * 0.2E-9 M) / 6.67E-8 M

$[Volume]_1$ = 2.9985E-5 L = **29.9985 ul**

Thus, add **29.9985ul** of 66.7nM I-125 Sauvagine into 8mL of 1X Tracer Buffer to get 0.2nM I-125 Sauvagine for *total* CRF1 & CRF2 receptor binding.

***Paraformaldehyde Solutions:**

4% Buffered Paraformaldehyde
For 1 liter – adjust for volume needed:
40 grams paraformaldehyde
800 mL ddH2O
3-5 pellets NaOH (or dissolved concentrated NaOH) *(Young Lab: Powdered Chemical Shelf: Sodium Hydroxide)*
100mL 10xx PBS
more ddH2O to bring to 1000mL (1L) total

- **Safety Issues**- Use caution when weighing para – do NOT breathe in powdered para – this is a tissue fixative, it may harm your lungs if exposed. Wear a mask or respirator and try to gently scoop para onto weigh boat to prevent a cloud of powder from blowing up. Prepare para solution in a ventilated hood for the same reason – while heating the para fumes are being released which are harmful to be exposed to. For extra safety, pH para in the hood also. Once the para cools – it is not as volatile and there are less fumes.

  Heat 800ml dH2O to 56º C in a 2L flask. Slowly add 40g Para. When dissolved add either pellets or concentrated NaOH dropwise until solution is clear. When completely dissolved cool to at least room temp. (At this point – you can leave in fridge overnight to complete and use the next day). Once cooled, add 100ml 10xPBS, pH the solution to 6.8-7.5 with concentrated HCl depending on needs. (Young lab - generally pH to 7.5 +/- 0.2 for in situ or autoradiography, Anne Murphy's perfusion uses pH 6.8) Once pHed, bring up to 1 liter in graduated cylinder.Vacuum filter in the hood using vacuum flask, ceramic filter funnel and round paper filters to remove any large undissolved particles.

  Keep at 4º C until used.
  For in situ, use within 24 hrs.
  For perfusion, use within one month.
  For binding, use within three months diluted to 0.1%

  For autoradiography/binding use 0 .1% para:
  For 1L of 0.1%: (V1)(C1) = (V2)(C2)
  (1000ml) (0.1%) = (xml) (4%)
  100ml* % / 4% = xml
  25ml = xml ➔ x = 25ml
  So: Add 25ml 4% para to a graduated cylinder and add ddH2O to 1000ml to make 1000ml of 0.1%

0.1% Buffered Paraformaledhyde
For 1 liter – adjust for volume needed:
1 gram paraformaldehyde
800 mL ddH2O
1-2 pellets NaOH
100mL 10xx PBS
more ddH2O to bring to 1000mL (1L) total

†PBS (Saline Buffer Solution) (1L):
800mL ddH2O
80g NaCl
2g KCl
11.5g Na2HPO4 * 7 H2O
2g KH2PO4
Add 1 pellet NaOH | pH to 7.3
Fill to 1L with ddH2O

Identical to 4% except – no need to heat dH2O, after pHing, no need to filter.

APPENDIX 3

Riboprobe *In Situ* Hybridization Protocol

Todd Ahern | October 2008

Adapted from Lisa Stanek's 2004 Protocol
(based on methodology from Ressler & Sullivan, originally acquired from Giovanna Marazzi and David Sassoon)

BRAIN TISSUE ACQUISITION

Brain Extraction: *(It was found that using fresh frozen brains gives lower background than paraformaldehyde perfused brains)*

1. Crush **dry-ice** to a fine powder and place in a styrofoam box
2. Cut **aluminum foil** (Fisher 01-213-105) into enough 3in x 3in squares so that each brain will be wrapped by one square. Use Sharpie to label each square to identify the brain.
3. Over-anethetize each animal: pour a small amount of **Isoflurane** onto a cotton ball and put in closed container; place animal in container; wait until the animal stops breathing
4. Ensure dry-ice is still well powdered; dig a small hole and mound some powdered dry-ice next to the hole
5. Check to animal for responsiveness by using a toe-pinch
6. Use **scissors** to cut cleanly through the neck
7. (Collect trunk blood if necessary)
8. Use **tweezers** remove the skull
9. Use a small curved **spatula** to scoop out the brain
10. Quickly drop the brain (dorsal side down) into the dry-ice hole and quickly cover with the mound of powdered dry-ice. [This freezes the brain much faster than letting it sit on top of the cold aluminum foil]
11. Ensure the appropriate aluminum foil square is labeled and cooled, then wrap the brain in it.
12. Store in a **-80C Freezer** until use.

20 min TO SETUP
4-6 min PER BRAIN
5 min BREAKDOWN

Brain Slicing:

13. Set cryostat (Leica CM 1800) to appropriate or desired temperature (e.g., -19C)

14. Ensure there is a clean, un-chipped **glass roll-plate** (Leica 0.419.30402: Anti-roll Plate Glass) and a sharp **Low Profile Microtome Disposable Blade** (Accu-Edge, 4689)
15. Ensure the cryostat **chuck**, **tweezers**, **a super-fine**, **a fine**, **and a larger paint-brush**, and **razor blade** are clean and cold in the cryostat
16. Ensure there is **Embedding Media** (Tissue-Tek O.C.T. Compound, 4583)
17. Remove the brain from the -80C
18. Place the brain in the cryostat and let equilibrate for *15 min*
19. Mount the brain on the chuck using the Embedding Media; let freeze
20. Apply more **Embedding Media** if necessary
21. Situate the cryostat chuck in the bore of the cryostat and bring brain to blade
22. Set **Slice Thickness = 20uM**
23. Thaw mount on pre-cleaned **SuperFrost/Plus Microscope Slides** (Fisher, 12-550-15)
24. Once a single slide is full, it can sit in a slide box at Room Temperature for a few hours.
25. After slides are ready, store at -80C

TIME is variable depending on what parts of the brain need to be cut for analysis

RIBOPROBE IN SITU HYBRIDIZATION: SOLUTIONS + EQUIPMENT *(make + find these ahead of time)*

DAY 1 Solutions:

☐ **1L – ddH2O, pH:7.8**
900mL ddH2O, pH:7.8
Fill to 1L ddH2O

☐ Circular Plasmid Template
☐ 10X BSA
☐ Restriction Enzyme (e.g., NotI)
☐ Restriction Enzyme Buffer (e.g., NEB 3)
☐ Small PCR Tubes
☐ PCR Machine (with program for 37C)
☐ ~1 Hour

DAY 2 Solutions:

