OPIOID REGULATION OF PAIR BONDING IN THE SOCIALLY MONOGAMOUS PRAIRIE VOLE

by

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DEDICATION

This dissertation is dedicated to my loving father,

Ivan Harkey.

Without his guidance and continued support,

my sisters and I would not have achieved our goals

and (sometimes crazy) dreams that we set out to accomplish.

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CHAPTER 1
INTRODUCTION

GENERAL INTRODUCTION

Humans have an extraordinary propensity for social bonding that is quite rare among mammals (Kleiman, 1977). The occurrence of this rare social trait within our species (as well as other cooperatively breeding species) serves a very important evolutionary purpose and that is to facilitate mating and the successful rearing of offspring (Kleiman, 1977). This cooperative breeding strategy is theorized to be particularly advantageous under harsh environments where resources are scarce and predation risks are high, similar to the environment in which early humans evolved (Dunbar & Shultz, 2007; Gowlett, 2009; James, 2009). Additionally, human offspring have long periods of development and under the environmental conditions described above, two parents may have been required to raise the offspring: one to protect the offspring and one to gather food for nourishment (Traulsen & Nowak, 2006; Nowak *et al.*, 2010; Shultz *et al.*, 2011). Thus, to keep mating pairs together, social monogamy evolved.

Under environmental conditions where social monogamy is adaptive, selection will act on behavioral traits characteristic of this breeding strategy, such

as biparental care and mate guarding, thereby increasing the number of animals displaying behaviors representative of cooperative breeding (Darwin, 1859). Moreover, if a monogamous social strategy has an adaptive advantage within a given species, than selection should wire the brains of individuals to exhibit a high propensity for social bonding (Tinbergen, 1953). To understand the biological mechanisms underlying social bonding, and, ultimately, human behavior, we need an animal model that is capable of both forming and maintaining social bonds. The socially monogamous prairie voles (*Michrotus ochrogaster*) is such a species and permits an understanding of neural mechanisms of pair bonding (Insel *et al.*, 1998) (Carter & Keverne, 2002; Young & Wang, 2004; Young *et al.*, 2005; Aragona & Wang, 2009).

The Prairie Vole as an Animal Model of Social Bonding

Unlike most mammalian species, prairie voles form species-typical pair bonds that are characterized by the sharing of a home territory, biparental care, and mate guarding (Getz *et al.*, 1981; Getz *et al.*, 1993). Importantly, ecological studies of prairie vole social behavior have provided a great deal of information regarding the environmental conditions that have lead to the evolution of a monogamous mating system in this species (Jameson, 1947; Getz *et al.*, 1987). Knowledge regarding the social behavior of prairie voles in nature has been utilized to develop laboratory measures of pair bonding that can be broken down into two distinct phases, pair bond formation and maintenance.

Breaking pair bonding down into stages of formation and maintenance is important because they are associated with dramatically different social

behaviors: specifically, pair bond formation is associated with affiliative social behaviors, while pair bond maintenance is associated with aggressive social encounters (discussed below) (Aragona & Wang, 2009). To study the neural mechanisms that mediate pair bond formation and maintenance, two behavioral assays have been developed, tests of partner preference and selective aggression, respectively (Carter & Getz, 1993). These studies can be combined with pharmacological, anatomical, as well as genetic techniques to determine the underlying neural mechanisms that mediate these behaviors (Carter *et al.*, 1995; Carter *et al.*, 1997; Young *et al.*, 1998; Young *et al.*, 2008; Aragona & Wang, 2009) and, importantly, perhaps the neural mechanisms that mediate bond formation and maintenance in our own species (Insel, 2003; 2010).

SOCIAL BONDING AND NEURAL MECHANISMS OF REWARD

Previous studies examining the neural mechanism that mediate pair bonding in prairie voles have identified that neural mechanism that are important for the processing of primary rewards are also important for pair bonding (Aragona & Wang, 2009). More specifically, primary rewards, such as food, sex, and water, are all components of the environment that are necessary for species survival (Marks, 2011). As such, mechanisms that promote the expression of reward seeking behaviors have evolved (Kringelbach, 2010; Kringelbach & Berridge, 2010; Smith *et al.*, 2010). These mechanisms include the induction of a positive affective state upon experiencing the sensory components of a reward, such as the pleasure associated with eating a highly palatable food item or the

comfort felt in the presence of close friends (Thorndike, 1911; Cabanac, 1971; Richard *et al.*, 2013), that acts to reinforce reward-seeking behavior. Moreover, learned associations between reward cues and the induction of a positive affective state can result in cues that predict reward to also gain motivational value and promote reward seeking behaviors (Berridge & Kringelbach, 2008; Dickinson & Balleine, 2010; Watson *et al.*, 2010). Thus, reward-seeking behavior involves the integration of positive hedonics, learning, and motivation (Berridge & Robinson, 2003). Importantly, social bond formation is hypothesized to utilize similar mechanisms of reward learning to positively reinforce social interactions with the partner and, consequently, to motivate the maintenance of proximity with the partner (Dunbar, 2012).

While the formation of social bonds is hypothesized to be regulated by he association of a positive hedonic state with partner contact (Bowler *et al.*, 2002; Aragona & Wang, 2009; Burkett *et al.*, 2011; Dunbar, 2012), the maintenance of a social bond requires that a threat to the social relationship to be perceived as aversive and motivate behaviors that protect the initial bond (Resendez *et al.*, 2012). For example, a critical aspect of pair bond maintenance in monogamous mammals is mate guarding (i.e., preventing access to the mate by aggressively defending the mate and or/territory). This behavior acts to maintain the pair bond in two ways: 1.) by preventing the partner from engaging in extra-pair copulations and 2.) by denying the partner the opportunity to form a bond with a novel mating partner (Carter & Getz, 1993). Importantly, in prairie voles, this type of aggressive behavior only emerges after the establishment of a pair bond

as sexually naïve prairie voles will not only approach, but will engage in affiliative social interactions with novel social stimuli of both sexes (Carter & Getz, 1993). Thus, the establishment of a pair bond dramatically transitions social behavior directed at novel social stimuli from approach and engage to avoidance through aggressive rejection. This transition is theorized to occur by translating the valence of novel social stimuli from rewarding to aversive (Resendez & Aragona, 2013).

In addition to processing novel social stimuli as aversive, pair bond maintenance requires that the absence of the partner induce a negative affective state that acts to motivate partner reunion (Bosch *et al.*, 2009; Cacioppo *et al.*, 2011). Therefore, while the presence of an attachment figure (i.e. a mother-infant bond or a mating partner) induces feelings of pleasure and comfort that promote proximity to the partner (Cacioppo *et al.*, 2004), the absence of an attachment figure is associated with a state of psychological distress and emotional pain that motivates contact seeking behavior (Panksepp, 2003; Cacioppo *et al.*, 2011; Eisenberger, 2012). Together, a balance between positive and negative hedonic states acts to reinforce positive social interactions between potential mating partners in sexually naive prairie voles and negatively reinforce interactions with this same social stimulus following the establishment of a pair bond.

POSITIVE AFFECT AND INITIAL PAIR BOND FORMATION IN PRAIRIE VOLES

Social Behaviors Associated with Pair Bond Formation

Pair bond formation is associated with positive social behaviors and requires the willingness to approach potential mating partners. Specifically, sexually naïve prairie voles are reproductively suppressed within their natal group and must first leave their natal nest to begin the mating process (Thomas & Birney, 1979; DeVries *et al.*, 1996). Upon leaving the natal nest, a chance encounter with an unmated opposite-sex conspecific may spark the appropriate social interactions to ignite the initial development of a pair bond (Resendez & Aragona, 2013). These interactions begin with approach toward a novel individual and are followed by olfactory investigation, the latter of which is usually initiated by the female (Gavish *et al.*, 1983). However, these initial social interactions are only the first steps in the development of a pair bond and the induction of mating behavior. This is because female prairie voles require the presence of a male to initiate an estrous cycle and they are induced ovulators, requiring extended periods of contact with a male to become sexually receptive (Carter *et al.*, 1980). Specifically, in nature, female prairie voles require 24-48 hours of exposure to a male to induce behavioral estrus (Shapiro & Dewsbury, 1990; Williams *et al.*, 1992a). In other words, following the initial meeting of a mating partner, male and female pairs must remain together for at least 48 hours before the mating process can begin.

Remaining in contact with a mating partner may seem may like an easy and enjoyable task to a naturally social species such as ourselves, but the propensity for social contact is actually quite rare among mammalian species

(Carter *et al.*, 1992). For example, the closely related nonmonagmous meadow vole actively avoids (often aggressively) contact with novel individuals (Getz, 1962). When placed in a chamber together, meadow voles will occupy opposite corners of the enclosure as to maintain the maximum amount of distance, while prairie voles readily approach new individuals and eventually engage in side-by-side contact (Aragona *et al.*, 2006). Moreover, vole species that do not engage in affiliative contact with conspecifics do not form pair bonds suggesting that a propensity for social contact is a critical component of initial pair bond formation. Indeed, in nature, newly formed pairs of prairie voles spend a great deal of time in contact with one another (Thomas & Birney, 1979). Given that female prairie voles require extended periods of contact with a male to become sexually receptive (Carter *et al.*, 1980), this attraction to the partner and high propensity for affiliation may serve an important reproductive purpose, and that is to keep the pair together long enough to induce estrus in the female.

Social Reward Processing and Pair Bond Formation

In general, for an environmental stimulus to elicit approach or reliably evoke seeking behavior, it must be processed as rewarding (Schultz, 2006). As mentioned above, the computation of reward within the brain requires the integration of multiple psychological constructs (Berridge & Robinson, 2003). Specifically, for a stimulus to be rewarding the sensory processing of the stimulus must induce a positive affective state, have attractive motivational value (i.e., elicit approach behavior), and an animal must be able to learn associations between cues that predict the reward and the reward itself (Berridge & Robinson,

2003). In other words, objects that are rewarding are those that have some primary reinforcing in that they induce a positive affective state and the learned association between positive affect and the reward promotes future seeking behavior (Thorndike, 1911; Dickinson & Balleine, 1994). Environmental stimuli that serve as primary reinforcers are those that play an integral role in the survival of a species, such as food, water, sex, and shelter (Schultz, 2006) and one method that is commonly used to test the reward value of such stimuli is the conditioned place preference test (Bardo & Bevins, 2000; Tzschentke, 2007).

In the conditioned place preference paradigm, a rewarding stimulus serves as an unconditioned stimulus and is repeatedly paired with a discrete contextual environment (Bardo & Bevins, 2000). Conditioning sessions are usually carried out in a two-chambered apparatus in which the environmental cues of one chamber or paired with the rewarding (or unconditioned) stimulus, while the environmental cues of the other chamber or paired with a neutral stimulus. Over the course of conditioning, the repeated pairing of the of the unconditioned rewarding stimulus with a previously neutral set of environmental cues results in the environment acquiring secondary rewarding values that can act as a conditioned stimulus and elicit approach behaviors. In contrast, the repeated pairing of a neutral stimulus with the neutral cues of the other chamber does not elicit reward-seeking behavior. On the test day, the animal is given the choice to spend time in either the reward paired environment or the neutral environment (Tzschentke, 2007). If the unconditioned stimulus was indeed rewarding, the animal will prefer to spend more time in the reward-paired

environment indicating the development of a conditioned place preference (Bardo & Bevins, 2000; Tzschentke, 2007). Interestingly, a similar paradigm has been developed in the study of pair bond formation and is referred to as the partner preference test (Williams *et al.*, 1992a). Moreover, this paradigm has been used to identify components of reward circuitry that mediate pair bond formation suggesting the social attachments formation is indeed a form of reward learning (Insel, 2003).

Similar to the conditioned place preference paradigm, the partner preference test entails placing the test subject (male or female) in a three-chamber apparatus where they have the choice to spend time in contact with an opposite-sex conspecific that they have previously cohabitated with, referred to as the partner, or a novel opposite-sex conspecific, referred to as the stranger (Williams *et al.*, 1992b). While most mammals would choose a novel mating partner (i.e., the stranger), monogamous species will prefer to spend more time in contact with the partner that have previously mated with (Shapiro & Dewsbury, 1990). This rare occurrence among mammals requires an affinity for contact with the partner and is quantified by measuring the duration of time spent in contact with the partner as well as the stranger (Williams *et al.*, 1992a). A greater amount of time spent in contact with the partner over the stranger is referred to as a partner preference and suggest that socially monogamous species find their original mating partners to be more rewarding than a novel mating partner (Insel, 2003). Importantly, this preference is theorized to be the earliest behavioral indicator of pair bond formation (Insel & Hulihan, 1995) and is mediated by

reward processing regions of the brain (Aragona & Wang, 2009; Burkett & Young, 2012; Resendez & Aragona, 2013).

As discussed above, reward processing in the brain requires integration of multiple psychological constructs, such as learning, hedonics, and motivation, (Berridge & Robinson, 2003) and this integration occurs through interactions between neural circuits that in part regulate each of these constructs (Resendez & Aragona, 2013). For example, the initial development of a pair bond requires first the motivation to approach a potential mating partner and second the desire to remain in contact with, or actively seek out, this individual. In general approach and seeking behaviors are regulated by motivational circuitry (Weeks, 1962; Bozarth & Wise, 1981) and behavioral pharmacology studies of pair bond formation have indicated that neural regulators of motivation, such as dopamine within the mesocorticolimbic circuit (Ikemoto & Panksepp, 1999; Lammel *et al.*, 2011) is important for the initial formation of a bond (Young *et al.*, 2008; Aragona & Wang, 2009; Young *et al.*, 2011).

Motivational Systems and Pair Bond Formation

The mesocorticolimbic dopamine system is important for reward processing and the generation of motivated behavior (Berridge & Robinson, 1998; Kelley & Berridge, 2002; Everitt & Robbins, 2005). The release of dopamine within this circuit activates two classes of dopamine receptors, the low-affinity D2-like dopamine receptors (i.e., D2, D3, D4) or the high affinity D1-like dopamine receptors (i.e., D1, D5) (Richfield *et al.*, 1989). Low levels of dopamine release preferentially activate the low affinity D2-like class of

dopamine receptors, while D1-like receptors require high levels of dopamine release to be activated, such as that which occurs during burst firing of dopamine neurons (Richfield *et al.*, 1989). Interestingly, peripheral blockade of D2-like receptors, but not D1-like receptors, inhibits the development of mating induced partner preferences in both sexes (Wang *et al.*, 1999; Aragona *et al.*, 2003). Moreover, activation of D2-like receptors induces partner preference formation in the absence of mating (Wang *et al.*, 1999). Together, these data suggest that low levels of dopamine release that preferentially activate D2-like receptors are important for pair bond formation.

Given that the nucleus accumbens is an important brain region for dopaminergic regulation over reward (DiChiara *et al.*, 2004), it was hypothesized that activation of D2-like receptors within this brain region would be important for pair bond formation. Indeed, blockade of D2-like receptors within the nucleus accumbens, but not the medial prefrontal cortex, blocked the formation of mating-induced partner preferences in female prairie voles (Gingrich *et al.*, 2000) indicating that D2-mediation of partner preferences is specific to the nucleus accumbens. Furthermore, administration of low, but not high, doses of the non-selective dopamine agonist, apomorphine, into the nucleus accumbens induced pair bond formation in the absence of mating (Aragona *et al.*, 2003). Importantly, low doses of apomorphine preferentially activate D2-like receptors (Missale *et al.*, 1998) suggesting that dopaminergic regulation of partner preference formation is the result of activation of D2-like receptors within the nucleus accumbens.

The nucleus accumbens is composed of two compartments, the core and shell and each of these compartments has a distinct role in reward processing (Heimer *et al.*, 1991; Zahm, 2000). Specifically, dopamine within the nucleus accumbens shell is important in the processing of unconditioned salient stimuli, while dopamine transmission within the nucleus accumbens core is important for the processing of cues that predict reward (i.e. learned associations between rewards and reward predictive stimuli). This regional specificity has been demonstrated by the ability of both positively and negatively valenced stimuli to preferentially increase dopamine release within the nucleus accumbens shell in the absence of prior conditioning (Kalivas & Duffy, 1995; Aragona *et al.*, 2009). In other words, the first time exposure to a salient stimulus can enhance dopamine transmission within the nucleus accumbens shell resulting in the generation of motivated behavior (Ikemoto & Panksepp, 1999). In contrast, discriminative reward predicative cues preferentially enhance dopamine release within the nucleus core and this increase occurs only after conditioning (Aragona *et al.*, 2009). Moreover, lesions of the nucleus accumbens core attenuate approach behavior toward reward predictive cues (Parkinson *et al.*, 1999), while the release of dopamine within this region is positively correlated with investigation of a reward predicative stimulus (Uslaner *et al.*, 2006). Thus, dopamine within each region of the nucleus accumbens has different roles in reward processing with nucleus accumbens shell dopamine primarily mediating responses to unconditioned stimuli and nucleus accumbens core dopamine primarily mediating responses to conditioned stimuli.