☐ **150mL - 0.7% Agarose Gel**
1.05g Agarose (Seakem LE Agarose, 50004)
150mL 1X TBE
Heat for ~4 min in microwave (until clear)
1.5ul Ethidium Bromide (Sigma, E1510-10mL)
Pour into mold

☐ 6x Loading Dye (Promega, G190A)
☐ 1kb Ladder (NEB, N3232S)
☐ Razor Blade
☐ 1.5mL Centrifuge Tubes
☐ Zymoclean Gel Kit (D4001)
☐ Nanodrop (ThermoScientific)
☐ ~3 Hours

DAY 3: (*Must be Clean and RNase Free)*

☐ **19.5mL – Hybridization Buffer**
See page 7

☐ **1mL – 10mg/mL Yeast tRNA**
10mg Yeast tRNA (Sigma, R-8759 10,000)
1mL ddH2O

☐ **500mL - 4% Paraformaldehyde:** ***Make fresh***
20g Paraformaldehyde (Polysciences, 00380)
250mL ddH2O
4-5 NaOH Pellets
Heat Solutions and mix until pellets dissolve
100mL 10x PBS
Adjust pH to 7.3
Add ddH2O to 500mL
Store at 4C (for up to 24 hours)

☐ **1L - 10X PBS**
1 Packet (Fisher, BP665-1)
1000mL ddH2O (clean, RNase free)

☐ **3L - 1X PBS**
300mL 10X PBS
2700mL ddH2O (clean, RNase free)

☐ **250mL - 0.5M EDTA**
36.5 g EDTA (Fisher, BP118-500; MW: ~292)
200 mL ddH2O
5g NaOH Pellets (add slowly; stir with stir bar)
Slowly add more NaOH, until pH:8

☐ **500mL - Proteinase K Buffer**
5 mL 0.5M EDTA (see above)
25 mL 1M Tris-HCl, pH:8 (Ambion, 9856)
470 mL ddH2O (clean, RNase free)

☐ **10mL – 10mg/mL Proteinase K**

100mg Proteinase K (Promega, V302B)
10mL ddH2O
Aliquot 500ul into separate 1.5mL Tubes

☐ **250mL – Proteinase K Solution**
500ul 10mg/mL Proteinase K (see above; just before use)
250mL Proteinase K Buffer (see above)

☐ **50mL – 1M DTT**
7.71 g -20C DTT, (Fisher Biotech, BP172-25, MW: 154.25)
50mL ddH2O

☐ **500mL – Acetylation Sol'n**
3.75g Triethanolamine (liquid; Sigma, T1377-500mL)
500 ul Acetic Anhydride (Fisher Scientific, A10-100)
398 mL ddH2O (clean, RNase free)
Stir ahead of time in Tissue Tek Chamber using stir bar

☐ **1L – 5M NaCl**
292.2g NaCl (Fisher, S671-10)
1000mL ddH2O

Continues on next page…

DAY 3: *Continued….Again, maintain RNase Free*

☐ **500mL – 50% Formamide / 4X SSC (Humidity Soln)**
250mL Formamide (general use; Sigma, F7503-1L)
100mL 20X SSC (Ambion, 9763)
150mL ddH2O

☐ **3L - 0.2 PBS (Kiyoshi)**
165g Na2HPO4
21g NaH2PO4
2880mL ddH2O (clean, RNase free)

☐ Promega Riboprobe T3/T7 Kit (P1540) -20C
☐ 1mCi S35-UTP (PerkinElmer, NEG739H001MC) -20C
☐ DEPC-treated ddH2O (Ambion, 9915G)
☐ RNASE ZAP (RNASEZAP, 724000)
☐ Dextran Sulfate (Omnipur, 3710)
☐ 1M Tris-HCl, pH:8 (Ambion, 9856)
☐ Deionized Formamide (Sigma, F9037-100ML) -20C
☐ 50x Denhardts (Sigma, D2532-5mL) -20C
☐ 10mg/mL Salmon Testis DNA (Invitrogen, 15632-011) -20C
☐ Triethanolamine (liquid; Sigma, T1377-500mL)
☐ 37C Block Incubator
☐ 37C Air Incubator
☐ ProbeQuant 96 G-50 Micro Columns (GE, 28-9034-08)
☐ 1.5mL Centrifuge Tubes
☐ Eppendorf Centrifuge (5415C)
☐ Scintillation Fluid (Fisher, SX 17-4)
☐ Scintillation Vials (Kimble, 74503-20)
☐ RNase free gray, 48-slide in situ rack
☐ RNase free Tissue Tek Chambers (> 15 chambers)
☐ RNase free small magnetic stir bar
☐ 80C Block Incubator
☐ Blow-Drier (with *cool* setting)
☐ 200 Proof EtOH (Decon Labs, 2701)
☐ Chloroform (Fisher, C297-4)
☐ 96-well-plate lids (Fisher, 12-565-511)
☐ Glass cover-slips (Corning, 2935-246)
☐ 55C Oven 3 Waterbaths (37C, 55C, 60C)
☐ Lab Tape
☐ Aluminum Foil + Saran Wrap (maybe)
☐ 25mL Conical Tubes (maybe)
☐ 50mL Conical Tubes (maybe)
☐ Time: All Day

DAY 4: *(Don't have to be RNase Free)*

☐ **1L – 20X SSC** (Ambion, 9763; or recipe)
175g NaCl
88g NaCitrate
800mL ddH2O, pH: ~ 7
Fill to 1L ddH2O

☐ **2L - 5X SSC**
500mL 20X SSC (Ambion, 9763)
1500mL ddH2O

☐ **2L - 2X SSC**
200mL 20X SSC (Ambion, 9763)
1800mL ddH2O

☐ **500mL - 0.1X SSC**
2.5mL 20X SSC (Ambion, 9763)
498mL ddH2O

☐ **1L - 50% Formamide / 2x SSC**
500mL Formamide (general use; Sigma, F7503-1L)
100mL 20X SSC (Ambion, 9763)
400mL ddH2O

☐ **1L - 10X RNase (Wash) Buffer**
233.8g NaCl (Fisher, S671-10)
100mL 1M Tris-HCl, pH:8 (Ambion, 9856)
100mL 0.5M EDTA, pH:8 (see above)
Fill to 1L ddH2O

☐ Above Solutions – Equilibrated to Temp.
☐ -20C DTT, (Fisher Biotech, BP172-25, MW: 154.25)
☐ Diaper (Versi-Dry, 74018)
☐ 30% EtOH + 0.3M NH4Ac
(1L: 300mL 200 Proof EtOH + 700mL ddH2O + 23.12g Ammonium Acetate [MW = 77.08; Fisher, BP326-500])
☐ 60% EtOH + 0.3M NH4Ac
☐ 80% EtOH + 0.3M NH4Ac
☐ 90% EtOH + 0.3M NH4Ac
☐ 95% EtOH each + 0.3M NH4Ac
☐ 100% EtOH
☐ 96-well-plate Lids (Fisher, 12-565-511)
☐ Paper Towel
☐ Film Cassette (Fisher Biotech, FBXC 1417)
☐ Lab Tape
☐ Chromotography Paper (Fisher, 05-714-4)
☐ C14 microscale standard (GE Healthcare, RPA 511)
☐ MR Film (Kodak BioMax MR Film, 871-5187)
☐ Time: 4-5 hours

RIBOPROBE IN SITU HYBRIDIZATION: DAY 1 *(this can be done months in advance of Days 3-4)*

Linearize Plasmid Template: *(Circular plasmid is available for linearization after a mini- or maxi-prep*)

26. Thaw eluted circular plasmid, 10x BSA, 10x Enzyme Buffer
27. Locate appropriate Restriction Enzyme
28. Mix up the following Digests in small PCR tubes

Linear Digest:	
5 ul	Circular Plasmid
2 ul	Enzyme Buffer
2 ul	10x BSA (if necessary)
1 ul	Restriction Enzyme
10 ul	ddH2O (pH: 7.8)
20 ul	TOTAL MIX

EXAMPLE: *20-45 min to locate, thaw, and mix*	
5 ul	PLTA028 (Prairie GR: NotI + T3 = Anti-Sense)
2 ul	10x Buffer 3 (NEB, B7003S)
2 ul	10x BSA (yes)
1 ul	NotI (NEB, R0189S)
10 ul	ddH2O (pH: 7.8)
20 ul	TOTAL MIX

29. Place digests in PCR machine set to 37C (top-plate set to 104C to prevent condensation)
30. Let incubate overnight (if necessary, 3 hours is usually sufficient)

RIBOPROBE IN SITU HYBRIDIZATION: DAY 2 *(this can be done months in advance of Days 3-4)*

Purify Linearized Template: *(Stanek protocol suggests Proteinase K treatment and Phenol-Chloroform extraction to purify the template; a gel is easier as long as you have access to a gel extraction kit*)

31. Make a **0.7% Electrophoresis gel** with **Ethidium Bromide** (Sigma, E1510-10ML)

32. Add 3-5ul of **6x loading dye** (Promega, G190A) to the digest from DAY 1
33. Load the entire digest + dye to 0.7% gel
34. Add a **1kb DNA ladder** (NEB N3232S) to a distant lane (to prevent contamination)
35. Run gel slowly for about *1 hour*
36. Get a clean **razor blade**
37. On **UV lamp box**, cut out linearized template
38. Place chunk of gel containing the linearized template in a fresh 1.5mL tube
39. Run through **Gel Extraction Kit** (e.g., ZymoC Research: Zymoclean Gel DNA recovery kit, D4001)
40. Make sure to elute using ddH2O (pH: ~7.8).
41. Quantify concentration using **Nanodrop** (ThermoScientific, NanoDrop 1000)
42. Store in **-20C freezer** until needed (can be stored years)

~3 hours

RIBOPROBE IN SITU HYBRIDIZATION: DAY 3 *(Days 3-4 must be performed one after another)*

Probe Labeling: *(Labeling is performed with Promega Riboprobe Combination System T3/T7, Cat # P1540*)

43. Get **Promega Riboprobe Combination System Kit** (Promega, P1540)
44. Get radioactive **1mCi S35-UTP** (PerkinElmer, NEG739H001MC) and ensure it is not too far beyond the calibration date
45. Fill 37C **block incubator** holes with ddH2O; start 37C air incubator
46. Using the Kit's contents and the S35-UTP, make the following mixes *(adding solutions in the following order)*:

Reaction Mix: *(multiply each value below by the number of different probes to be made + 1)*

1.25 ul Nuclease-free ddH2O (from Kit)
2 ul 5x Trans Buffer (from Kit)
0.25 ul 100mM DTT (from Kit)
0.25 ul 10mM ATP (from Kit)
0.25 ul 10mM CTP (from Kit)
0.25 ul 10mM GTP (from Kit)
0.25 ul Rnasin (RNase Inhibitor, from Kit)
4.5 ul TOTAL REACTION MIX

Probe Mix: (*make a separate probe mix for each probe)*

4.5 ul Aliquot of TOTAL REACTION MIX
2 ul Unique Linearized DNA Template (50-200ng total)
1 ul Appropriate RNA polymerase (T3, T7, Sp6)
2.5 ul S35-UTP (see conversion sheet for exact amount
10 ul PROBE Mix *(Can sit at Room Temperature until all Probe Mixes are made.*

With high counts, 10ul PROBE should be enough for ~45 slides)

47. Put PROBES in 37C block incubator for *1-2 min*
48. Move PROBES to 37C air incubator for *1.5 hours*
49. Add 1ul **Dnase RQ1** to each PROBE (from Kit; 1 unit/ul)
50. Return PROBES to air incubator for *15 min*
51. Add 3ul **10mg/mL Yeast tRNA** to each PROBE
52. Proceed to Probe Purification

Probe Purification: *(GE Healthcare ProbeQuant 96 G-50 Micro Columns Radiolabeled Probe Purification Kit, 28-9034-08*)

53. Get **illustra ProbeQuant 96 G-50 Micro Columns Radiolabeled Probe Purification Kit** (GE Healthcare, 28-9034-08)
54. Follow included instructions (re-capitulated here):
    a. Resuspend the column resin by vortexing
    b. Loosen the cap one-quarter turn
    c. Snap off the bottom closure
    d. Place column with the resin in supplied collection tube
    e. Centrifuge (Eppendorf 5415C) at 3000rpm for: *1 min*
    f. Add 40ul Probe Elution and Storage Buffer (from Kit) to 10ul PROBE: makes 50ul PROBE Sol'n
    g. Label new 1.5mL microcentrifuge tube
    h. Put resin column in labeled 1.5mL microcentrifuge tube
    i. Slowly add 50ul PROBE Sol'n to resin (do not touch resin or let sol'n drip down sides)
    j. Centrifuge (Eppendorf 5415C) at 3000rpm for: *2 min*
    k. Figure out approximate eluted volume