In addition to compartments within the nucleus accumbens playing distinct roles in general motivated behavior, the role of nucleus accumbens dopamine in pair bond formation also has regional specificity (Aragona & Wang, 2009). Specifically, site-specific administration of a D2-like receptor agonist into the nucleus accumbens shell, but not the core, induces partner preference formation in the absence of mating (Aragona *et al.*, 2006). Moreover, this effect is specific to the rostral portion of the nucleus accumbens shell as activation of D2-like receptors in caudal regions failed to induce pair bond formation (Aragona *et al.*, 2006) suggesting that mating induced partner preferences are mediated by low levels of dopamine release that preferentially activate D2-like dopamine receptors specifically within the rostral nucleus accumbens shell. The specificity of the nucleus accumbens shell is significant as dopamine within this region mediates the rewarding properties of unconditioned primary rewards (Di Chiara & Bassareo, 2007; Ikemoto, 2007; Aragona *et al.*, 2008), while the nucleus accumbens core is important for conditioned motivated behaviors, such as approach towards a learned reward predicting cue (Aragona *et al.*, 2009). Given that an encounter with a potential mating partner cannot be predicted and requires the propensity to respond without prior conditioning, it makes sense that dopaminergic mediation of pair bond formation is specific to the nucleus accumbens shell.

As described above, pair bond formation is induced by the activation of D2-like dopamine receptors that, under natural conditions, occurs as a result of extended periods of contact and mating with a potential bonding partner.

Interestingly, in estrous females, the presence of a male induces a 30% rise in baseline dopamine levels within the nucleus accumbens, but only a 17% increase within the nucleus accumbens of females that are not sexually receptive (Gingrich *et al.*, 2000). Perhaps the more robust increase following mating accounts for the facilitation of partner preference formation by mating (i.e., partner preferences formed under shorter cohabitation periods), while the small increases in dopamine induced solely by cohabitation may reflect initial increases in dopamine that occur under natural conditions upon the first meeting of a potential mating partner. Additionally, it is also possible that the presence of a male may have greater motivational value to a sexually receptive female and thus account for the greater level of dopamine release. Nonetheless, these data provide convincing evidence that a surge in dopamine release and the consequent activation of D2-like receptors within the rostral nucleus accumbens shell is required for pair bond formation. Given that mating induced increases in dopamine is rewarding (Everitt, 1990; Mermelstein & Becker, 1995; Pfaus, 2009), it is theorized that this increase in dopamine partially accounts for the processing of a mate as rewarding and consequently, pair bond formation.

Positive Hedonics and Pair Bond Formation

However, the mesolimbic dopamine system only mediates motivational (Berridge & Robinson, 1998) and learning (Schultz, 2000) aspects of reward and does not account for the hedonic component (Richard *et al.*, 2013). Instead, the positive hedonic aspects of reward are mediated by the activation of mu-opioid receptors within discrete regions of motivational circuitry, specifically, the ventral

pallidum and the rostral dorso-medial nucleus shell of the nucleus accumbens (Smith & Berridge, 2007). During reward consumption (e.g., eating a palatable food item), activation of mu-opioid receptors specifically within these brain regions induces a positive affective state that is important for reinforcing adaptive behaviors, including those of a social nature (Aldridge & Berridge, 2010; Cabanac, 2010; Dickinson & Balleine, 2010; Frijda, 2010; Komisaruk *et al.*, 2010; Kringelbach, 2010). For example, activation of mu-opioid receptors within motivational circuitry mediates the rewarding aspects of social affiliation and mating (Panksepp *et al.*, 1980; Szechtman *et al.*, 1981; Keverne *et al.*, 1989; Shapiro *et al.*, 1989; Stein *et al.*, 2007; Curley, 2011; Trezza *et al.*, 2011)—two behaviors that are important during the early stages of pair bond formation. Given that mu-opioid receptors mediate positive hedonics as well as affiliative social interactions, it was hypothesized that activation of these receptors would be important for pair bond formation.

Affiliation and mating contribute to pair bond formation and activation of mu-opioid receptors is required for both of these behaviors (Panksepp *et al.*, 1980; Szechtman *et al.*, 1981). Specifically, peripheral administration of a mu-opioid receptor agonist increases affiliation in sexually naïve prairie voles (Shapiro & Dewsbury, 1990) and blockade of these receptors decreases the frequency of mating bouts as well as inhibits pair bond formation (Burkett *et al.*, 2011). Given that the hedonic component of reward is mediated by mu-opioid receptors within the nucleus accumbens (Pecina & Berridge, 2000; Pecina *et al.*, 2006) it was hypothesize that mu-opioid receptors within this region would be

important for pair bond formation. However, blockade mu-opioid receptors within the nucleus accumbens failed to inhibit pair bond formation, while blockade of mu-opioid receptors within the dorsal striatum that do not mediate positive hedonics did inhibit pair bond formation (Burkett *et al.*, 2011). From these data, it was concluded that mu-opioid receptors within the nucleus accumbens, and, therefore, those that mediate positive hedonics, are not important for pair bonding.

The nucleus accumbens shell is a heterogeneous region (Reynolds & Berridge, 2002) and this heterogeneity is partially due to the anatomical and functional diversity of mu-opioid receptors within this region (Pecina & Berridge, 2005). Within the nucleus accumbens shell, mu-opioid receptors are densely expressed within the dorso-medial area of the nucleus accumbens (Mansour *et al.*, 1987; Voorn *et al.*, 1996) and activation of this patch of receptors mediates positive hedonics (Pecina *et al.*, 2006), while activation of mu-opioid receptors throughout the entire striatum (dorsal and ventral) mediates appetitive motivation (Difeliceantonio *et al.*, 2012). A similar binding pattern of mu-opioid receptors is found within the nucleus accumbens shell of prairie voles (Burkett *et al.*, 2011) suggesting that a similar functional architecture of mu-opioid receptor regulation of motivation and hedonics may occur within this species.

Blockade of mu-opioid receptors within the nucleus accumbens shell was previously shown to not affect partner preference formation (Burkett *et al.*, 2011). However, examination of the injections sites from this study shows that the injections were made within the ventral nucleus accumbens shell (Burkett *et al.*,

2011), a region of the shell where mu-opioid receptors mediate motivation, but not positive hedonics (Pecina & Berridge, 2005). Additionally, examination of the partner preference data reveals that subjects treated with a mu-opioid receptor antagonist spent more time in contact with the stranger than control subjects (Burkett *et al.*, 2011) suggesting that blockade of mu-opioid receptors within the ventral nucleus shell had a small non-significant effect on partner preference formation, perhaps due to blockade of a small percent of mu-opioid receptors within the dorso-medial nucleus shell. Together, these data suggest that precise targeting of mu-opioid receptors within the dorso-medial shell may have a more robust effect on partner preference formation; however, more studies are necessary to determine if mu-opioid receptors that mediate positive hedonics are important for pair bond formation.

In summary, previous data have demonstrated that neural regulators of motivated behavior are important for pair bond formation (Aragona & Wang, 2009; Burkett & Young, 2012) and we suggest that neural regulators of positive hedonics are also involved. Together, motivational and hedonic processing systems likely interact to reinforce social interactions with a mating partner and therefore promote continuous proximity to the partner (Leknes & Tracey, 2010). A high motivation to remain in contact with the partner is especially important during the early stages of social attachment formation (Lim & Young, 2006), when positive hedonics associated with the attachment figure are typically at their highest (Kringelbach, 2010).

AVERSIVE MOTIVATION AND THE MAINTENANCE OF SOCIAL ATTACHMENTS

Social Behaviors Associated with Pair Bond Maintenance

In contrast to the early stages of pair bond formation, pair bond maintenance is associated with aversive social encounters, such as the aggressive rejection of novel conspecifics (Getz *et al.*, 1981; Aragona *et al.*, 2006; Aragona & Wang, 2009). These aversive social encounters are critical to pair bond maintenance because they act to guard the mate and the territory, which also prevents the formation of a new pair bond (Getz *et al.*, 1993; Aragona *et al.*, 2006). Therefore, following the formation of a pair bond, the previous approach behavior and potentially rewarding impact upon encountering a novel social stimulus is not only attenuated, but the valence is switched. Now, instead of the social stimulus inducing a positive affective state that reinforces proximity, it induces an aversive state resulting in the same social stimulus to be aggressively rejected.

Selective aggression occurs in both sexes (Bowler *et al.*, 2002) and is studied in the laboratory with a resident-intruder test. In general, this procedure entails pairing a sexually naïve male and female for two-weeks of cohabitation (Winslow *et al.*, 1993; Wang *et al.*, 1997; Aragona *et al.*, 2006; Gobrogge *et al.*, 2007; Gobrogge *et al.*, 2009; Resendez *et al.*, 2012). During this period, the female will be induced into estrus and mating will begin about three days into the pairing period (Williams *et al.*, 1992a). The pair will continue to live in this

environment and establish a 'home territory' that they actively defend, similar to territory defense behaviors seen in the wild (Getz, 1962). After two-weeks of cohabitation, the resident-intruder test is conducted by removing the partner (male or female) from the cage and placing an intruder into the pair's home cage. Behavioral interactions with the intruder are than recorded for a period of 5-10 minutes and scored for aggressive (lunge, bites, attacks, offensive rearing, and chasing) and affiliative (sniffing and side-by side contact) social interactions (Aragona *et al.*, 2006; Gobrogge & Wang, 2011). High levels of aggression as well as low levels of affiliation with an intruder indicate the establishment of a pair bond.

Similar to tests of pair bond formation, tests of selective aggression can be combined with pharmacological and anatomical techniques to identify the neural mechanisms underlying selective aggression and therefore pair bond maintenance (Young *et al.*, 2008; Aragona *et al.*, 2009). We will discuss the known mechanisms below, however, it is important to note, that while many studies have focused on the neurobiology of pair bond formation (Williams *et al.*, 1992a; Williams *et al.*, 1992b; Winslow *et al.*, 1993; DeVries *et al.*, 1995; Insel & Hulihan, 1995; DeVries *et al.*, 1996; Bilbo *et al.*, 1999; Cho *et al.*, 1999; Wang *et al.*, 1999; Cushing & Carter, 2000; Gingrich *et al.*, 2000; Curtis *et al.*, 2001; Liu *et al.*, 2001; Pitkow *et al.*, 2001; DeVries *et al.*, 2002; Aragona *et al.*, 2003; Cushing *et al.*, 2003; Liu & Wang, 2003; Lim & Young, 2004; Curtis & Wang, 2005a; b; Aragona *et al.*, 2006; Aragona & Wang, 2007; Bales *et al.*, 2007; Lim *et al.*, 2007; Ahern *et al.*, 2009; Ahern & Young, 2009; Ross *et al.*, 2009a; Ross *et al.*, 2009b;

Liu *et al.*, 2010; Burkett *et al.*, 2011), far fewer have examined the neural mechanisms that mediate pair bond maintenance (Aragona *et al.*, 2006; Gobrogge *et al.*, 2009). Additionally, although both sexes exhibit selective aggression (Bowler *et al.*, 2002), most studies have focused on males resulting in very little information on neural mechanisms that mediate pair bond maintenance in females.

Motivational Systems and Pair Bond Maintenance

Selective aggression can be described as an aversive motivated behavior and activation of dopamine receptors is also required for the expression of this behavior. However, selective aggression is mediated by a different class of dopamine receptors than those that mediate pair bond formation (Aragona & Wang, 2009). Specifically, pair bond maintenance is mediated by D1-like receptors within the nucleus accumbens as blockade of these receptors prevents the aggressive rejection of novel conspecifics and activation of D1-like receptors within the nucleus accumbens of sexually naïve males prevents pair bond formation (Aragona *et al.*, 2006). Additionally, D1-like receptors become up regulated within the nucleus accumbens, but not the dorsal striatum, following the establishment of a pair bond (Aragona *et al.*, 2006). Importantly, this up-regulation acts to maintain the pair bond by preventing affiliative social interactions with potential mating partners as well as the establishment of a second partner preference (Aragona *et al.*, 2009).

While D2-like receptors that mediate pair bond formation are activated by low-levels of dopamine release, D1-like receptors are the low-affinity type

receptor and require high levels of dopamine release, such as that which occurs during burst firing, to be activated (Richfield *et al.*, 1989). It is therefore hypothesized that the presence of an intruder evokes high levels of dopamine release within the nucleus shell resulting in activation of D1-like receptors (Aragona & Wang, 2009). D1-like receptors are expressed on medium spiny neurons that contain dynorphin, the endogenous ligand for kappa-opioid receptors (Chavkin *et al.*, 1982) and activation of D_1-like receptors increases expression and release of dynorphin (Gerfen *et al.*, 1991; Carlezon *et al.*, 1998; Tejeda *et al.*, 2012). We therefore hypothesized that D_1-like and kappa-opioid receptors interact to mediate bond maintenance.

Negative Hedonics and Pair Bond Maintenance

In contrast to mu-opioid receptors, activation of kappa-opioid receptors is associated with aversion, negative affect, and the attenuation of reward (Mucha & Herz, 1985; Shippenberg & Herz, 1986; Di Chiara & Imperato, 1988; Bals-Kubik *et al.*, 1989; Spanagel *et al.*, 1990; Bals-Kubik *et al.*, 1993; Heidbreder *et al.*, 1993; Maisonneuve *et al.*, 1994; Carlezon *et al.*, 1998; Shirayama *et al.*, 2004; Todtenkopf *et al.*, 2004; Carlezon *et al.*, 2006; McLaughlin *et al.*, 2006; Bruchas *et al.*, 2007; Land *et al.*, 2009; Ebner *et al.*, 2010; Smith *et al.*, 2012). Moreover, aversive stimuli activate kappa-opioid receptors specifically within the nucleus accumbens (Land *et al.*, 2009) and activation of this population of kappa-opioid receptors induces conditioned place aversions (Mucha & Herz, 1985) as well as attenuates the perceived value of previously rewarding stimuli (Shippenberg & Herz, 1986)—similar hedonic states that are hypothesized to be

important for pair bond maintenance (Resendez & Aragona, 2013). Specifically, pair bond maintenance requires that the reward value of other potential mating partners is attenuated as well as social threats to the pair bond are processed as aversive and subsequently aggressively rejected (Resendez & Aragona, 2013). We hypothesize that this occurs through negative valence signaling by kappa-opioid receptors within the nucleus accumbens and the direct interaction of this system with D1-like receptors to mediate aversive motivation.

SPECIFIC AIMS OF THIS DISSERTATION

Biological adaptations that facilitate social bonding likely arise because they improve reproductive fitness and, consequently, species survival. A similar affinity for social bonding between socially monogamous mammals and humans suggest that the propensity for social bonding has adaptive value in our species (Shultz *et al.*, 2011). Perhaps by increasing our understanding of neural mechanisms that mediate social attachment behavior in prairie voles, it will increase our understanding of human sociality. For this reason, much work has been conducted to understand the neural mechanisms underlying social bonding in prairie voles. However, very little is known about the role of the endogenous opioid system in pair bond behavior in prairie voles or selective social attachment in general.

The primary focus of this dissertation will be to identify the role of the endogenous opioid systems in social bond formation. We will begin by

identifying the role of the endogenous opioid system in pair bond formation. Given that this stage of pair bonding is associated with affiliative social interactions that are generally categorized as positive (Resendez & Aragona, 2013), we hypothesize that activation of the mu-opioid receptors that mediates positive hedonics (i.e., those in the dorso-medial nucleus accumbens shell) (Smith *et al.*, 2010) will be critical to pair bond formation. In contrast, pair bond maintenance is associated with aversive social encounters (Resendez & Aragona, 2013) and we therefore hypothesize that activation of kappa-opioid receptors that signal aversion (Bruchas *et al.*, 2010) will be important for this stage of pair bonding. Additionally, given the close association of this system with components of motivational circuitry that are also involved in pair bond maintenance, we hypothesize that interactions between D1-like and kappa-opioid receptors are also important for pair bond maintenance. The following experiments presented in the next three chapters (listed below) have therefore been designed to examine these hypothesized mechanisms.

Summary of following chapters:

- **Chapter 2**: This chapter will begin by characterizing the distribution of mu-opioid receptors throughout the striatum of female prairie voles. In this characterization, we identify that the mu-opioid receptor binding is heterogeneous within the nucleus accumbens shell and the densest binding occurs within the rostral dorso-medial region. Importantly, we show that this dense patch of mu-opioid receptors is important for partner preference formation and this

patch has previously been identified as a neural regulator of positive hedonics (Pecina & Berridge, 2005). Therefore, these data identify for the first time a role for positive hedonics in pair bond formation.