55. Take 1ul of each eluted, purified probe and add it to 1 squirt of **scintillation fluid** (Fisher, SX 17-4) in a **scintillation vial** (Kimble, 74503-20)
56. Mix thoroughly with brief vortexing
57. Count using a **scintillation counter** (Biomarkers Core Lab) with **Miranda's S35 program**; the output will look like following at the bottom of the page:

Cycle 1 Results

S#	Count Time	CPMA	SIS
1	0.50	1087740	88.52
2	0.50	1423860	89.15
3	0.50	992900	88.73

58. Look at the CPMA column: only 1ul was added to the scintillation, so CPMA = counts/ul for each PROBE
59. Multiply counts (e.g., 1087740) by your estimated number of eluted uls for total PROBE counts
60. Ultimately, you will aim use between 250,000 – 1,000,000 counts per slide
61. Anything less than 100,000 counts/ul is likely to be no good.
    a. If less, try re-running your eluted probe back over the column
    b. Or simply start over

Prehybridization: *(This can be performed in parallel with Probe Labeling & Purification procedures outlined above – see accompanying timeline*)

62. Find all RNase-Free solutions (see Day 3 Solutions & Equipment)
63. Remove slides from -80C
64. Place slides in **gray in situ rack** *(holds 48 slides back-to-back)*: *~15 - 30 min*
65. Let air-dry (or blow cool air from a **blow-drier**) until dry: *~ 30 min*
66. Ensure each of the **Tissue Tek chambers** has the appropriate wash/treatment solutions: *because the 4% Para steps are the bottle-necks, have separate 4% Para chambers for each rack to be treated.*

67. Begin the following series of treatments and washes (START TIMER for each):

a.	250mL 4% Paraformaldehyde	*30 min* (can be used again in subsequent step)
b.	250mL 1x PBS (0.1M)	*5 min*
c.	250mL 1x PBS (0.1M)	*5 min*
d.	250mL Proteinase K	*5 min* (fresh: 250mL PK buffer + 500ul 10mg/mL PK)
e.	250mL 1x PBS (0.1M)	*5 min*
f.	250mL 4% Paraformaldehyde	*30 min* (can be same 4% Para from previous step)
g.	250mL DEPC-treated ddH2O	*Dip*
h.	250mL Aceylation Sol'n	*10 min* (3.75g TEA + 500ul Acetic Anhydride [to slides])
i.	250mL 1x PBS (0.1M)	*5 min*
j.	250mL 50% EtOH	*2 min*
k.	250mL 70% EtOH	*2 min*
l.	250mL 85% EtOH	*2 min*
m.	250mL 95% EtOH	*2 min*
n.	250mL 100% EtOH	*2 min*
o.	250mL 100% EtOH	*3 min*
p.	250mL 100% Chloroform	*3 min* (do under hood; Chloroform removes all the fats)
q.	250mL 100% EtOH	*3 min*
r.	250mL 100% EtOH	*3 min*
s.	Air-dry slides	*30 min*

68. Use slides immediately (proceed to Hybridization) or wrap in saran wrap + aluminum foil and store at 4C for 1-3 days

Hybridization:

69. Make hybridization buffer using the following recipe; add in order + vortex to liquid after each addition:

Hybridization Buffer Mix: *(makes enough for ~80 slides)*

2 g	Dextran Sulfate	(Amersham Pharmacia 17-0340-01)
7 mL	DEPC-treated ddH2O	(Ambion, 9915G)
400 ul	1M Tris-HCl, pH: 8	(Ambion, 9856)
1.2 mL	5M NaCl	(see Recipe in Day 3 Solutions)
10 mL	Deionized Formamide	(aliquoted @ -20C; Sigma, F9037-100ML)
400 ul	50x Denhardts	(Sigma, D2532-5mL)
400 ul	10mg/mL -20C Salmon Testis DNA	(tap; spin; Invitrogen, 15632-011)
20.0 mL	TOTAL HYBRIDIZATION BUFFER MIX	

(250ul for each slide = 1mL / 4 slides.
This mix can be stored at -20C for 6 months)

70. Aliquot enough Hybridization Buffer Mix for each probe
    a. e.g., 4 different probes * 4 slides each = 16 slides
    b. Aliquot 1 mL of Hybridization Buffer into 4 different 1.5 mL Tubes
    c. Each probe will be added to one of these aliquots in the Probe Aliquots step
    d. Each tube will contain enough Hot Probe Hybridization Solution to add ~200 ul to each of 4 slides.

71. Make Probe Aliquots using the following recipe:

Probe Aliquots: *(take the number of slides to be probed and divide by 4 = multiplier)*

1 mL * *multiplier*	Hybridization Buffer Mix
20 ul * *multiplier*	Yeast tRNA (25mg/mL)
20 ul * *multiplier*	1M DTT
1,000,000 counts * *multiplier*	PROBE
~1.05 mL * *mulitplier*	Hot Probe Hybridization Solution

72. Denature Probe Aliquots in 80C incubation block (small tubes) or water (conical tubes) for: *2 min*
73. Quickly put Probe Aliquots on ice
74. Lay slides out flat
75. Lay a cover-slip (Corning, 2935-246) out flat next to each slide
76. Pipet ~200ul of Hot Probe Aliquot down the center of each cover-slip
77. Gently and slowly flip the slides with tissue onto the cover-slips and then flip so tissue is facing up

78. Ensure there are no air-bubbles (use a razor blade to adjust the cover-slip if necessary)
79. Completely moisten 1 piece of paper-towel (cut in half) with **50% Formamide/4x SSC solution** (~5mL)
80. Place moistened paper-towel piece in the lid of a 96-well-plate (Fisher, 12-565-511)
81. Place 4 slides into each lid
82. Stack lids until all slides are in the 96-well-plate lid tower
83. Add one empty lid as a top
84. Completely wrap the entire lid tower with tape to ensure no air/moisture escapes
85. Place in oven set to 55C for *Overnight*
86. Clean up
87. Set waterbaths to 37C, 55C, and 60C and allow appropriate solutions to equilibrate overnight:
    a. 55C : 5x SSC
    b. 60C : 50% Formamide / 2x SSC
    c. 37C : RNase Buffer
    d. Room Temperature : 2x SSC and 0.1x SSC

RIBOPROBE IN SITU HYBRIDIZATION: DAY 4 *(Days 3-4 must be performed one after another)*

Re-Racking Slides: *(make sure all solutions have equilibrated to the proper temperatures*)

88. Remove the lid-tower with the slides from the oven
89. Remove the tape
90. Hold each slide vertical and thrust down vertically to force cover-slip off the slide (do not pull on cover-slip)
    a. Perform this in the hood, over a **diaper** that can be easily discarded in the Radioactive Dry Waste
    b. Ensure that all radioactive solids and liquids are disposed of properly
91. Put slides into **gray, in situ rack** situated in 55C 250mL 5x SSC + 0.4g DTT (add DTT just before start)

Washing: *(make sure all solutions have equilibrated to the proper temperatures*)

92. 250mL 55C 5x SSC + 0.4g DTT *30 min* (low stringency; start 30 when rack is full)
93. 250mL 60C 50%Formamide/2xSSC + 0.4g DTT *30 min* (high stringency wash)
94. 250mL 37C RNase Buffer *10 min*
95. 250mL 37C RNase Buffer + 25 ul RNase A *10 min* (RNase A solution: 1ug/mL)
96. 250mL 37C RNase Buffer *10 min*
97. 250mL 60C 50%Formamide/2xSSC + 0.4g DTT *30 min* (high stringency wash)
98. 250mL Room Temp. 2x SSC *10 min*
99. 250mL Room Temp. 0.1x SSC *10 min*
100. 250mL 30% EtOH + 0.3M NH4Ac *2 min* (each of the following can be reused)
101. 250mL 60% EtOH + 0.3M NH4Ac *2 min*
102. 250mL 80% EtOH + 0.3M NH4Ac *2 min*
103. 250mL 90% EtOH + 0.3M NH4Ac *2 min*
104. 250mL 95% EtOH + 0.3M NH4Ac *2 min*
105. 250mL 100% EtOH *2 min*
106. Allow to air-dry in clean, dry place *> 30 min*

Autoradiography:

107. Remove slides from rack and lay flat on filter paper to air-dry more
108. Tape the slides to a filter paper backing, cut to the size of the cassette
109. Add a C14 microscale standard (GE Healthcare, RPA 511)
110. Go to Darkroom

111. Appose slides to MR Film (Kodak BioMax MR Film, 871-5187)
112. Close film cassette and record ON time.
113. Appose to film for *5 – 21 days*
114. Proceed to film development (and Emulsion for individual cellular resolution if necessary)

APPENDIX 4

Immunohistochemistry Protocol: Donald Rainnie, Joanna Dubrowski

Abcam CRF2 antibody and Chemicon OT Antibody

1. Rinse sections in PBS × 3 (>30 min)
2. Incubate in 0.014% phenylhydrazine (10 µl in 10 ml PBS-Triton X-100 0.5% ≥ 20 min)
3. Rinse in PBS × 3 (>30 min)
4. Incubate sections in PBS + Triton-X 100 for 30 min
5. Transfer sections to 1º antibody solution at required concentration in 500 µl PBS + Triton-X 100/well. Put parafilm over wells and place on shaker in 4 ºC for 48 hrs. – In case of CRF2R (Abcam) – 1:1000 for DAB, 1:500 for fluorescense

6. Rinse with PBS × 3 (>30 min)
7. 2º antibody in PBS – Triton X-100 for 2 hrs at room (usually 1:500 dilution)

8. Rinse in PBS × 3 (>30 min) – prepare ABC solution ABC Elite Kit, Vector) in PBS/ Triton X-100 30 minutes before applying (4.5 µl of A and B per 1 ml needed)
9. Transfer sections to ABC solution – 500 µl per well for 1 hr
10. Rinse in PBS × 2 (≥ 20 min)
11. Rinse in sodium acetate (0.175 M = 7.18 g anhydrous form in 500 ml H_2O)
12. Incubate in Ni-DAB or 'normal' DAB for as long as needed. Usually it takes longer for 'normal'

2 mg DAB + 250 mg $NiSO_4$ in 10 ml sodium acetate – then add 8.3 µl 3% H_2O_2)
Or 2 mg DAB in 10 ml sodium acetate (then add 8.3 µl 3% H2O2 before applying to the wells)

13. Wash sections in PBS × 3 (30 min)
14. Wash in PB before mounting on slides
15. Let slides air dry over night
16. Process through alcohols
    - H_2O – 5 min
    - 75% ethanol – 5 min
    - 95% ethanol – 5 min
    - 100 % ethanol – 5 min
    - Histoclear or Xylene – at least 10 min
17. Coverslip with DPX or permount and let dry 1 hr

For fluorescence technique – protocol is exactly the same until washing after primary. Then, you want to apply secondary fluorescent antibody of course :) (Molecular Probes, for CRF2R I use goat anti-rabbit AlexaFluor 568) or cocktail of secondary antibodies. After 2hours incubation you wash sections exactly the same way and then mount them on subbed slides and keep them in darkness. Then I closed the slides with Vectashield Mounting Medium for fluorescence (H-1000, Vector).

APPENDIX 5

Western Blotting: Tissue Homogenization, Protein Gel Electrophoresis, Membrane Transfer, and Imagine

Todd H. Ahern (12/2009) with the Invitrogen NuPAGE System

TISSUE HOMOGENIZATION

1. Add tissue punches to Homogenization Buffer

   Homogenization Buffer
   0.32M sucrose
   5mM HEPES
   (In English: 109.4 g sucrose, 4 g HEPES in 1L ddih20, final pH 7.4.)
   Note: Make up only 50 ml at time if you don't plan on using a lot. The sucrose tends to attract mold.

   For the initial homogenization step, you must use cOmplete, Mini, EDTA-free 25 tablets in a glass vial (for 10 ml each) protease inhibitors with the buffer. For the protease inhibitors (Roche: 11 836 170 001), take one tablet, and place in centrifuge tube. Add 1.5 mL of ddH20 to the tablet, and vortex. This results in a 7 x concentration of protease inhibitors. For example, to create 7 ml of final homogenization buffer, add 1 mL of 7 x protease inhibitors, plus 6 mL of homogenization buffer.