- **Chapter 3**: While chapter two focuses on opioid regulation of pair bond formation, chapter three will focus on opioid regulation of pair bond maintenance. The data presented in this chapter demonstrates that activation of kappa-, but not mu-, opioid receptor activation is required for the display of selective aggression toward a resident-intruder and therefore pair bond maintenance. Additionally, we identify that kappa-opioid receptor regulation of pair bond maintenance is specific to the nucleus accumbens shell as blockade of kappa-opioid receptors within the nucleus accumbens shell, but not the nucleus accumbens core or ventral pallidum, attenuates selective aggression in both males and females. Together, these data suggest that the aversive processing of novel social stimuli within the nucleus accumbens shell is important for pair bond maintenance.
- **Chapter 4**: In this chapter, I present data demonstrating that pair bonding enhances dopamine transmission within the nucleus accumbens shell of males and females. This enhancement likely facilitates the activation of low-affinity D1-like dopamine receptors that are also important for selective aggression. Finally, we

demonstrate for the first time that D1-mediated aggression occurs through downstream activation of kappa-opioid receptors. Thus, interactions between motivational and hedonic processing systems are required for the appropriate display of social behaviors that are important for pair bond maintenance.

- **Chapter 5**: In this final chapter, I will summarize the data presented in chapters two through four as well as discuss the limitations of the data. The limitations will primarily be discussed in terms of a lack of in vivo measures of opioid and dopamine transmission during affiliative and aggressive behaviors that are important for pair bond formation and maintenance, respectively. I will end the chapter by proposing important future directions of this work related to examining the role of opioids in interactions between drug and social reward.

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CHAPTER 2

MU-OPIOID RECEPTORS WITHIN DISTINCT SUB-REGIONS OF THE STRIATUM MEDIATE PAIR BOND FORMATION THROUGH PARALLEL YET DISTINCT REWARD MECHANISMS

ABSTRACT

The prairie vole is a socially monogamous rodent that is an excellent animal model for studies of the neurobiology of social attachment. Such studies have demonstrated that activation of reward circuitry during social interactions facilitates pair bond formation. Within this circuitry, mu-opioid receptors (MORs) modulate naturally rewarding behavior in an anatomically segregated manner; MORs located throughout the striatum (dorsal striatum, nucleus accumbens (NAc) core, and the entire NAc shell) are implicated in general motivational processes while those located specifically within the dorso-medial NAc shell mediate positive hedonics (and are referred to as a "hedonic hotspot"). The purpose of the present study was to determine if MORs within these distinct sub-regions differentially mediate pair bond formation. We first used receptor autoradiography to compare MOR binding densities between these regions. MOR binding was significantly higher in the NAc core and dorso-medial NAc

shell compared to the ventral NAc shell. We next used partner preference testing to determine if MORs within these sub-regions differentially mediate pair bonding. Blockade of MORs using 1 or 3 μg of H-D-Phe-Cys-Tyr-D-Trp-Arg-thr-Pen-Thr-NH2 (CTAP) within the dorsal striatum decreased mating during the cohabitation period and inhibited partner preference formation. In contrast, blockade of MORs within dorso-medial NAc shell inhibited partner preference formation without effecting mating behavior while other regions were not involved. Thus, MORs within the dorsal striatum mediate partner preference formation via impairment of mating while those in the dorso-medial NAc shell appear to mediate pair bond formation through the positive hedonics associated with mating.

INTRODUCTION

The socially monogamous prairie vole (*Microtus ochrogaster*) is an excellent animal model for studying the neurobiology of social attachment (Young *et al.*, 2005). Prairie vole mating partners form selective pair bonds that begin with an initial preference for a mating partner. This 'partner preference' is associated with positive social interactions (Williams *et al.*, 1992; Winslow *et al.*, 1993) that are regulated by reward circuitry (Aragona and Wang, 2009). Importantly, this circuitry is partly comprised of hedonic processing systems that code the valence of environmental stimuli and, together, coordinate goal-seeking behaviors (Dickinson and Balleine, 2010; Leknes and Tracey, 2010). For

example, positive hedonics is important for appetitive behavior (Cacioppo *et al.*, 2004; Watson *et al.*, 2010), including that of a social nature (Komisaruk *et al.*, 2010). An essential neural mechanism for mediating positive hedonics is the activation of mu-opioid receptors (MORs) (Panksepp et al., 1980; Bakshi and Kelley, 1993a; Pecina and Berridge, 2000) within the dorso-medial portion of the nucleus accumbens (NAc) shell (i.e. a hedonic hotspot), a sub-portion of the striatum with distinct functional/anatomical characteristics (Pecina and Berridge, 2005; Britt and McGehee, 2008; Smith et al., 2010; Britt et al., 2012; Watabe-Uchida et al., 2012).

While neural regulators of reward are commonly hypothesized to mediate appetitive social behavior (Trezza *et al.*, 2011), 'reward' is not a unitary psychological concept; it has been suggested that 'reward' may encompass at least three psychological components: hedonics, motivation, and learning (Berridge and Robinson, 2003). Importantly, studies of food reward have identified that MORs distributed throughout the striatum mediate motivational and hedonic components of food reward in an anatomically segregated manner (Kelley and Berridge, 2002). Specifically, stimulation of MORs throughout the striatum (dorsal striatum, NAc core, and the entire NAc shell) increases general motivational state (Bakshi and Kelley, 1993b; Zhang and Kelley, 2000; DiFeliceantonio et al., 2012), whereas only stimulation of MORs within the dorso-medial NAc shell mediates the positive hedonic responses associated with the consumption of highly palatable foods (Kelley et al., 2005; Pecina and Berridge, 2005; Smith and Berridge, 2007). This anatomical framework can be used as a

tool to test the neurochemical and neuroanatomical correlates that mediate other types of reward, such as attachment formation.

In the current study, we have used this well-established functional/anatomical mapping of MOR regulation of reward to determine if specific sub-regions of the striatum, and therefore, possibly specific psychological components of reward, regulate partner preference formation. This study is especially important because it was recently suggested that MORs in the dorsal striatum, but not the NAc, are important for partner preference formation because blockade of MORs within dorsal striatum, but not within the ventral NAc shell, prevented partner preference formation (Burkett *et al.*, 2011). However, this previous study did not examine the role of MORs within the dorso-medial NAc shell (i.e., the region critical for hedonics). Thus, in the present study we used receptor autoradiography and site-directed behavioral pharmacology to compare the involvement of MORs within four regions of the striatum in partner preference formation.

METHODS

Subjects: Subjects for partner preference tests were adult female prairie voles bred at the University of Michigan (Resendez *et al.*, 2012). Adult male prairie voles were used as stimulus animals. Subjects were weaned and housed as previously described (Resendez *et al.*, 2012). All procedures were conducted in accordance with the animal care guidelines of the University of Michigan. Adult female prairie and meadow voles used for MOR autoradiography were bred at

Florida State University (FSU) and all procedures were conducted in accordance with FSU animal care guidelines.

Receptor autoradiography: Subjects were sacrificed via rapid decapitation, and brains of female prairie (*n* = 5) and meadow voles (n = 5) were removed, immediately frozen on dry ice, and stored at -80 °C (Aragona *et al.*, 2006; Lim *et al.*, 2006; Resendez *et al.*, 2012). Brains were sectioned on a cryostat at 15 μm in four serial sections and stored at -80 °C until processing (Liu *et al.*, 2010). MOR autoradiography (DAMGO; Perkin Elmer, catalog #NET 902; lot #3615807) was conducted as previously described (Resendez *et al.*, 2012). Kodak BioMAx MS film was laid on the slides and exposed for six months (Resendez *et al.*, 2012). After completion of the exposure period, film images were captured using a Scan Maker 1000XL Microtek scanner. The density of MOR binding within the dorsal striatum, the NAc core, the dorso-medial NAc shell, and the ventral NAc shell (Figure 1A and B) was analyzed with NIH ImageJ 64 (Bales et al., 2007b). MOR binding densities within each region were measured in 4 serial rostral coronal sections (prior to the corpus callosum fusing; (Aragona *et al.*, 2006)) as well as 4 serial coronal sections caudal to the callosum fusions (when the anterior commissure is aligned with the ventricle). These rostral and caudal regions were averaged for each respective region of the striatum.

The above anatomical markers of rostral and caudal striatum were chosen to be consistent with those that have been previously described in voles (Aragona *et al.*, 2006) as well as those that are currently used to describe the

location of the NAc hedonic hotspot in rats (Richard *et al.*, 2013). Mean densities of all regions of interest were background subtracted from white matter tracts (anterior commissure) (Olazabal and Young, 2006). ImageJ 64 was also used to generate composite images of the average MOR binding density of five female prairie voles within the rostral and caudal striatum (Figure 1C and D). Images were made by stacking either the rostral or caudal sections used for analysis for each female prairie vole (n = 20; 4 sections/female) and than the binding density was averaged across the images.

Stereotaxic cannulation: Female prairie voles were implanted with a 26-gauge bilateral guide cannula aimed at the dorsal striatum (+1.6 mm A/P; ± 1.5 mm M/L; -3.0 mm D/V), NAc Core (+1.6 mm A/P; ± 1.2 mm M/L; -3.5 mm D/V), or NAc shell (+1.7 mm A/P; ±1mm M/L; dorso-medial: -4.2 mm D/V; ventral: -4.5 mm D/V) (Aragona *et al.*, 2003; Burkett *et al.*, 2011) and given 3-5 days to recover in their home cage with their cage mate.

Cohabitation and Partner Preference Tests: MOR regulation of pair bond formation was examined utilizing site-directed pharmacological manipulation of mating-induced partner preferences (Liu and Wang, 2003; Cushing et al., 2008). Following surgery, female subjects were estrogen primed with 2.0 μg estradiol benzoate for three days prior to cohabitation with a male (Fowler *et al.*, 2005; Burkett *et al.*, 2011). On the day of the experiment, artificial cerebral spinal fluid (aCSF) (n = 11) or aCSF containing 1 or 3 μg of the specific MOR antagonist H-

D-Phe-Cys-Tyr-D-Trp-Arg-thr-Pen-Thr-NH2 (CTAP) (Burkett *et al.*, 2011; Trezza *et al.*, 2011) was infused into one of four regions of the striatum (Dorsal striatum - *n* = 6 to 10; NAc core *n* = 5 to 6; dorso-medial NAc shell: *n* = 4 to 8; ventral NAc shell: *n* = 8 to 9).

Immediately following injections, female subjects were placed in a cage with a novel male (referred to as the 'partner') and allowed to cohabitate and mate for 24-hrs, which reliably induces partner preference formation (Williams *et al.*, 1992). The first 6-hrs of the cohabitation were analyzed for mating and only subjects who mated during this period were included in the study (Carter and Keverne, 2002; Aragona et al., 2003; Liu and Wang, 2003; Curtis and Wang, 2005a). The first 10-mins of each hour during this 6-hr period were also scored to quantify affiliative behavior (olfactory investigation and side-by-side contact) as well as locomotor activity (cage crosses) during the cohabitation period.

Following the 24-hr cohabitation period, partner preference testing was conducted using a modified partner preference apparatus (Ahern and Young, 2009; Burkett et al., 2011). Briefly, the partner preference apparatus was composed of three equally sized compartments divided by partial barriers. Male partners were loosely tethered in one compartment while novel males (referred to as 'strangers') were loosely tethered in the opposite compartment (Donaldson et al., 2010; Keebaugh and Young, 2011). At the beginning of the test, the female subjects were placed in the center (neutral) compartment and allowed to freely roam between compartments for 3-hrs (Curtis et al., 2001; Bales et al., 2007a). A significant partner preference was determined by statistically comparing (see

below) the duration of mean time spent in contact with the partners to the duration of time spent in contact with the strangers (Cho et al., 1999; Cushing et al., 2003; Bales et al., 2007a). Cannulae placements were confirmed after testing and only subjects with correct placements were used for analysis. All striatal placements were in rostral portions of the nuclei (i.e. regions previously proven to be important for pair bond formation; (Aragona *et al.*, 2006).

Statistics: A one-way ANOVA was used to compare the densities of MORs between the four regions the striatum (Heinz *et al.*, 2005). A paired t-test was used to compare MOR binding density between the rostral and caudal portions of each region. A two-way ANOVA (species x region) was used to compare MOR binding density between prairie and meadow voles (Insel and Shapiro, 1992). A one-way ANOVA was used to compare differences in mating bouts between treatment groups as well as total contact time (partner contact + stranger contact) during the partner preference test (Burkett *et al.*, 2011). A two-way ANOVA (treatment x time) was used to determine if inhibition of MORs impacted affiliative behaviors or locomotor activity during the first 6-hrs of cohabitation (Curtis *et al.*, 2001; Aragona *et al.*, 2003). A two-way ANOVA (treatment x chamber) was also used to determine if MOR blockade effected the time spent in each chamber of the partner preference apparatus. All ANOVA's were followed by a Tukeys *post hoc* test. A partner preference was determined with a paired t-test by comparing the duration of time spent in contact with the partner to that of the stranger

(Cushing et al., 2003; Curtis and Wang, 2005a). In all cases, statistical significance was determined using an alpha level of $p < 0.05$.

RESULTS

Quantification of MOR Binding throughout the Striatum

Previous studies have identified MORs within the dorso-medial NAc shell as important for positive hedonics (i.e. a "hedonic hotspot") and it has been postulated that this may be associated with a higher density of MORs in this region (Pecina and Berridge, 2000; Smith and Berridge, 2007). In our previous study, we noticed (qualitatively) that prairie voles showed a higher density of MORs in the dorso-medial NAc shell (see Figure 6 of (Resendez *et al.*, 2012)) and this is also evident in a recently paper published by another group (see Figure 4 of (Burkett *et al.*, 2011)). In the present study, we provide the first quantification of MOR density across the striatum (Figure 1A-D) and demonstrate that MOR density varies by region $F_{(3, 19)} = 4.70$, $p = 0.02$. Specifically, within rostral regions of the striatum, MOR binding within the dorso-medial NAc shell is significantly higher than the ventral NAc shell $p = 0.05$ (Figure 1E). MOR binding within the NAc core was also significantly higher than the ventral NAc shell $p = 0.01$ (Figure 1E). The density of MOR binding did not significantly differ between any other regions of the striatum. While MORs within the dorsal striatum were not significantly higher than the ventral striatum in this paper, it is very important to understand that this is likely due to variation in patch/matrix distribution

because MOR density is very high in patches (or striosomes) and low in the matrix (Graybiel and Chesselet, 1984; Johnston et al., 1990; Gerfen, 1992).

MOR binding densities also varied along a rostral-caudal gradient. Within all regions of the striatum, the binding density was significantly higher in rostral regions compared to caudal portions: dorsal striatum $t_{(4)} = 4.69$, $p = 0.009$; NAc core $t_{(4)} = 3.41$, $p = 0.03$; dorso-medial NAc shell $t_{(4)} = 3.77$, $p = 0.02$, ventral NAc shell $t_{(4)} = 3.48$, $p = 0.03$ (Figure 1F). Together, these data demonstrate that within the striatum, MOR binding density is significantly higher within the rostral regions. Moreover, within the NAc shell, MOR binding density is highest within rostral dorso-medial region (Figure 1C and D).

To determine if there are any differences in striatal MOR binding density between monogamous and non-monogamous vole species, we compared MOR binding density between female prairie voles and female meadow voles, a non-monogamous voles species (Beery and Zucker, 2010). This comparison was made because previous studies have identified relationships between receptor binding patterns and the social organizations of a vole species (Insel and Shapiro, 1992; Insel et al., 1994; Wang et al., 1997; Young et al., 1997; Young et al., 1999; Lim and Young, 2004; Aragona et al., 2006; Barrett et al., 2013). Similar to above, MOR binding was measured in the dorsal striatum, the NAc core, the dorsomedial NAc shell, and the ventral NAc shell. Consistent with previous studies (Insel and Shapiro, 1992), there were no species differences in binding density between any regions $F(2, 32) = 0.41$, $p = 0.53$ (Figure 1G). Within the caudal striatum, the overall ANOVA for MOR binding was significant

$F(2, 32) = 4.12$, $p = 0.05$, but post hoc test did not reveal any specific species differences between any region of the striatum (Figure 1H).

The consistent binding pattern of these receptors across vole species suggest that MORs within the striatum do not play a direct role in species specific social organization, but rather appear to play a more general role in natural reward processing. Consistent with this, within the NAc of other species, a high density of MOR binding can be seen in the dorso-medial NAc shell of the rat (Herkenham *et al.*, 1984) and MOR binding within the human NAc shell is also reported to be highly heterogeneous (Voorn *et al.*, 1996) further suggesting that MORs within specific striatal regions may act as a common neural currency of reward. To test if MORs are important for social reward and therefore important for social bonding, we conducted a thorough analysis of MORs throughout the striatum for their role in pair bond formation.