2. Homogenize the sample thoroughly with Sonic Dismembrator (Fisher, Model 100) on setting 2.
3. Vortex vigorously
4. Store at -20C (will go bad eventually) or -80C

See Invitrogen NuPAGE Technical Guide
(http://tools.invitrogen.com/content/sfs/manuals/nupage_tech_man.pdf)

DAY 1

SAMPLE PREPARATION: *time is variable depending on how many samples* (*15 - 45 min*)

1. Figure out how many samples you want to run
   a. Young Lab currently orders 15 Well Gels (1.5mm holds 25ul; Invitrogen NuPAGE 4-12% Bis-Tris Gel, 1.5mm x 15well, Cat#: NP0336BOX)
   b. Can run two gels simultaneously, so maximum samples = 28 (14 samples/gel + 1 protein-ladder/gel)
   c. Plan accordingly

2. Ensure that samples are quantified appropriately (e.g., Using Pierce BCA Protein Quantification Kit, microplate instructions):
    a. See the following link for a cheat sheet for 96-well plate processing:
    b. "BCA Quantification" worksheet @ https://spreadsheets.google.com/ccc?key=0AhZaIwTarMXHdENEdGpTWUZ4cS13V0ZONVlsb2ZhNnc&hl=en
3. Before mixing samples, set PCR Thermocycler to HOLD @ 70C (w/ lid at 104C)
4. On page 13 of the NuPAGE Technical Guide are instructions for preparing the samples to load in the gel:
    a. Run "reduced samples" unless otherwise required (due to sensitivity, etc.)
    b. Find strings of PCR tubes
    c. Place PCR tubes in holder on ice
    d. Thaw all homogenized protein samples
    e. In each tube, add the following (see table below):

REAGENT	**REDUCED SAMPLE**	**NON-REDUCED SAMPLE**
Sample	x.x ul	x.x ul
NuPAGE LDS Sample Buffer (4x)	2.5 ul	2.5 ul
NuPAGE Reducing Agent (10x)	1.0 ul	--
Deionized H2O (not ddH2O)	up to 6.5 ul	up to 7.5 ul
TOTAL VOLUME	10.0 ul	10.0 ul

NuPAGE LDS Sample Buffer (4x) - Invitrogen (Cat#: NP0007, 10mL, store at RT or 4C)
NuPAGE Reducing Agen (10x) - Invitrogen (Cat#: NP0004, 250ul, store at 4C)

Each gel-well can hold up to 25ul, so the volumes can be adjusted based on the the concentration of each protein sample.

For assistance with calculations, see "Western Blot Sample Calc Sheet" @ https://spreadsheets.google.com/ccc?key=0AhZaIwTarMXHdENEdGpTWUZ4cS13V0ZONVlsb2ZhNnc&hl=en

Only change the information in each of the blue cells, the white cells will auto-calculate.

1. Close the tops of the PCR tubes
2. Mix tubes
3. Quickly centrifuge

4. Place PCR tubes in 70C Thermocycler for *10 min* (set timer!)
5. While tubes incubate at 70C, make NuPAGE SDS Running Buffer (MOPS)
    a. Get a 1L twist-top, glass bottles
    b. Get a 200mL Flask
    c. Get NuPAGE SDS Running Buffer (MOPS) (20X)
    d. Mix **50mL** NuPAGE SDS Running Buffer (MOPS) (20X) with **950mL** dH2O (not ddH2O)
    e. Mix thoroughly to make 1X NuPAGE SDS Running Buffer (MOPS)
    f. Poor 200mL of the 1X NuPAGE SDS Running Buffer (MOPS) into 200mL Flask
    g. Get NuPAGE Antioxidant (Invitrogen, Cat#: NP0005)
    h. Get 1000ul Pipet with tip, set to 500ul
6. Proceed to Gel Loading

GEL LOADING (page 15-18 of Technical Guide) (*20-40 min*)

1. Find all X-Cell SureLock Western Blotting apparatus pieces (ADD PICTURES)
2. Get 1 (or 2) NuPAGE 4-12% Bis-Tris (1.5mm x 15 well) gels (Invitrogen, Cat# NP0336BOX) from 4C fridge
3. Over the sink, cut open plastic pouch containing NuPAGE gel; drain liquid
4. Remove gel cassette
5. Rinse gel cassette with dH2O
6. Bring gel cassette to bench with X-Cell SureLock apparatus
7. Remove white tape from gel cassette (in one smooth motion)
8. Carefully, but firmly, remove the gel-well rake
9. [LISTEN FOR 70C TIMER; PUT SAMPLES IN PCR TUBE HOLDER AND PLACE ON ICE]
10. Ensure that wells haven't been compromised by removal of the rake
    a. If they have use a long pipet in and straighten well sides
11. Place gels firmly against white wire-electrode piece, with wells facing IN
12. Place in Western box and use Gel Tension Wedge to lock in place
13. Pour about 100mL of 1X NuPAGE SDS Running Buffer (MOPS) in between the two gel cassettes (this is the Upper Buffer Chamber).
14. Ensure that this amount doesn't leak out
    a. If it does, unlock the Wedge, reset, and try again
    b. Once seal is tight, move on
15. Dump out Running Buffer
16. Add the 200mL of Antioxidant treated 1X NuPAGE SDS Running Buffer (MOPS) to the Upper Buffer Chamber (between the two gels)
17. Ensure the wells are covered
18. Using long, Western-gel loading pipets, load 24.5ul of each sample into each well
19. Make sure to add 5-15ul of standard band ladder (e.g., Invitrogen [Cat# LC5925] or BioRad [Cat# 161-0373]) into one of the wells.

20. Add ~600mL of 1X NuPAGE SDS Running Buffer (MOPS) to the Lower Buffer Chamber (outside of gels) to dissipate heat
21. Put the top on the X-Cell SureLock apparatus
22. Set the Volt-Meter to:
    a. MOPS SDS Running Buffer: 125 volts constant (~100 > ~60mA/gel) for *1:10 Hrs*
    b. *(Invitrogen recommends 200V for 50 min, but this may cause the samples to bow)*
    c. *(For more options [e.g., MES buffer], refer to page 18 of the Technical Guide)*
23. Set timer!
    a. START DATE | TIME:

Move on to the first steps of the Gel Transfer

GEL TRANSFER (page 32-35 of Technical Guide) (*1.5 hrs*)

1. In preparation for membrane transfer, make up 1X NuPAGE Transfer Buffer about 15 minutes before gel run is complete
    a. Get a 1L twist-top, glass bottles
    b. Get 3 glass cassorole dishes
    c. Get NuPAGE Transfer Buffer (20X)
    d. Mix **50mL** NuPAGE Transfer Buffer (20X) with **200mL Methanol** (for 2 gels) or **100mL Methanol** (for 1 gel) in 1L glass bottle
    e. Fill glass bottle to 1L (1000mL) with dH2O to make 1X NuPAGE Transfer Buffer
    f. Get NuPAGE Antioxidant (Invitrogen, Cat#: NP0005)
    g. Get 1000ul Pipet with tip, set to 1000ul
    h. Right before using the Transfer Buffer, add **1000ul** NuPAGE Antioxidant to the 1X Transfer Buffer
2. Locate a clean metal spatula
3. Locate a clean razor blade
4. When Gel Running is complete, perform the following
    a. Set out 3 glass dishes
    b. Fill bottom of each glass dish with 1X Transfer Buffer (enough to cover the white transfer pads)
    c. Find 1 or 2 Invitrogen Nitrocellulose Membrane Filter Paper Sandwich 0.45um Pore Size, 20/pk, (Invitrogen, Cat# LC2001)
5. Turn off the Volt Meter
6. Move 1 or 2 membrane sandwiches (filter-paper/membrane/filter-paper) to second dish with 1X Transfer Buffer
7. Remove X-Cell SureLock apparatus top
8. Release the Gel Tension Wedge
9. Remove the first gel cassette