MORs and Partner Preference Formation

Dorsal striatum

It was recently demonstrated that blockade of MORs in the dorsal striatum with 1 μg CTAP prevented the formation partner preferences in female prairie voles (Burkett *et al.*, 2011). Therefore, we first set out to replicate this finding. As previously described (Burkett *et al.*, 2011), control females that received aCSF showed significant partner preferences $t_{(10)} = 2.895$, $p = 0.02$ (Figure 2A). Further, we also replicated this study by demonstrating that blockade of MORs within the dorsal striatum with CTAP inhibits partner preference formation

(Burkett *et al.*, 2011) although our dose response differed. Specifically, we did not replicate the finding that blockade of MORs within the dorsal striatum with 1 μg CTAP inhibits partner preference formation $t_{(8)}$ = 3.34, p = 0.01 (Figure 2A). However, the higher dose of CTAP (3 μg) used in the present study prevented partner preference formation $t_{(5)}$ = 0.72, p = 0.50 (Figure 2A). Blockade of MORs in the dorsal striatum did not effect the total contact time $F(2, 26) = 2.38$, p = 0.114 (Figure 2B) or the amount of time spent in each chamber $F(2, 72) = 9.41$, p = 0.97 (Figure 2C) during the partner preference test. Thus, our overall finding that blockade of MORs within the dorsal striatum inhibits partner preference formation is consistent with that published in a previous report (Burkett *et al.*, 2011) and the difference in effective dose may be due to slight variations in probe placement, especially given the variation in patch/matrix activation (Graybiel, 1990; Gerfen, 1992) (see inset in Figure 2A) or, it is always possible for there to be slight differences between subjects from two different colonies.

Importantly, blockade of MORs in the dorsal striatum significantly decreased the total number of mating bouts during the habituation period $F(2, 26) = 3.58$, p = 0.04 (Figure 2D) without effecting the level of affiliative social interactions during the habituation period $F(2, 120) = 0.97$, p = 0.40 (Table 1). Post hoc tests revealed that subjects who received the high dose of CTAP into the dorsal striatum mated significantly less than control subjects p = 0.05 (Figure 2D). Importantly, MOR regulation over prairie vole mating behavior is consistent with a previous study (Burkett *et al.*, 2011) and as mating is important for partner preference formation, these data suggest that administration of a dose of CTAP

into the dorsal striatum that attenuates mating is the mechanism by which partner preference formation is disrupted. This decrease in mating behavior is not secondary to a general decrease in motor activity as blockade of MORS in the dorsal striatum had no effect on locomotor activity during the habituation period $F(2, 120) = 1.37$, $p = 0.27$ (Table 2).

NAc Core

We next tested a possible role for MORs within the NAc core and blockade of MORs within this region with the low $t_{(5)} = 3.07$, $p = 0.03$ or high $t_{(5)} = 3.07$, $p = 0.03$ dose of CTAP did not inhibit partner preference formation (Figure 3A). There was also no overall effect on the time spent in each chamber $F(2, 57) = 0.03$, $p = 0.97$ (Figure 3B) or total contact time $F(2, 21) = 0.18$, $p = 0.88$ (Figure 3C) during the partner preference test. During the cohabitation period, there was also no effect on affiliative behavior $F(2, 108) = 0.06$, $p = 0.94$ (Table 1) or locmotor activity $F(2, 108) = 0.87$, $p = 0.43$ (Table 2). However, there was a trend for a decrease in mating behavior $F(2, 21) = 3.00$, $p = 0.07$ (Figure 3D).

The inability of MOR blockade within the NAc core to significantly effect partner preference formation is consistent with previous studies of pair bonding that have not identified a role for the NAc core in this behavior (Aragona et al., 2006; Aragona and Wang, 2007; Resendez et al., 2012). However, when the trend for a decrease in mating is considered in relation to the significant decrease in the dorsal striatum and lack of an effect on mating in the NAc shell (see below), the present data are consistent with the notion that the striatum is

functionally connected via a ventromedial to dorsolateral spiraling system which would make the NAc core a striatal transition zone between the NAc shell and dorsal striatum (Haber et al., 2000; Haber, 2003; Everitt and Robbins, 2005; Vanderschuren and Everitt, 2005). Therefore, MORs within this region may have intermediate effects on mating that are not sufficient to impact partner preference behavior. Intermediate pharmacological effects within the NAc core on partner preference behavior are consistent with the view that the striatum functions in a topographic manner and intermediate effects on social reward behavior can be found in transition zones, such as the NAc core.

NAc Shell

Previous studies have demonstrated that the NAc shell is a highly heterogonous region (Ikemoto, 2007; Britt and McGehee, 2008; Resendez et al., 2012; Watabe-Uchida et al., 2012), especially in regards to function (Pecina and Berridge, 2005; Smith et al., 2010; Lammel et al., 2011; Britt et al., 2012; Richard et al., 2013). For example, the rostral dorso-medial NAc shell modulates positive hedonics, whereas the ventral NAc shell does not (Pecina and Berridge, 2005; Mahler et al., 2007; Faure et al., 2010; Smith et al., 2010). These regions are also anatomically distinct; they significantly differ in MOR binding with the dorso-medial NAc shell having significantly greater binding compared to the ventral NAc shell (Figure I; Burkett et al., 2011). Therefore, we next tested if MORs within these sub-regions differentially regulate partner preferences (Figure 2A).

We first replicated a recent study (Burkett *et al.*, 2011) by demonstrating that blockade of MORs within the ventral NAc shell with a low $t_{(8)} = 3.62$, $p = 0.007$ or high dose of CTAP $t_{(7)} = 3.31$, $p = 0.03$ did not influence partner preference formation (Figure 4A). However, unlike the ventral NAc shell, blockade of MORs within the rostral dorso-medial NAc shell with either the low $t_{(7)} = 0.80$, $p = 0.45$ or high $t_{(4)} = 0.46$, $p = 0.67$ dose of CTAP abolished partner preference formation (Figure 4A). This effect was not due to drug effects on general social behavior or locomotor activity since blockade of MORs within any region of the NAc shell did not impact affiliative behavior $F_{(4, 180)} = 0.81$, $p = 0.53$ (Table 1) or locomotor activity during the cohabitation period $F_{(4, 180)} = 0.90$, $p = 0.48$ (Table 2). During the partner preference test, there was no overall difference in the time spent in each chamber between treatment groups $F_{(4, 105)} = 0.17$, $p = 0.96$ (Figure 2C) or total contact time $F_{(4, 40)} = 2.23$, $p = 0.08$ (Figure 2D). Together, these data indicate that within the NAc, MOR regulation of partner preference formation is specific to the dorso-medial NAc shell — the region dense with MORs (Figure 1) and, perhaps most importantly, that has previously been implicated in positive hedonics (Pecina and Berridge, 2005).

Unlike the dorsal striatum, inhibition of mating-induced partner preferences in the dorso-medial NAc shell was not associated with decreased mating as administration of neither the low or high dose of CTAP within the dorso-medial NAc shell altered the total number of mating bouts $F_{(4,38)} = 1.14$, $p = 0.35$ (Figure 4D) during the cohabitation period. Moreover, since this inhibition of partner preference (via MOR blockade in the dorso-medial NAc shell) formation

does not act through the regulation of mating behavior, but is rather a consequence of mating, these data suggest that MORs within the dorso-medial NAc shell regulate partner preference formation through different psychological mechanisms than those located within the dorsal striatum that directly impact mating behavior, most likely, the positive hedonics associated with mating.

DISCUSSION

Partner preference formation is a powerful example of social reward and the current study is among many that show that brain reward circuitry is essential for this behavior (Wang et al., 1999; Gingrich et al., 2000; Aragona et al., 2003; Liu and Wang, 2003; Lim and Young, 2004; Curtis and Wang, 2005a, b; Aragona et al., 2006; Aragona and Wang, 2007; Lim et al., 2007; Burkett et al., 2011; Hostetler et al., 2011; Keebaugh and Young, 2011; Liu et al., 2011). The present study is the first to demonstrate that regional specificity in MORs within the striatum of pair bond formation is due to different underlying mechanisms associated with social reward.

We first replicated a recent finding (Burkett *et al.*, 2011) by demonstrating that partner preference formation requires the activation of MORs within the dorsal striatum, a region of the brain where MORs mediate motivated behavioral responses (DiFeliceantonio et al., 2012). Additionally, we extend this current knowledge by providing the first evidence that endogenous opioids within the NAc are also critical for partner preference formation — specifically, activation of MORs within the region of the NAc shell implicated in positive hedonics is

required for pair bond formation. Importantly, these data provide the first evidence that this dense patch of MORs within the dorso-medial NAc shell not only mediates positive hedonics associated with food reward (Pecina and Berridge, 2000; Smith and Berridge, 2007), but may play a general role in the neural processing of all natural rewards, including social reward. Taken together, our data identify two potential parallel mechanisms in which MORs regulate partner preference formation: one in which MORs in the dorsal striatum regulate the motivation to engage in mating behavior that is central to pair bond formation and the second in which MORs in the dorso-medial NAc shell regulate the positive hedonic processing that are a consequence of socially rewarding acts, such as mating.

Motivation, the Dorsal Striatum, and Partner Preference Formation

Partner preference formation in prairie voles is facilitated by mating (Williams *et al.*, 1992) and the present study demonstrates that blockade of neural systems that mediate this behavior, such as the endogenous opioid system within the dorsal striatum, interferes with the initial formation process. While opioid regulation of prairie vole mating has only recently been examined (Burkett *et al.*, 2011), and is therefore not well understood, data from other species has directly implicated this system as important for both the act of mating (Coolen *et al.*, 2004; Parra-Gamez *et al.*, 2009; Komisaruk *et al.*, 2010) and the formation of preferences for environments in which mating has occurred (Coria-Avila *et al.*, 2008). During mating, endogenous opioids are released into reward processing regions of the brain (Szechtman *et al.*, 1981) and the release of these

peptides is critical for generating sexually motivated responses as peripheral blockade of MORs in rats increases the mating bout interval as well as decreases the frequency of bouts (Ismail *et al.*, 2009). Additionally, the expression of enkephalin, an endogenous ligand for MORs (Simantov *et al.*, 1977) increases in the dorsal striatum of female rats during proestrus (Roman *et al.*, 2006) – the period of the estrous cycle where lutenizing hormone and progesterone concentrations surge to induce ovulation, sexual receptivity and motivation (Smith *et al.*, 1975; Becker, 2009). Together, these results suggest that activation of MORs within the dorsal striatum mediates motivational aspects of sexual behavior that is necessary for partner preference formation.

Indeed, recent evidence from studies of food reward directly implicates MORs within the dorsal striatum in motivated behavior (DiFeliceantonio et al., 2012). Specifically, enkephalin, is released in the dorsal striatum during the onset of food consumption and is positively correlated with the speed at which food consumption begins (DiFeliceantonio et al., 2012). Together, these behavioral measures indicate that activation of MORs within this region is important for energizing appetitive response to a rewarding stimulus (Richard *et al.*, 2013) and, therefore, blockade of these receptors while in the presence of a highly salient social stimulus, such as a potential mating partner, may decrease the motivation to seek the reward. Interestingly, activation of the dorsal striatum is also thought to regulate the motivational aspects of partner preference formation in humans as this region is activated during the early stages of a romantic relationship, but this activation is not correlated with the positive

hedonic state induced by the partner (Aron *et al.*, 2005). Similarly, enkephalin released into the dorsal striatum during food consumption is not associated with the hedonic responses to this stimulus (DiFeliceantonio et al., 2012). Thus, activation of MORs within the dorsal striatum appears to be specific to the motivational aspects of reward seeking. When these findings are considered alongside the decrease in mating produced by dorsal striatal MOR blockade in the present study, these data suggest that MORs within the dorsal striatum may be critical to partner preference formation by generating socially motivated behavioral responses, such as mating and subsequent consequences of mating, such as partner preference formation.

Positive Hedonics, the Rostral Dorso-medial NAc shell, and Partner preference Formation

MORs within the dorso-medial NAc shell, have been implicated in positive hedonics (Berridge and Kringelbach, 2008) that is critical in the early stages of attachment formation (Panksepp et al., 1980; Resendez and Aragona, 2013). During the cohabitation period, male and female prairie voles engage in high levels of rewarding social interactions such as investigatory behavior, mating, and huddling (Carter and Getz, 1993). These interactions are important for the formation of a bond (Williams *et al.*, 1992) and data from the present study demonstrates that blockade of MORs within the dorso-mdedial NAc shell did not interfere with social contact and mating (i.e., consumatory behavior related to social reward). Instead, removing of a positive hedonic signal following mating by blocking MORs within the dorso-medial NAc shell disrupts social reward and

interferes with a positive motivated social decision (Aragona and Wang, 2009). These data are consistent with a previous study of social reward that demonstrated that activation of MORs within the NAc shell, and possibly hedonic signaling, is important for guiding socially motivated behavior (Trezza *et al.*, 2011).

A role for hedonics in social bonding is strongly supported by the human literature. In humans, social interactions with a mating partner are not only rewarding, but are indeed pleasurable (Fisher *et al.*, 2006). Social interactions with a mate or viewing photos of a romantic love interest activate reward circuitry (Panksepp et al., 2002; Curtis and Wang, 2005b; Fisher et al., 2006). Together, these data suggest that homologous reward circuits across mammalian species are involved in selective attachment formation. This speaks to the evolution of the role of positive affect in attachment and given that a common neural circuit may mediate pleasure, our work has implications for a 'common neural currency' important for general motivation, including socially motivated behaviors (Cabanac, 2002; Burgdorf and Panksepp, 2006; Kringelbach and Berridge, 2012).

Parallel Motivational and Hedonic Processing in Partner Preference Formation

Appetitive processing within the brain involves interactions between parallel processing striatal circuits associated with cognitive, motor, and limbic regions (Flaherty and Graybiel, 1994; Haber, 2003; Kreitzer and Berke, 2011). With respect to attachment formation, a lack of coupling between consummatory

motivated behavior, such as mating, and the subsequent positive hedonic encoding of that behavior may act to decrease future social reward seeking, such as contact with the mated partner during the partner preference test (Resendez and Aragona, 2013). Interestingly, inputs and outputs into the striatum are organized into a topographical, spiral pattern (Haber *et al.*, 2000; Belin *et al.*, 2009) that may account for the regional differences in MOR regulation of pair bond formation within the striatum. Specifically, blockade of MORs in the dorsal striatum may decrease the motivation to generate appropriate motor responses to a salient social stimulus (i.e., reduced mating), whereas those in the dorso-medial NAc shell appear to regulate the positive hedonic coding of that same social stimulus. Thus, coordination between distinct neural systems that differentially code psychological processes of behavior related to social reward and its importance for attachment formation.

Conclusion

Among monogamous prairie voles (Getz *et al.*, 1981), the choice of a mate that will result in successful reproduction is of critical importance (Curtis, 2010; Resendez *et al.*, 2012). Choosing an appropriate mate is therefore essential, and, the present study demonstrates that appropriate MOR activation within distinct regions of the striatum has evolved to facilitate social decision making necessary for the motivational and hedonic processes associated with successful pair bond formation (Resendez and Aragona, 2013). Within the striatum, MORs within dorsal and ventral sub-regions act in parallel to mediate mating and the hedonic consequences of mating, respectively. Together, the

present data and data from studies of food reward indicate that MORs within the striatum do not play a specific role in one type of reward (Richard et al., 2012; Berridge and Kringelbach, 2013), but rather act as general neural currency to motivate rewarding/adaptive behavioral responses, including the formation of a selective attachment.