10. Insert a metal spatula around the edges of the gel cassette and twist/lift-off enough to make a small pop.
11. Repeat this popping by moving the metal spatula along the outer edge and wedging the two halves of the cassette apart (the gel is relatively sturdy, but be very careful; work around the cassette several times if necessary so as to avoid putting too much pressure on the gel) [ADD PICTURES]
12. Remove one half of the plastic cassette carefully (making sure the gel isn't torn); use the metal spatula to help if necessary
13. Place the cassette-half with gel in first dish with 1X Transfer Buffer, gel facing UP
14. With Razor, cut off wells
15. Take a piece of pre-soaked filter paper (from the sandwich in second dish) and place on top of gel
16. Flip cassette with gel over into the gloved hand (palm) and slowly use metal spatula to separate the gel from the cassette
17. Filter paper and gel should settle into your hand (hand/filter-paper/gel) with gel facing up
18. Place filter-paper/gel into the second dish
19. Take the membrane and lay on top of the gel
20. Place the second piece of filter paper on top of the membrane:
    a. **[bottom]** filter-paper/gel/membrane/filter-paper
    b. Ensure all air-bubbles are removed
21. Repeat steps 9-20 with gel cassette 2
22. In the deep-half of the white Blot Module, place 2 pre-soaked blotting pads horizontally
23. Place your first gel sandwich (with membrane on top of gel, in between filter papers)
24. Place another pre-soaked blotting pad
25. Place the second gel sandwich (with membrane on top of gel, in between filter papers)
26. Add two more pre-soaked blotting pads
    a. The configuration should look like the following (see manual for pictures):
    b. Figure 1 = transferring 1 membrane
    c. Figure 2 = transferring 2 membranes

27. Clean out Gel Running buffer from X-Cell SureLock box
28. Clean Gel Tension Wedge
29. Add the top of the Blot Module
30. Squeeze together over blue-diaper or sink
31. Place in X-Cell SureLock box
32. Use Gel Tension Wedge to force tight seal
33. Poor more 1X NuPAGE Transfer Buffer into the Blot Module until white screws; don't overfill
34. Check for leaks

35. If no leaks, add enough dH2O in the Lower Buffer Chamber to come approximately 2cm below the top of the Blot Module
36. Add the top of the X-Cell SureLock apparatus (attached to Volt Meter)
37. Set the Volt-Meter to:
    a. 30 volts constant (~100 > ~60mA/gel) for *1 Hr*
    b. *(For more options, refer to page 35 of the Technical Guide)*
38. Set timer! and fill-in info sheet
    a. START DATE | TIME:

WESTERN BLOTTING > PRIMARY TREATMENT (*45-60 min*)

1. During Membrane Transfer, make up Kim's Blocking Buffer in 500mL glass bottle (see recipe below) - make this at the start of the 1hr-long transfer since dry-milk takes awhile to dissolve completely
    a. 5mL 1M HEPES
    b. 25mL 1M NaCl
    c. 10g Nonfat Dry Milk
    d. 500ul Tween 20
    e. Fill to 500mL w/ dH2O (not ddH2O)
2. When Membrane Transfer is completed, turn off machine, remove membrane
3. Place Membrane immediately in a square petri dish and add enough Blocking Buffer to submerge the membrane, even when rocking
4. Let Block while rocking for *30min - 1hr*
5. During blocking, dilute primary antibody in Blocking Buffer to desired concentration:
    a. E.g., 5mL of Blocking Buffer + 5ul of Primary Antibody (1:1000, AbCam CRFR2)
    b. 5mL is a good volume for a sealed bag.
6. Add membrane to plastic bag
7. Add 5mL of Primary Antibody in Blocking Buffer
8. Ensure minimal air-bubbles
9. Seal with Plastic Bag sealer (in Ressler Lab)
10. Let incubate in Primary Antibody Solution Bag while rocking at 4C *Overnight*
    a. START DATE | TIME:

DAY 2

WESTERN BLOTTING > SECONDARY TREATMENT AND EXPOSURE

1. With scissors, cut open bag over sink
2. Move membrane to a petri dish with Blocking Buffer
3. Wash membrane in Blocking Buffer 3 times for *5 min each* (*15 min total*)
4. While washing, dilute secondary antibody in Blocking Buffer to desired concentration:
    a. E.g., 5mL of Blocking Buffer + 10ul of Secondary Antibody (1:500, XXXX)
    b. 5mL is a good volume for a sealed bag.
5. After washes, move membrane to bag (or petri dish) with Secondary Antibody
6. Let membrane incubate in Secondary Antibody Solution for *1-2 hrs*
7. When Secondary Antibody incubation is complete, move membrane to a petri dish
8. Wash membrane 2 times in Blocking Buffer for *5 min each* (*10 min total*)
9. Wash membrane 1 time in 1X PBS for *5 min*
10. Get 15mL Conical Tube
11. Get a Transparency sheet and lay it on a lab bench
12. Just before membrane finishes with 1X PBS wash, mix Pierce SuperSignal West Pico Chemiluminescent Substrates together in a 1:1 ratio in a 15mL Conical Tube
    a. E.g., for 1 membrane, add 600ul of Reagent A and 600ul of Reagent B
13. Take membrane out of 1X PBS and touch membrane corner to paper-towel
14. Place membrane on transparency sheet, dark-band-side up.
15. Add Chemiluminescence solution
16. Cover membrane completely with Chemiluminescence solution, let sit at RT for *5 min*
17. While membrane sits, do the following:
    a. Get a piece of saran wrap and lay completely flat near the membrane
    b. Ensure that the Alpha Innotech camera is on
    c. Put whitish plate down
    d. Insert black target board
    e. Put camera on .95 based on white-line
    f. Click the luminescence to 1
    g. Insert a piece paper with text and focus using the top nob
    h. Open Alpha Innotech program on the computer
    i. Set sensitivity to medium/medium
    j. Unclick "Auto-exposure
18. After 5 min with Chemiluminescence solution, lift membrane with tweezers, touch corner to paper-towel, and lay flat on saran wrap (Chemiluminescence side up)
19. Cover upper surface of membrane with remaining saran wrap and make sure saran wrap is completely flat
20. Move saran-wrap-covered membrane to Alpha Innotech imager
21. [AVOID LIGHT EXPOSURE BY CLOSING THE IMAGER DOOR]