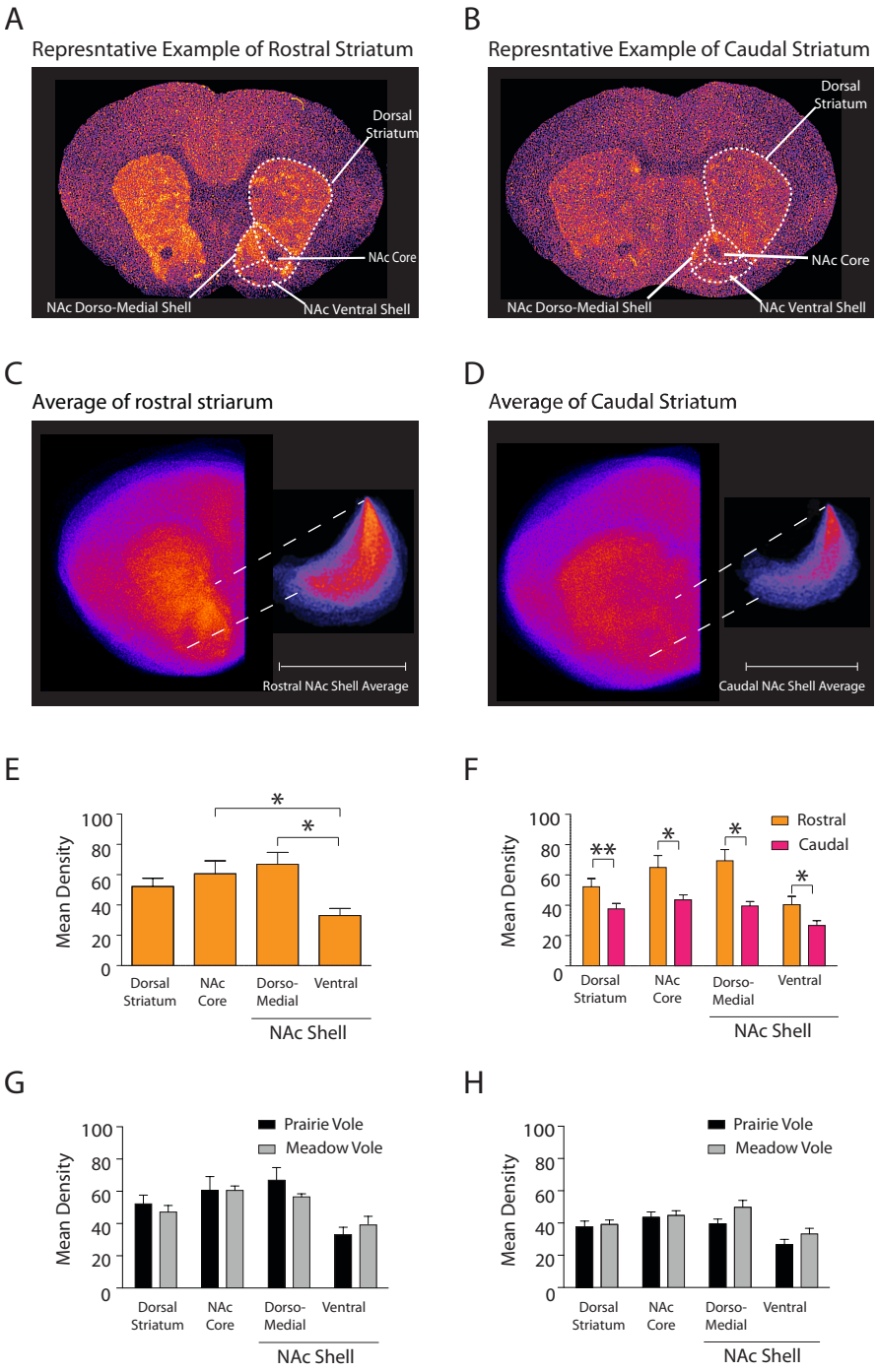

Figure 1

MOR binding within the striatum. A) On the left we show a representative image of MOR binding density within the rostral striatum and B) we show a representative image of MOR bonding within the caudal striatum. On the right side of each image, we outline the regions analyzed to obtain mean MOR binding density. C) A composite image of the rostral shell of female prairie voles and D) represents the caudal shell. E) MOR binding was significantly higher in the NAc core and dorso-medial NAc shell compared to the ventral NAc shell (n = 5). F) MOR binding was higher in all regions in the rostral striatum compared to the caudal striatum (*n* = 5). The was no difference in MOR binding density between prairie and meadow voles in G) rostral or H) caudal regions of the striatum (n = 5).

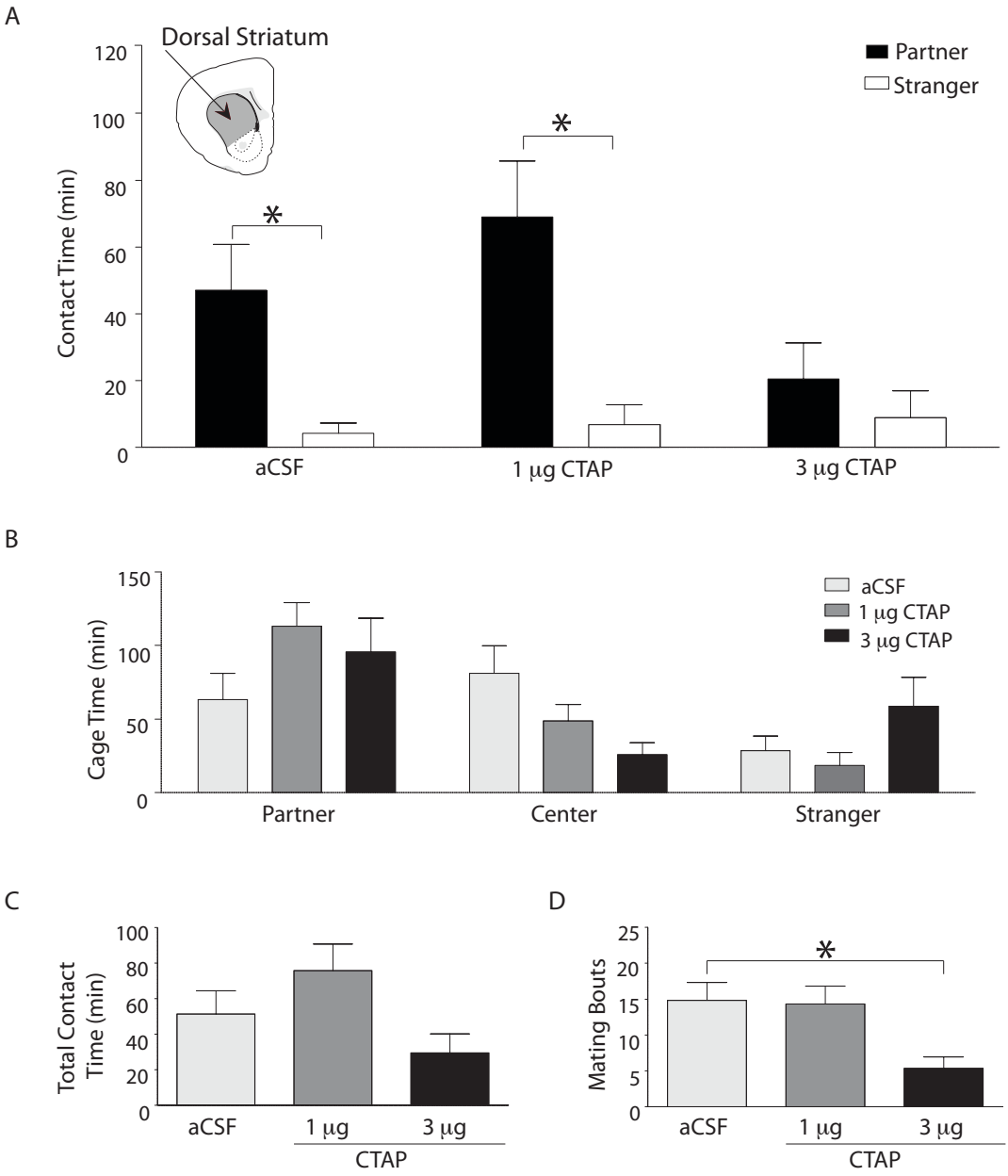

Figure 2

MORs within the dorsal striatum regulate pair bond formation via inhibition of mating. A) Injections of aCSF or the low dose of CTAP into the dorsal striatum did not inhibit partner preference formation, whereas injections of the high dose of CTAP into this region abolished partner preference (inset represents site of injection shaded in gray (left) and the binding of MOR within the dorsal striatum (right)). MOR blockade did not effect B) cage time or C) total contact time during the partner preference test. However, blockade of MORs with the high dose of CTAP decreased the total number of mating bouts during the cohabitation period (n = 6 to 10).

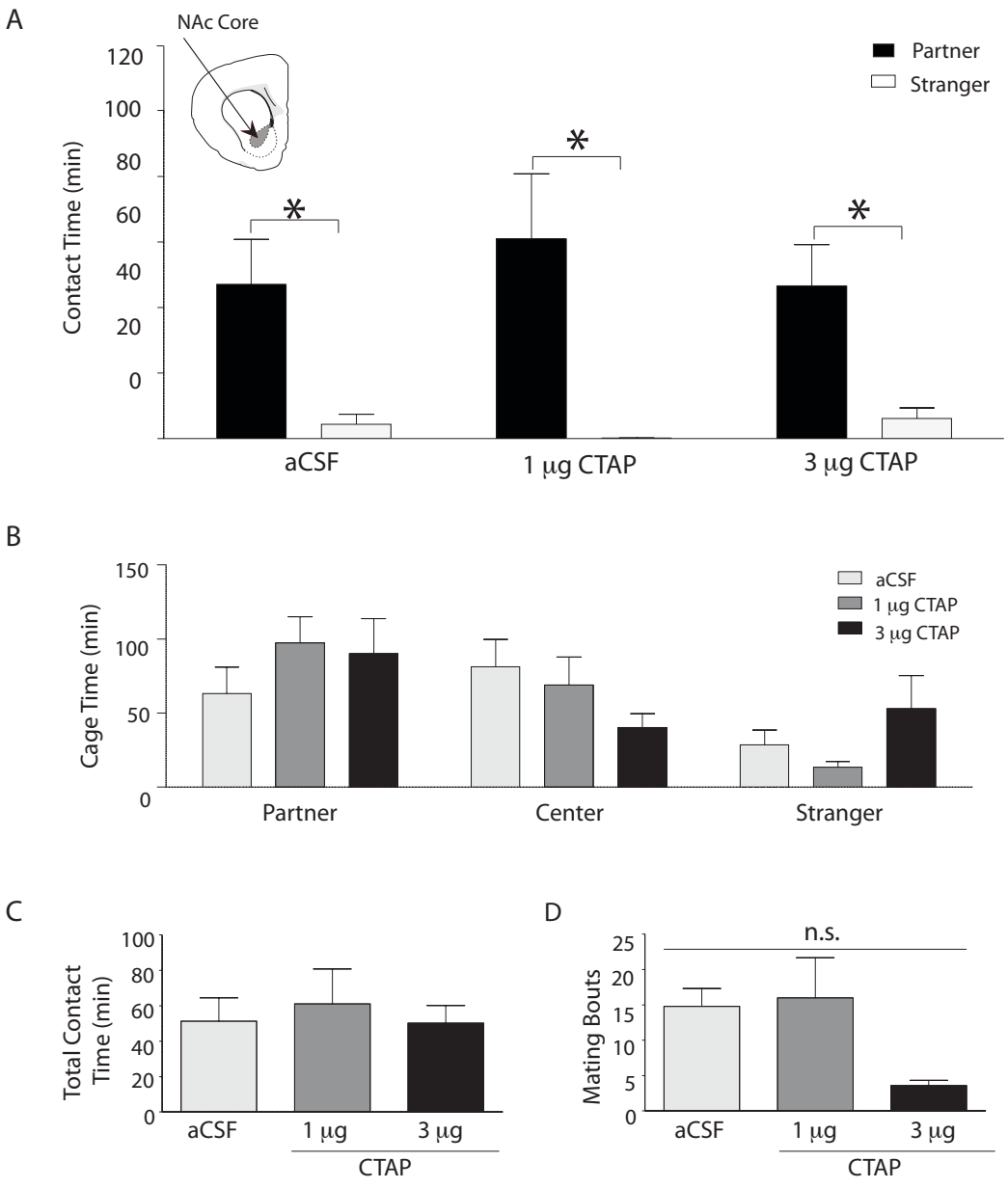

Figure 3

MORs within the NAc core do not play a significant role in partner preference formation. A) Neither injections of the low or high dose of CTAP into the NAc core impacted partner preference formation (inset represents site of injection shaded in gray (left) and the binding of MOR within the NAc core (right)). B) MOR blockade within the NAc did not effect on B) cage time or D) total contact time during the partner preference test, e) although there was a trend for a decrease in the number of mating bouts (n = 5 to 6).

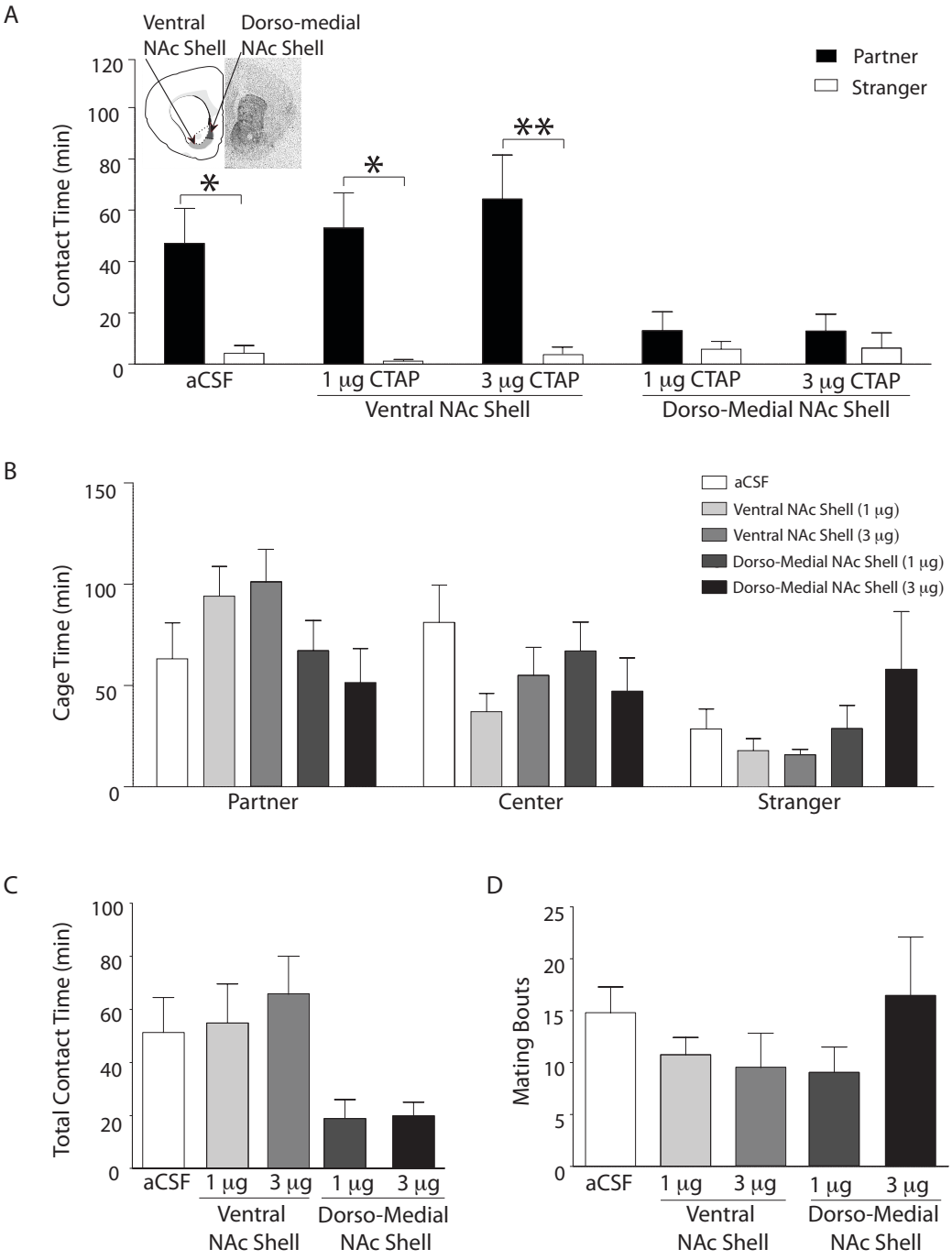

Figure 4

MORs within the dorso-medial, but not ventral, NAc shell are important for partner preference formation. A) Site-specific injection of both the low and high dose of CTAP into the dorso-medial NAc shell inhibited partner preference formation, while injections of either dose of CTAP were without effect in the ventral NAc shell (inset represents site of injection into the dorso-medial Nac shell (dark gray) or the ventral NAc shell (light gray) (left) and the binding of MOR within the NAc shell (right)). B) MOR blockade with either dose of CTAP into the NAc shell did not effect on B) cage time, C) total contact time, or D) the number of mating bouts (n = 4 to 9).