22. Use a short (3-5 sec) exposure to get a sense of how straight the membrane is (getting it straight at the start makes manipulating the photos later easier)
23. Make sure all light sources within the imager are OFF
24. Expose serially
    a. 00:30 sec
    b. 01:00 min
    c. Then judge
25. Save each file from each different exposure--make sure you get a really nice image and make sure you save the the modified version (under the File menu), since the program does some autoadjusting that won't be saved if just saving normally.
26. Now get an image of the standard while maintaining the membrane in exactly the same position:
    a. After acquiring the perfect image(s), open door a very little bit (unclick and squeeze finger between door and door brace)
    b. Change the exposure to 1 sec
    c. Acquire
    d. Change exposure if necessary
    e. Save multiple "Standard Band" files
    f. Close door completely
27. Use the Overlay Function (button)
    a. Add the "Standard Band" file to the red
    b. Add the Western band file to the green
    c. Adjust images appropriately so there is high-contrast
    d. Save modified image as a new file
28. Once you think you have a good (publishable) set of images, proceed immediately to Stripping (there is Pause Point below (*35 min. from now*), if pressed for time).

WESTERN BLOTTING > STRIPPING AND LOADING CONTROL PRIMARY ANTIBODY

1. Wear gloves
2. Find Pierce Stripping Buffer (above Kim's bench or above Meera's bench)
3. Remove the membrane from the Alpha Innotech imager
4. Remove saran wrap
5. Put membrane in a square petri dish
6. Add 10mL of Pierce Stripping Buffer
7. Let rock at RT for *15 min*
8. Remove stripping buffer
9. Add 1x PBS
10. Wash in 1x PBS for *15 min* (prevents the milk in the Blocking Buffer from curdling)
11. *[Here is a **Pause Point**: can leave membrane in fresh 1x PBS overnight or proceed to next step]*

12. Remove 1x PBS and add Blocking Buffer; set rock at RT for *30 - 60 min*
13. While blocking, make Loading Control (Beta-Actin or GAPDH) Primary Antibody Solution
    a. E.g., 5mL of Blocking Buffer + 1ul of Primary Antibody (1:5000, GAPDH)
    b. 5mL is a good volume for a sealed bag.
14. Add membrane to plastic bag
15. Add 5mL of Primary Antibody in Blocking Buffer
16. Ensure minimal air-bubbles
17. Seal with Plastic Bag sealer (in Ressler Lab)
18. Let incubate in Primary Antibody Solution Bag:
    a. If early, you can let this rock at 4C *1.5 hrs*
    b. If late, you can let this rock at 4C *Overnight*
    c. START DATE | TIME:

WESTERN BLOTTING > LOADING CONTROL SECONDARY TREATMENT AND EXPOSURE

1. With scissors, cut open bag over sink
2. Move membrane to a petri dish with Blocking Buffer
3. Wash membrane in Blocking Buffer 3 times for *5 min each* (*15 min total*)
4. While washing, dilute secondary antibody in Blocking Buffer to desired concentration:
    a. E.g., 5mL of Blocking Buffer + 10ul of Secondary Antibody (1:500, Anti-Mouse)
    b. 5mL is a good volume for a sealed bag.
5. After washes, move membrane to bag (or petri dish) with Secondary Antibody
6. Let membrane incubate in Secondary Antibody Solution for *1-1.5 hrs*
7. When Secondary Antibody incubation is complete, move membrane to a petri dish
8. Wash membrane 2 times in Blocking Buffer for *5 min each* (*10 min total*)
9. Wash membrane 1 time in 1X PBS for *5 min*
10. Get 15mL Conical Tube
11. Get a Transparency sheet and lay it on a lab bench
12. Just before membrane finishes with 1X PBS wash, mix Pierce SuperSignal West Pico Chemiluminescent Substrates together in a 1:1 ratio in a 15mL Conical Tube
    a. E.g., for 1 membrane, add 600ul of Reagent A and 600ul of Reagent B
13. Take membrane out of 1X PBS and touch membrane corner to paper-towel
14. Place membrane on transparency sheet, dark-band-side up.
15. Add Chemiluminescence solution
16. Cover membrane completely with Chemiluminescence solution, let sit at RT for *5 min*
17. While membrane sits, do the following:
    a. Get a piece of saran wrap and lay completely flat near the membrane

   b. Ensure that the Alpha Innotech camera is on
   c. Put whitish plate down
   d. Insert black target board
   e. Put camera on .95 based on white-line
   f. Click the luminescence to 1
   g. Insert a piece paper with text and focus using the top nob
   h. Open Alpha Innotech program on the computer
   i. Set sensitivity to medium/medium
   j. Unclick "Auto-exposure
18. After 5 min with Chemiluminescence solution, lift membrane with tweezers, touch corner to paper-towel, and lay flat on saran wrap (Chemiluminescence side up)
19. Cover upper surface of membrane with remaining saran wrap and make sure saran wrap is completely flat
20. Move saran-wrap-covered membrane to Alpha Innotech imager
21. [AVOID LIGHT EXPOSURE BY CLOSING THE IMAGER DOOR]
22. Use a short (3-5 sec) exposure to get a sense of how straight the membrane is (getting it straight at the start makes manipulating the photos later easier)
23. Make sure all light sources within the imager are OFF
24. Expose serially
   a. 00:05 sec, 00:10 sec, 00:20 sec, 00:30 sec, 01:30 min
25. Save each file from each different exposure--make sure you get a really nice image and make sure you save the the modified version (under the File menu), since the program does some autoadjusting that won't be saved if just saving normally.
26. Now get an image of the standard while maintaining the membrane in exactly the same position:
   a. After acquiring the perfect image(s), open door a very little bit (unclick and squeeze finger between door and door brace)
   b. Change the exposure to 1 sec
   c. Acquire
   d. Change exposure if necessary
   e. Save multiple "Standard Band" files
   f. Close door completely
27. Use the Overlay Function (button)
   a. Add the "Standard Band" file to the red
   b. Add the Western band file to the green
   c. Adjust images appropriately so there is high-contrast
   d. Save modified image as a new file
28. Save membranes in saran wrap for Standard Band reference (just in case)
29. Process all Western Images for Publication-quality figures

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