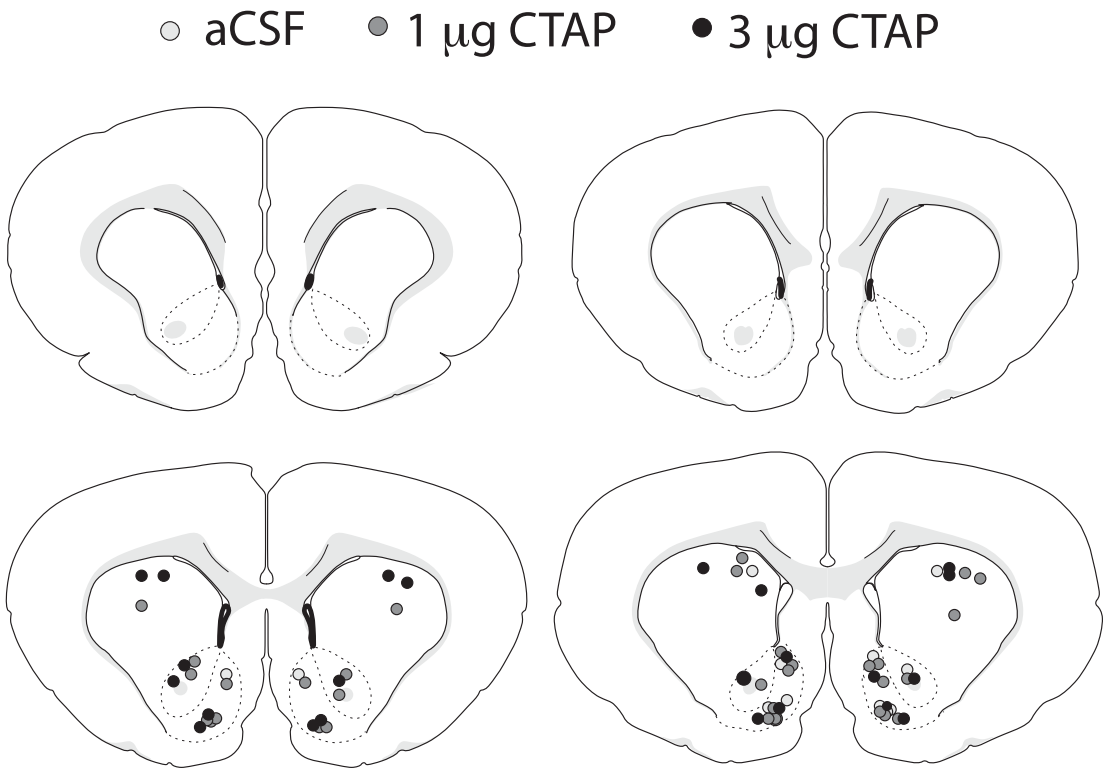

Figure 5

Cartoon images representing injection sites of aCSF, 1 μg CTAP, or 3 μg CTAP into the dorsal striatum, the NAc core, the dorso-medial NAc shell, or the ventral NAc shell.

Affiliative Behavior

	Hour					
Group	**1**	**2**	**3**	**4**	**5**	**6**
aCSF	1.71 ± 0.60	2.14 ± 0.77	1.83 ± 0.50	5.21 ± 0.95	2.80 ± 0.93	3.94 ± 1.07
Dorsal striatum						
1 μg CTAP	1.43 ± 0.42	3.15 ± 1.04	4.15 ± 1.29	3.64 ± 1.29	4.81 ± 1.56	6.12 ± 1.35
3 μg CTAP	2.08 ± 0.68	3.13 ± 1.55	3.03 ± 1.40	2.94 ± 0.96	3.40 ± 1.27	5.49 ± 1.03
NAc core						
1 μg CTAP	1.58 ± 0.63	1.08 ± 0.51	2.75 ± 2.17	2.6 ± 1.09	6.40 ± 1.51	3.18 ± 0.99
3 μg CTAP	1.01 ± 0.64	1.14 ± 0.55	3.11 ± 1.83	3.55 ± 1.67	3.94 ± 2.12	3.56 ± 1.64
NAc dorso-medial shell						
1 μg CTAP	1.97 ± 0.43	2.17 ± 1.23	0.22 ± 0.10	3.20 ± 1.40	4.27 ± 1.48	4.12 ± 1.40
3 μg CTAP	1.54 ± 0.59	2.07 ± 1.09	2.83 ± 1.59	2.38 ± 1.65	3.37 ± 1.11	7.10 ± 1.53
NAc ventral shell						
1 μg CTAP	2.15 ± 0.84	1.43 ± 0.59	2.41 ± 1.09	2.05 ± 1.21	3.45 ± 1.55	3.83 ± 1.35
3 μg CTAP	1.20 ± 0.44	2.00 ± 0.93	4.12 ± 1.34	1.93 ± 0.95	5.96 ± 0.74	6.66 ± 1.26

Table 1

Affiliative behavior during the cohabitation period. Site-specific blockade of MORs with 1 or 3 μg CTAP into any region of the striatum did not impact the duration of time (min) that female subjects spent engaging in affiliative behavior with the partner during the first 6 hours of cohabitation. Data presented as mean ± standard error of the mean.

Cage crosses

	Hour					
Group	1	2	3	4	5	6
aCSF	13.00 ± 2.78	11.25 ± 1.27	5.67 ± 1.61	4.83 ± 1.20	5.67 ± 1.24	3.83 ± 1.22
Dorsal striatum						
1 μg CTAP	10.50 ± 2.87	8.25 ± 1.80	4.75 ± 1.34	2.62 ± 0.92	5.50 ± 2.18	2.38 ± 1.05
3 μg CTAP	17.43 ± 3.82	7.57 ± 2.36	9.29 ± 3.23	7.86 ± 1.72	4.23 ± 2.20	5.57 ± 2.11
NAc core						
1 μg CTAP	7.5 ± 1.55	5.00 ± 2.12	7.25 ± 3.35	7.75 ± 0.48	6.00 ± 2.48	2.00 ± 1.15
3 μg CTAP	19.20 ± 3.94	7.40 ± 1.29	6.40 ± 2.62	4.00 ± 2.55	6.20 ± 2.91	8.00 ± 2.00
NAc dorso-medial shell						
1 μg CTAP	18.22 ± 4.93	7.89 ± 2.50	6.22 ± 0.97	7.00 ± 3.23	3.29 ± 1.60	8.86 ± 4.40
3 μg CTAP	11.40 ± 0.68	6.60 ± 1.60	3.20 ± 1.24	5.60 ± 1.40	0.80 ± 0.49	1.20 ± 0.73
NAc ventral shell						
1 μg CTAP	17.33 ± 5.67	13.89 ± 5.25	8.44 ± 4.07	7.88 ± 1.98	8.38 ± 2.96	6.25 ± 4.18
3 μg CTAP	12.11 ± 0.89	8.78 ± 0.97	3.33 ± 1.57	4.89 ± 1.24	3.33 ± 1.00	1.00 ± 0.71

Table 2

Locomotor activity during the cohabitation period. Site-specific blockade of MORs with 1 or 3 μg CTAP into any region of the striatum did not impact locomotor activity as measured by cage cross frequency during the first 6 hours of cohabitation. Data presented as mean ± standard error of the mean.

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CHAPTER 3

KAPPA-OPIOID RECEPTORS WITHIN THE NUCLEUS ACCUMBENS SHELL MEDIATE PAIR BOND MAINTENANCE

ABSTRACT

The prairie vole is a socially monogamous species in which breeder pairs typically show strong and selective pair bonds. The establishment of a pair bond is associated with a behavioral transition from general affiliation to aggressive rejection of novel conspecifics. This 'selective aggression' is indicative of mate guarding that is necessary to maintain the initial pair bond. In the laboratory, the neurobiology of this behavior is studied using resident-intruder testing. Although it is well established that social behaviors in other species are mediated by endogenous opioid systems, opioid regulation of pair bond maintenance has never been studied. Here, we used resident-intruder testing to determine if endogenous opioids within brain motivational circuitry mediate selective aggression in prairie voles. We first show that peripheral blockade of kappa-opioid receptors with the antagonist nor-BNI (100 mg/kg), but not with the preferential mu-opioid receptor antagonist naloxone (1, 10, or 30 mg/kg), decreased selective aggression in males. We then provide the first comprehensive characterization of kappa and mu-opioid receptors in the prairie vole brain. Finally, we demonstrate that blockade of kappa-opioid receptors (500

ng nor-BNI) within the nucleus accumbens (NAc) shell abolishes selective aggression in both sexes, but blockade of these receptors within the NAc core enhances this behavior specifically in females. Blockade of kappa-opioid receptors within the ventral pallidum or mu-opioid receptors (1 ng CTAP*)* within the NAc shell had no effect in either sex. Thus, kappa-opioid receptors within the NAc shell mediate aversive social motivation that is critical for pair bond maintenance.

INTRODUCTION

The socially monogamous prairie vole (*Microtus ochrogaster*) is an excellent model system to study the neurobiology of social attachment (Young & Wang, 2004; Young *et al.*, 2005; Aragona & Wang, 2009). Prairie voles show species typical pair bonds characterized by sharing territory, nests, and parental responsibilities (Getz *et al.*, 1981; Getz *et al.*, 1993). Initial pair bond formation involves pro-social behaviors that are reliably assayed with the partner preference test (Wilson, 1982; Williams *et al.*, 1992; Winslow *et al.*, 1993). This behavior is regulated by the processing of social reward within motivational circuitry (Aragona *et al.*, 2003; Liu & Wang, 2003; Lim & Young, 2004; Curtis & Wang, 2005). For example, pair bond formation is facilitated by activation of D_2-like dopamine (DA) receptors within the nucleus accumbens (NAc) shell during the initial hours of cohabitation and mating (Aragona *et al.*, 2006). In contrast, the long-term maintenance of the bond requires increases in aversive motivational behavior, such as mate guarding. This is studied in the lab using

tests of selective aggression toward unfamiliar conspecifics (Gavish *et al.*, 1983; Winslow *et al.*, 1993; Young *et al.*, 1997) and it is known that this behavior is regulated by D_1-like receptors within the NAc shell (Aragona *et al.*, 2006). Thus, D_1- and D_2-like receptors within the NAc shell (a region important for processing social incentives; (Newman, 1999; Champagne *et al.*, 2004)) exert differential regulation over the formation and maintenance of monogamous pair bonds (Aragona *et al.*, 2006).

This is significant because D_1- and D_2-like receptors are primarily expressed on distinct neuron populations (Le Moine & Bloch, 1995; Perreault *et al.*, 2011). D_2-like receptors are expressed on neurons that contain enkephalin, an endogenous ligand for mu-opioid receptors that mediate motivation and positive hedonics (Bozarth & Wise, 1981; Gerfen & Young, 1988; Curran & Watson, 1995; Pecina & Berridge, 2005**)**. Conversely, D_1-expressing neurons contain dynorphin, the endogenous ligand for kappa-opioid receptors that mediate aversion and negative affect (Chavkin et al., 1982; Mucha & Herz, 1985; Pfeiffer et al., 1986)**.** Moreover, activation of D_1-like receptors increases dynorphin levels (Gerfen *et al.*, 1990) indicating a direct interaction between these systems. Given this interaction and kappa-opioid receptor regulation over aversion (van Ree et al., 1999; Le Merrer et al., 2009)**,** we hypothesized that kappa-opioid receptors within the NAc shell mediate selective aggression and are therefore important for aversive social motivation.

Here, we conducted a series of experiments to examine opioid regulation of selective aggression in male and female prairie voles. We first tested the

effects of global kappa- and mu-opioid receptor blockade on this behavior and then characterized central opioid receptor distributions in prairie voles. Finally, we tested the involvement of opioid receptors specifically within the NAc shell, NAc core, or ventral pallidum on the expression of selective aggression. Our studies demonstrate that kappa-opioid receptors within the NAc shell mediate selective aggression and thus provide the first evidence that this region mediates the aversive social motivational processing critical for pair bond maintenance.

METHODS

Subjects: Subjects were adult male and female prairie voles (90-150 days old) initially obtained from Florida State University and bred in a laboratory colony at the University of Michigan. Subjects were weaned at 21 days of age and housed with same-sex cage mates (2 per cage; typically siblings) in a 14L/10D with *ad lib* food and water. All procedures were conducted in accordance with the animal care guidelines of the University of Michigan.

Cohabitation, mating, and pregnancy: Adult males and intact, non-estrogen primed, females were paired for a two-week cohabitation period (Aragona *et al.*, 2006; Gobrogge *et al.*, 2009). This length of cohabitation, as well as the occurrence of mating during this period, has been used to infer pair bonding as characterized by selective affiliation toward the familiar partner and selective aggression toward novel conspecifics (Aragona *et al.*, 2006; Gobrogge *et al.*, 2009). However, recent data show that mating alone is not sufficient for reliable

pair bonding (Curtis, 2010). This study demonstrates that for a pair bond to be consistently established, the pair must achieve estrus, mating, ovulation and impregnation within an optimal time frame (Curtis, 2010). For this reason, the present study characterized the relationships between mating onset and pregnancy as well as the relationship between pregnancy status of the female and selective aggression (i.e. the behavioral index of pair bond maintenance) (Carter & Getz, 1993; Aragona & Wang, 2009).

For the first three days of the cohabitation, social interactions were recorded and latency to initiate mating, as well as the number of mating bouts, were quantified. Once behavioral testing was completed, we determined if females were pregnant and estimated the duration of the pregnancy by measuring neonatal weight. While mating onset and neonatal weight were positively correlated $r(52) = -0.40$, $p = 0.0024$ (data not shown), there were instances wherein mating occurred but no pregnancy resulted. Importantly, males in these pairs were less likely to show selective aggression (below). It was therefore determined that rapid and successful establishment of pregnancy as measured by neonatal weight, is a better method to assess if a cohabitation is likely to be associated with pair bonding rather than relying on the onset of mating behavior during the cohabitation. Thus, the current and previous studies (Curtis, 2010) indicate that future studies of pair bonding should quantify pregnancy, rather than simply determining copulatory behavior during the cohabitation period when attempting to approximate the likelihood of a pair bond.

In order to directly determine the relationship between pregnancy and selective aggression, the stage of pregnancy was estimated based on neonatal weight and characterized as optimally pregnant (pregnancy achieved shortly following the onset of cohabitation) or sub-optimally (delayed pregnancy onset) (Curtis, 2010). Previous studies have used neonatal length to categorize the stage of pregnancy in prairie voles (Curtis, 2010), therefore, in a sub-group of animals we also measured the length of the fetus at the time of testing and plotted these values against the weight of the fetus. We found that weights greater than 0.3g corresponded to lengths of 10 mm (greater than 10 days pregnant) and therefore were indicative of optimal impregnation, while ~ 0.165g corresponded to lengths of 5 mm (3-5 days pregnant) and were thus indicative of sub-optimal pregnancy (Curtis, 2010). Subjects were then categorized as either optimally (neonatal weight greater than 0.3g) or sub-optimally (neonatal weight of 0-0.3g) pregnant and mean selective aggression frequencies between these groups were compared with a t-test.

Resident-intruder test: Resident-intruder testing was conducted between 14 and 17 days into the cohabitation. Both members of the breeder pair were tested; males and females were tested in counterbalanced fashion and there was no order effect. Prior to the resident-intruder test, the member of the breeder pair that was not being tested first, was removed from the home cage and placed in a novel cage for no more than 30-mins. The test subject received either a peripheral or site-directed injection of an opiate antagonist (see below) and was

returned to the home cage for 1-hr, which was followed by a 10-min habituation in the testing room. During the habituation period, locomotor activity was recorded and the number of cage crossings during the last 10-min of this period was scored by an experimentally blind observer using Behavior Tracker 1.0 Software to determine if opiate antagonists altered general locomotor activity.

Following the habituation period, the resident-intruder test was initiated by placing a same-sex intruder into the test subject's home cage and behavioral interactions were recorded for 10-min (Gobrogge *et al.*, 2009). Same-sex intruders were chosen because previous research has shown that pair bonded animals show consistently high levels of aggression toward same-sex intruders whereas this behavior is more modest and variable with opposite-sex intruders (Firestone *et al.*, 1991b; Wang *et al.*, 1997; Aragona *et al.*, 2006). The frequency of aggressive interactions (lunges, bites, chases, offensive rears) and duration of affiliative behaviors (olfactory investigation, anogenital sniff, side by side contact) were quantified using Behavior Tracker 1.0 software. Immediately following the test, stimulus animals were removed and subjects were sacrificed via rapid decapitation and trunk blood was collected and for animals in site-specific study, brains were rapidly removed and frozen.

Peripheral drug administration: To determine if global blockade of kappa- or mu-opioid receptors alter selective aggression, either the kappa-opioid receptor antagonist (norbinaltorphimine; nor-BNI) (Portoghese *et al.*, 1987) or the preferential mu-opioid receptor antagonist (naloxone) (Magnan *et al.*, 1982) were

administered intraperitoneally (i.p.) 1-hr prior to the resident-intruder test. To control for vehicle injection, a 0.2 ml injection of sterile saline was given (n = 9 male; n = 11 female). All drugs were dissolved in sterile saline and mixed up fresh on the day of use. In addition to saline controls, we ran a separate group of handled, but not injected, controls (n = 9 male; n = 9 female) and found no behavioral difference between saline or non-injected controls (male $t(16) = 0.92$, $p = 0.37$; female $t(18) = 0.63$, $p = 0.57$) (control groups were therefore combined for statistical analysis).

The doses of nor-BNI tested were as follows: 10 mg/kg (n = 10 male; n = 10 female), 32 mg/kg (n = 10 male; n = 11 female), 50 mg/kg (n = 10 male; n = 9 female), or 100 mg/kg (n = 7 male; n = 10 female) (Broadbear *et al.*, 1994). While, the higher doses used in this study (50 and 100 mg/kg) are higher than those used in rats and mice, prairie voles remain ambulatory and show species typical social interactions at these doses. Additionally, to validate the use of high doses of nor-BNI for behavioral studies in prairie voles, we conducted additional behavioral tests using behavioral assays for which the effects of opioid drugs in other species have been well established (tests for locomotor activity and analgesia (described below).

The doses of naloxone used were as follows: 1 mg/kg (n = 11 male; n = 12 female), 10 mg/kg (n = 10 male; n = 10 female), or 30 mg/kg (n = 11 male; n = 12 female). These doses were chosen because 1 mg/kg (but not lower doses) of naloxone has been shown to decrease aggression in mice (Haug *et al.*, 1986). While this dose (1 mg/kg) has also been shown to decrease locomotor behavior

in rats (Sisti & Lewis, 2001), this dose had no effect on either locomotor activity or selective aggression in prairie voles (see below). We therefore used two higher doses, 10 mg/kg (Grimm *et al.*, 2007) and 30 mg/kg and these doses ensured that the lack of effect on selective aggression by naloxone treatment was not due to using a behaviorally ineffective dose because these higher doses significantly decreased general locomotor activity in females (see table 1).

Stereotaxic cannulation and microinfusion: Following 14 days of cohabitation, both males and females received stereotaxic surgery and then recovered together for 3 days in their home cage. Consistent with the methods established by previous studies (Liu & Wang, 2003; Aragona *et al.*, 2006), subjects were implanted with 26-guage bilateral guide cannulae (Plastics One, Roanoke, VA) anchored to the skull with stainless steel screws and dental cement and aimed at the NAc shell (+1.6 mm A/P; ±1mm M/L; -4.5 mm D/V), NAc core (+1.6 mm A/P; ±1mm M/L; -3.5 mm D/V), or the VP (+0.45 mm A/P; ± 1 mm M/L; -4.5 mm D/V). Injection sites for both the NAc and the VP corresponded to regions in which autoradiography determined that kappa-opioid receptor binding was dense (see figure 5 and 6).

On the test day, a 33-guage injector was used to infuse either artificial cerebral spinal fluid (aCSF) (male: n = 8 NAc shell, n = 6 NAc core; female: n = 6 NAc core, n = 7 NAc shell), aCSF containing 500 ng of nor-BNI (male: n = 7 NAc core, n = 8 NAc shell, n = 8 NAc VP; female: n = 7 shell, n = 6 core, n = 6 VP) or aCSF containing 1 ng of the highly selective mu-opioid receptor antagonist CTAP

(Pelton *et al.*, 1986) into the NAc shell (male n = 5; female n = 5) (Simmons and Self, 2009). Following behavioral testing, stimulus animals were removed, subjects were sacrificed via rapid decapitation, and trunk blood was obtained and brains were extracted and frozen for subsequent histological verification of cannulae placements. Only subjects whose cannulae placements were in the target region were included in the study.

Receptor autoradiography: At 90 days of age, sexually naïve male and female prairie voles were sacrificed via rapid decapitation. Brains were rapidly removed, frozen on dry ice, and stored at -80 °C. Brains were sectioned on a cryostat at 15 μm in four serial sections (i.e. 60 μm intervals) and placed back in the -80 freezer until all samples were ready to be processed. On the day of processing, slides were washed twice in room temperature 50 mM Tris-HCL (pH 7.4) for 10-mins. Samples were then incubated in either mu-opioid receptor ligand (DAMGO; cat# NET 902; lot # 3615807) for 1-hr or with the kappa-opioid receptor ligand (U69,593; cat# NET 952; lot #3615650) for 2-hrs. The incubation period was followed by a series of washes that are listed as follows: ice-cold Tris-buffer (2 x 5 minutes), chilled Tris-buffer while stirring (2 x10 minutes), dip in ice-cold distilled water (3xs), and then dried under a cool stream of air. Non-specific binding was determined by incubating a subset of slides with 1 μM naloxone for mu receptors or 1 μM nor-BNI for kappa receptors. Kodak BioMax MS Film was then laid on the slides and exposed for six months. Film images were captured using a Scan Maker 1000XL Microtek scanner.

Cortisol Assay: Previous studies have demonstrated that although nor-BNI blocks the negative behavioral and physiological consequences of stress, it does not itself decrease corticosterone levels (McLaughlin *et al.*, 2006a). Given that this was the first study using nor-BNI in prairie voles and the aversive nature of selective aggression, we determined the relationship between the actions of nor-BNI on corticosterone in prairie voles as there is in mice. Following rapid decapitation, trunk blood was collected from each subject in tubes containing heparin-coated rings and placed on ice until all behavioral tests for that day were complete (maximum of 4-hrs). Samples were centrifuged at 2000g for 20-mins at 4°c and plasma was immediately frozen. Plasma (5μl plasma/10mL buffer) was later assayed for corticosterone using a radioimmunoassay (MP Biomedicals Corticosterone (Rat) Cat. No. 07-120103). Given that this is the first time the MP Biomedical corticosterone antibody has been used for prairie voles it was analytically validated for use on this species. The slope of the line for serial dilutions of vole serum (plotted as dose per unit volume) was both linear (R^2 = 0.994, $p < 0.0001$) and parallel to the standard curve. Mean accuracy (determined by spiking kit standards with a high and a low vole serum sample) was 95.9% for the high sample (n = 6) and 94.0% for the low sample (n = 6). The intraassay CV (determined by running 6 duplicates in the same assay) for a high kit control was 3.1% (n = 6) and a low kit control was 5.1% (n = 6). The interassay CV for a high and low kit control was 5.7% (n = 4) and 6.3% (n = 4), respectively.

Testosterone Assay: In other species, testosterone is known to regulate aggressive behavior (Edwards, 1968; Bronson & Desjardins, 1969; Schuurman, 1980). To determine if testosterone is important for the display of selective aggression in pair bonded prairie voles, plasma samples were collected from control subjects (male n = 14; female n = 18) and processed as described above and assayed for testosterone (Calbiotech Testosterone 96 well ELISA kit. (mouse/rat) Cat. No. TE187S-100).

Validation of kappa-opioid receptor drug dosing: As our initial behavioral pharmacology testing indicated that prairie voles require high doses of kappa-opioid receptor drugs, we determined the behaviorally effective dose of nor-BNI necessary to block well established motor inhibitory and analgesic effects of a selective kappa-opioid receptor agonist in prairie voles. The use of these validated behavioral assays in prairie voles allowed us to compare the response to kappa-opioid receptor drugs in prairie voles to those of other species with established dose response curves.

The selective kappa-opioid receptor agonist, U50,488 (Von Voigtlander & Lewis, 1982), was dissolved in sterile saline and administered by i.p. injection at a dose of 0 mg/kg (male n = 7; female n = 7), 5 mg/kg (male n = 7; female n = 7), 10 mg/kg (male n = 8; female n = 6), or 25 mg/kg (male n = 5). The 25 mg/kg dose was not tested in females because a significant effect was achieved following a 10 mg/kg administration of U50,488. The doses of U50,488 used in the present study were chosen because they have previously been demonstrated to decrease motor activity (Ukai & Kameyama, 1985; Schnur & Walker, 1990;

Leyton & Stewart, 1992; Brent, 1993; Kuzmin et al., 2000; Mague et al., 2003) and enhance analgesia (Kuzmin et al., 2000; McLaughlin et al., 2006a) in other species.

Following identification of the dose of U50,488 that significantly decreased locomotor activity (25 mg/kg for males and 10 mg/g for females), subjects were administered i.p. injections of nor-BNI 1-hr prior to U50,488 at a dose of 0 mg/kg (male n = 6; female n = 6), 10 mg/kg (male n = 6; female n = 6), 50 mg/kg (male n = 9; female n = 6), or 100 mg/kg (male n = 7, female n =6). Control subjects received saline injections 1-hr prior to and immediately before testing (male n = 11; female n = 6). Locomotor activity was assessed with an open field test and analgesia was measured with a tail flick assay.

Open field and tail flick: For open field testing, subjects were placed in a 75 x 25 cm open field chamber for1-hr immediately following drug administration (Kuzmin et al., 2000; Mague et al., 2003). Behavior during the open field test was recorded and later scored for the duration of time spent in forward locomotion and wall climbing. The total duration spent in forward locomotion and wall climbing were combined for a measure of total activity. For tail flick, antinociceptive measurements were made 1-hr following administration of U50, 488 by immersing the tail in 55° Celsius water for a maximum of 15 s (McLaughlin et al., 2006a; McLaughlin et al., 2006b). Because baseline analgesia measures of male and female prairie voles were higher than that reported for other species (Butelman et al., 1993; McLaughlin et al., 2006a) C57BL/6 mice were also tested under control conditions (i.e. 1-hr following an i.p.

injection of saline). Adult male (n = 5) and female (n = 7) C57BL/6 mice were housed in a 12L/12D reverser light cycle with *ad lib* food and water. See tables 4 and 5 as well as figure 8 for detailed analysis. Briefly, these experiments confirm that high doses of kappa-opioid receptor drugs are needed for behavioral manipulations using prairie voles.

Statistics: A linear regression analysis was computed to determine the relationship between neonatal weight and mating and to determine if neonatal weight was associated with levels of selective aggression. A linear regression analysis was also used to determine the relationship between neonatal weight and mating onset as well as the relationship between hormone (corticosterone and testosterone) levels and aggression and pregnancy stage. Since previous studies have demonstrated that male prairie voles are generally more aggressive than females (Winslow *et al.*, 1993), we used a t-test to compare attack frequencies during resident-intruder testing between control males and females. Regarding pharmacoloigical manipulations of selective aggression, it was hypothesized that blockade of kappa, but not mu, opioid receptors would interfere with selective aggression. Therefore, a one-way ANOVA followed by a Tukeys post-hoc test was used to assess the pharmacological effects of peripheral opioid receptor blockade on selective aggression. Additionally, since multiple doses were used, a series of planned contrast was used to determine if peripheral opioid receptor blockade decreased aggression in a dose-dependent manner (Zhang & Kelley, 1997). For site-specific comparisons, it was hypothesized that blockade of kappa-opioid receptors within the NAc shell, but

not other regions, would significantly decrease aggression compared to saline controls. Given the Levene test of homogeneity of variances failed for site-specific data, a one-way ANOVA could not be used and thus a U Mann-Whitney test (Stribley & Carter, 1999) was conducted to test if site-directed blockade of opioid receptors decreased aggression levels compared to controls. Plasma corticosterone levels following nor-BNI treatments were compared to controls using a one-way ANOVA followed by a Tukeys post hoc-test. A one-way ANOVA followed by a Tukeys post hoc test was also used to determine the effects of a kappa-opioid receptor agonist or anatagonist on locomotor activity and analgesia. A t-test was used to compare sex and species differences in baseline analgesia. All analysis was performed with the use of SPSS Statistics 17.0 software.

RESULTS

Fecundity and Pair Bonding

While prairie voles are socially monogamous, males of this species display important individual variation in reproductive strategy (Getz *et al.*, 1993; Solomon *et al.*, 2009). In natural prairie vole populations, approximately one-half of males 'wander' across multiple female territories attempting to mate with multiple females (Getz *et al.*, 1993; Solomon & Jacquot, 2002) and the biological basis of this variation continues to be studied (Fink *et al.*, 2006; Young & Hammock, 2007; Ophir *et al.*, 2008; Mabry *et al.*, 2011). It has recently been shown that environmental factors also contribute to mating strategy in this

species as male prairie voles do not show partner preferences after long term cohabitation unless pregnancy is established soon after pairing (Curtis, 2010). However, the relationship between fertility and selective aggression has never been formally assessed. In previous studies of selective aggression, pairs that failed to achieve pregnancy following a two-week cohabitation were simply excluded from the studies (Aragona *et al.*, 2006; Gobrogge *et al.*, 2009). Here, we provided the first examination of the relationship between pregnancy and selective aggression by comparing neonatal weight at the time of testing to attack frequency in both male and female subjects.

In males, neonatal weight was positively correlated with attack frequency, $R^2 = 0.28$, $F(1,19) = 7.55$, $p = 0.01$ (Fig 6A). Consistent with a previous study (Curtis, 2010), pregnancy was considered 'optimal' if offspring size indicated that the pair achieved behavioral estrus, ovulation, and fertilization with minimal delay following the onset of the cohabitation. This corresponded to an average neonatal weight of greater than 0.30g at the time of resident-intruder testing. Males whose females were in an optimal stage of pregnancy were significantly more aggressive than males whose females were sub-optimally pregnant $t(19) = 2.67$, $p = 0.02$ (Fig 6B). However, there was no difference in selective aggression in female subjects depending on optimal vs. sub-optimal pregnancy $t(22) = 0.78$, $p = 0.44$ (Fig 6D). There was also no correlation between neonatal weight and aggression in females $R^2 = 0.01$, $F(1,22)$ $p = 0.59$ (Fig 6C). This is similar to previous laboratory studies showing that long-term cohabitation, but not mating, was correlated with aggression in females (Bowler et al., 2002) as well

as data from the field which show no correlation between stage of pregnancy and wounding (a proxy for aggressive encounters) (Rose & Gaines, 1976). These data suggest that it is adaptive for males, but not females, to form pair bonds depending on reproductive success. This is reasonable if indeed the function of selective aggression is primarily mate guarding to increase assurance of paternity. Conversely, the decision for females to bond may be heavily based on the degree of male investment, which is held constant under the preset experimental conditions (a continual presence for two weeks). Thus, more naturalistic circumstances may be needed to examine variation in selective aggression shown by females.

As we describe below, an important component of this study was to determine if opioid receptor manipulations altered corticosterone levels. Additionally, the presence of acute aversive stimuli, including those of a social nature, increase plasma corticosterone levels (Schuurman, 1980; Buwalda et al., 2011). The resident-intruder paradigm can also be aversive in nature and this also provided a need for the relationship between selective aggression and corticosterone levels to be determined in the present study. In males, attack frequency was positively correlated with plasma corticosterone levels $R^2 = 0.33$, $F(1,17) = 8.23$, $p = 0.01$. However, there was no relationship between attack frequency and plasma corticosterone in females $R^2 = 0.005$, $F(1,21) = 0.12$, $p = 0.74$ (data not shown).

Additionally, because testosterone has been implicated in general aggression in other species (Beeman, 1947; Edwards, 1968; Bronson &

Desjardins, 1969; Schuurman, 1980), the relationship between selective aggression and plasma testosterone was also assessed. There was no relationship between plasma testosterone and attack frequency in males R^2 = 0.004, $F(1,13) = 0.06$, $p = 0.82$ or females $R^2 = 0.16$, $F(1,17) = 3.15$, $p = 0.09$ (data not shown). This is consistent with previous reports that testosterone does not mediate selective aggression in pair bonded prairie vole (Carter and Getz, 1993). These data support the contention that this behavior is not representative of generalized aggression. Rather, selective aggression is indicative of the behavioral transformation that is associated with the development of a pair bond.

Effect of peripheral blockade of opioid receptor on selective aggression

Although there are quantitative differences in selective aggression between male and female prairie voles, both sexes show this behavior (Getz *et al.*, 1981). Initially, sexually naïve prairie voles are quite affiliative toward novel conspecifics (Aragona et al., 2006). However, once pair bonded, such affiliation is directed more selectively toward familiar conspecifics, especially the breeding partner. Thus, there is a behavioral transition in selective affiliation and aggression with non-pair bonded voles being generally affiliative and then becoming more aggressive once pair bonded (Fig 7) (Getz, 1978; Carter & Getz, 1993; Young *et al.*, 1998; Aragona *et al.*, 2006; Aragona & Wang, 2009). In the laboratory, resident-intruder testing using unfamiliar same sex stimulus animals is used to quantify selective aggression in both male and female prairie voles (Carter *et al.*, 1997) and provides a quantification of mate guarding and thus pair bond maintenance. Consistent with previous studies that have shown that males

are more aggressive than females (Gavish *et al.*, 1981; Firestone *et al.*, 1991a; Carter *et al.*, 1997), we found a significant sex difference in the magnitude of selective aggression with control males showing significantly greater attack frequency compared to control female subjects (Fig 7D; $t(58) = 2.97$, $p = 0.004$).

Given that selective aggression is a form of aversive motivation and that kappa-opioid receptors mediate aversion (van Ree *et al.*, 1999; Le Merrer *et al.*, 2009), we hypothesized that these receptors would be important for selective aggression whereas mu-opioid receptors (i.e. receptors that mediate reward) would not be involved in this behavior. Consistent with this hypothesis, peripheral injections of the kappa-opioid receptor antagonist nor-BNI dose dependently reduced the frequency of attacks in male prairie voles $F(4,50) = 3.22$, $p = 0.02$ (Fig 8A). Post hoc tests revealed that animals receiving the highest dose of nor-BNI showed significantly lower levels of selective aggression $p = 0.02$ and planned contrast comparisons indicated that nor-BNI decreased aggression in a linear manner $t(50) = -3.40$, $p = 0.001$ (Fig 8A). Groups did not differ in affiliation $F(4,50) = 1.30$, $p = 0.28$ (Fig 8C) or general locomotor activity $F(4,50) = 0.69$, $p = 0.08$ (Table 3). In contrast to kappa-opioid receptor blockade, blockade of mu-opioid receptors in males with the preferential mu-opioid receptor antagonist, naloxone, had no effect on attack frequency $F(3,46) = 0.71$, $p = 0.55$ (Fig 9A), attack latency $F(3,46) = 2.3$, $p = 0.90$ (Fig 9B), affiliative behavior $F(3,46) = 0.33$, $p = 0.80$ (Fig 9C), or locomotor behavior $F(3,46) = 0.21$, $p = 0.89$ (Table 3).

In females, peripheral administration of nor-BNI did not significantly alter attack frequency $F(4,54) = 1.65$, $p = 0.19$ (Fig 8D). However, nor-BNI caused a significant increase in latency to attack $F(4,54) = 2.89$, $p = 0.03$ (Fig 8E), although post hoc tests did not identify a specific dose that was most effective. There was no effect on affiliative behavior $F(4,54) = 0.60$, $p = 0.67$ (Fig 8F) or locomotor activity $F(4,54) = 2.07$, $p = 0.10$ (table 3). Naloxone had no effect on selective aggression in females $F(3,50) = 1.03$, $p = 0.40$ (Fig 9D) and this was despite that the higher dose of naloxone caused a significant decrease in locomotor activity in females $F(3,50) = 4.75$, $p = 0.005$ (table 3).

The behaviorally effective dose of nor-BNI (100 mg/kg) (i.e. the dose that decreased selective aggression) was 10-fold higher than doses reported to be effective in other rodent species (mice and rats) (Lindholm et al., 2001; McLaughlin et al., 2003; McCurdy et al., 2006; Zhang et al., 2007). To validate the need for higher doses in prairie voles, we determined the dose of nor-BNI that reversed the inhibitory motor effects of the kappa-opioid receptor agonist U50,4888 in an open field apparatus.

In males, the highest dose of U50,488 (25 mg/kg) significantly decreased total activity in the open field test $F(3,26) = 5.69$, $p = 0.009$ (Fig 8C) and this was reversed by 50 mg/kg $F(4,38) = 9.335$, $p = 0.328$ and 100 mg/kg nor-BNI $p = 0.246$, but not 10 mg/kg nor-BNI $p > 0.0001$ (Fig 8E). Thus, higher doses of nor-BNI are needed to alter motor activity compared to other rodent species (Lindholm et al., 2001; McLaughlin et al., 2003; McCurdy et al., 2006; Zhang et al., 2007). Behavioral assays of analgesia also demonstrated species

differences in kappa pharmacology. Voles have higher baseline analgesia compared to mice (Table 4) and higher doses of the kappa agonist were needed to enhance anti-nociception (McLaughlin et al., 2006a).

In females, a lower dose of U50,488 was needed to decrease locomotor activity than was necessary compared to how this drug impacted male prairie voles. Specifically, 10 mg/kg U50,488 significantly decreased total activity $F(2,19) = 7.05$, $p = 0.02$ (Fig 8H) and this decrease was reversed by 50 mg/kg $F(4,29) = 10.14$ $p = 0.46$ and 100 mg/kg nor-BNI $p = 0.76$, but not 10 mg/kg $p > 0.0001$ (Fig 8J). With respect to the tail withdrawal assay, female prairie voles showed a significantly higher baseline level of analgesia compared to male prairie voles $t(12) = 2.322$, $p = 0.0386$ (table 4) as well as female C57BL/6 mice $t(12) = 3.380$, $p = 0.0055$ (Table 4). Moreover, in contrast to males, peripheral administration of the kappa-opioid receptor agonist had no effect on the tail withdraw latency at any of the doses tested $F(2,19) = 1.97$, $p = 0.17$ (table 4) and this sex difference is consistent with rats (Craft & Bernal, 2001) and rhesus monkeys (Negus & Mello, 1999). Together, these data demonstrate that male and female prairie voles require higher doses of nor-BNI compared to rats and mice for behavioral studies.

Kappa- and mu-opioid receptor binding patterns in prairie voles

The peripheral manipulations of selective aggression described above demonstrate that kappa, but not mu-opioid receptors mediate this behavior. To ultimately identify the central location of kappa-opioid receptor regulation of selective aggression, it was first necessary to determine the distribution of opioid

receptors within prairie vole brains. Since very limited knowledge exists with respect to opioid receptor distributions in the vole brain (Insel & Shapiro, 1992; Burkett *et al.*, 2011), we conducted a detailed analysis of mu- and kappa-opioid receptor binding for this species.

Overall, kappa-opioid receptor binding is quite sparse compared to mu-opioid receptor expression (compare Figs 10 and 11). However, consistent with other species, including humans (Mansour *et al.*, 1987; Quirion *et al.*, 1987; Mansour *et al.*, 1988), there is dense kappa-opioid receptor binding throughout the striatum. Kappa-opioid receptor binding in prairie voles is similar to that of other rodents (guinea pig, rabbit, mouse, and rat) (Robson *et al.*, 1985).

In rostral portions of the striatum (i.e. rostral to the corpus callosum genu), kappa-opioid receptor binding is prominent within the dorsal striatum and very dense within the ventral striatum, in particular the NAc shell and olfactory tubercle (Fig 10A). There is also kappa-opioid receptor binding in the claustrum at this rostro-caudal level (Fig 10A). Within more caudal regions of the striatum (nearing the genu of the corpus callosum), kappa-opioid receptor binding within the dorsal striatum is less pronounced (particularly within the dorsomedial striatum) whereas binding within the NAc shell and OT remains quite dense (Fig 10B).

There is also a moderate level of kappa-opioid receptor binding within the rostral VP (Fig 10C, D), which is notable since this region is important for vasopressin regulation of pair bonding (Lim & Young, 2004). Kappa-opioid receptor binding is also present in caudal VP as well as the external globus

pallidus (aka the dorsal pallidum) (Fig 10E). In contrast to rats, prairie voles do not have high densities of kappa-opioid receptor binding in the hypothalamus (Robson *et al.*, 1985). Additionally, kappa-opioid receptors are quite strongly expressed within the substantia nigra pars reticulata (Fig 10G) and posterior medial cortical amygdala (Fig 10G). Finally, within cortical regions, light kappa-opioid receptor binding occurs in the insular cortex and regions of the somatosensory cortex (Fig 10A-F). Overall, these data suggest that the kappa-opioid receptor binding pattern of prairie voles is more similar to that of guinea pigs whose kappa-opioid receptor binding sites are found primarily in the striatum and cortex and differ from mice and rats who have high densities of kappa-binding in the midbrain and hypothalamus (Robson *et al.*, 1985).

Relative to kappa-opioid receptor expression, mu-opioid receptors are much more widely spread throughout the prairie vole brain. A previous study provided a very cursory initial description of mu-opioid receptor distributions for prairie voles (Insel & Shapiro, 1992) and a more recent study describes the distribution of mu-opioid receptors within the striatum (Burkett *et al.*, 2011). Here, we significantly extend these previous findings by providing the first description of mu-opioid receptor binding throughout the prairie vole brain (Figure 11). Within the rostral striatum there is dense mu-opioid receptor binding in the dorsal striatum as well as the NAc core and NAc shell (Fig 11). Unlike kappa-opioid receptors, there is no mu-opioid receptor binding in the claustrum or olfactory tubercle (Fig 11A, B). Importantly, as in other rodent species (Pert *et al.*, 1976; Herkenham & Pert, 1981; Gerfen & Young, 1988; Mansour *et al.*, 1994;

Brown *et al.*, 2002; Crittenden & Graybiel, 2011), a clear patch-matrix pattern of mu-opioid receptor distribution can be seen throughout the striatum (Fig 11A, B, C). Voles also show typical variability in patch size and most patches located in the dorsal striatum. There is also variability of mu-opioid receptor density in the ventral striatum, including robust mu-opioid receptor binding in the dorsomedial NAc shell (Fig 11B). In contrast to a previous study which claimed that mu-opioid receptor binding was found within the VP (Insel & Shapiro, 1992), we saw no mu-opioid receptor binding within this region (Fig 11C, D). Moderate mu-opioid receptor binding was present in the lateral septum, which is of interest because this region is involved in pair bonding (Fig 11B, C) (Liu *et al.*, 2001). This binding pattern contrast that of other rodent species, such as rats, who have mu-opioid receptors in the medial septum, but not in the lateral septum (Mansour *et al.*, 1987). Mu-opioid receptor binding was also seen in the interstital nucleus of the posterior limb of the anterior commissure, amygdala/striatum transition zone (Fig 11D, E), and the endopiriform nucleus (Fig 11).

Consistent with rats, mu-opioid binding within the hypothalamus is light and is present in the ventromedial nucleus, dorsomedial nucleus, and lateral hypothalamic area (Fig 11F) (Mansour *et al.*, 1987). The general distribution of mu-opioid receptors in the thalamus overlaps with that of rats (Mansour *et al.*, 1987) and can be seen in the mediodorsal, intermediodorsal, centromedial, paracentral, rhomboid, reuniens, and ventromedial thalamic nuclei as well as light opioid receptor binding within the zona incerta (Fig 11E,F). There is dense mu-opioid receptor binding within the medial habenula and light mu binding

within the lateral habenual and fasciculus retroflexus of the habenula (Fig 1 F). There is also substantial mu-opioid receptor binding in the posterior medial cortical amygdaloid nucleus of the midbrain (Fig 11G). Mu-opioid receptor binding is also found within sensory processing systems, the superior colliculus (visual) and medial geniculate nucleus (auditory) (Fig 11G). There is very dense expression of mu-opioid receptors within sub-regions of the ventral tegmental area including the paranigral nucleus (Fig 11G), the caudal and lateral interpeduncular nuclei (Fig 11G) and additional binding within the medial nuclei of the A10 region, such as the interfascicular nucleus (Fig 11G). There is only light binding within the pariaquiductal gray and substantia nigra, which is consistent with other rodent species (Mansour *et al.*, 1988) (Fig 11G). Similar to rabbits and guinea pigs, very little mu-opioid receptor binding is seen in the hippocampus (Robson *et al.*, 1985). Finally, within cortical regions, mu-opioid receptor binding occurs in the cingulate, entorhinal, and striate cortex (Fig 11G).

Region specific kappa regulation of selective aggression

The peripheral behavioral pharmacology experiments described above indicate that kappa- but not mu-opioid receptors mediate selective aggression. However, it is difficult to interpret data resulting from peripheral injections of antagonists because this manipulation blocks receptors globally in both the peripheral and the central nervous system (Wittert *et al.*, 1996). Further, opioid receptors are distributed across many brain regions that differ greatly in their regulation of behavior (Mansour *et al.*, 1987; 1988; Mansour *et al.*, 1994). In prairie voles, kappa-opioid receptors are densely expressed within two brain

regions that are very important for pair bonding, the NAc and VP (Fig 10). To determine if kappa-opioid receptors within these brain regions mediate selective-aggression, we selectively blocked kappa-opioid receptors within these regions (Fig 12A) of pair bonded prairie voles prior to resident-intruder tests and measured the corresponding effects on selective aggression.

Control subjects that received aCSF infusions into the NAc shell, NAc core, or the VP showed robust selective aggression and males showed significantly higher attack frequency compared to females (Fig 12B; $t(30) = 2.32$, $p = 0.03$). There was no difference between control injections of aCSF between these brain regions in males $F(2,15) = 0.68$, $p = 0.52$ or females $F(2,15) = 1.52$, $p = 0.26$. Therefore, data from these regions were combined to generate an aCSF control group (male: n = 16; female: n = 16).

In males, Mann-Whitney U Test for nonparametric data revealed a significant decrease in aggression when kappa-opioid receptors were blocked in the NAc shell $U = 20.50$, $p = 0.008$ (Fig 12C). However, the kappa-opioid receptor antagonist had no effect when infused into the NAc core $U = 46.50$, $p = 0.55$, or VP $U = 44.00$, $p = 0.24$ (Fig 12C). Site-directed infusion of nor-BNI had no effect on attack latency $U = 49.50$, $p = 0.40$ (Fig 12D), affiliative behavior $U = 49.00$, $p = 0.37$ (Fig 12E), or locomotor activity $U = 43.50$, $p = 0.92$ (table 6). Thus, site-specific behavioral pharmacology identified kappa-opioid receptors within the NAc shell — a key brain region in mediating unconditioned motivational responses (Ikemoto & Panksepp, 1999; Kelley & Berridge, 2002) — as important for aversive social motivation in pair bonded prairie voles.

In contrast to the sex differences following global blockade of kappa-opioid receptors (Fig 12), blockade of kappa-opioid receptors within the NAc shell U = 21.00, p = 0.02 also prevented selective aggression in females. However, unlike males, nor-BNI injections into the NAc core significantly increased this behavior (Fig 12F; U = 20.5, p = 0.05). As with males, kappa-opioid receptor blockade within the VP had no effect on selective aggression in females U = 45.50, p = 0.88. In females, central infusions of nor-BNI showed no significant effects on attack latency U = 32.00, p = 0.15 (Fig 12G), affiliation levels U = 32.00, P = 0.82 (Fig 12H), or locomotor behavior U = 33.50, p = 0.32 (table 6). Together, these data show that kappa-opioid receptors within the NAc shell mediate selective aggression in both male and female prairie voles.

The kappa-opioid receptor antagonist used in this study, nor-BNI, also has affinity for mu-opioid receptors initially following its delivery (Endoh *et al.*, 1992). Thus, our site-directed nor-BNI data alone do not rule out the possible involvement of mu-opioid receptors within the NAc shell. To test if the reduction of selective aggression by nor-BNI was due to blockade of mu-opioid receptors, a separate experiment was conducted in which the highly selective mu-opioid receptor anatagonist, CTAP (Crain & Shen, 1992; Nestler, 1993), was infused into the NAc shell prior to resident-intruder testing. Consistent with the peripheral study using naloxone, blockade of mu-opioid receptors directly in the NAc shell had no effect on selective aggression in males (U = 35.00, p = 0.36) or females (U = 32.50, p = 0.56) (data not shown).

Finally, since our data indicate that kappa-opioid receptors within the NAc shell mediates selective aggression through modulation of aversive social motivation, and aversive stimuli are known to increase corticosterone signaling, we determined if blockade of kappa-opioid receptors alters selective aggression indirectly though a reduction in corticosterone (DeVries *et al.*, 1996; Bosch *et al.*, 2009). As determined previously, plasma coticocsterone levels of male and female prairie voles were high compared to other rodent species (DeVries *et al.*, 1997; Taymans *et al.*, 1997; Campbell *et al.*, 2009) (Table 7 and 8). Consistent with studies in other rodent species (McLaughlin *et al.*, 2006a), nor-BNI had minimal to no effect on plasma corticosterone levels. In males, there was no difference between the plasma corticosterone levels of control subjects and those that had received peripheral nor-BNI $F(2,26)$, $p = 0.15$ (table 7) or nor-BNI infused centrally $F(2,23)$, $p = 0.66$ (table 8). In females, due to a slight increase in corticosterone following peripheral nor-BNI, the overall ANOVA for subjects in the peripheral study was significant $F(2,28)$, $p = 0.04$ (table 7), but post hoc tests revealed no significant differences between control subjects and those treated with nor-BNI. Similar to males, there was no difference in plasma corticosterone between females who had received site-specific administration of aCSF or nor-BNI in the NAc core or shell $F(2,22)$, $p = 0.24$ (Table 8). Together, these data suggest that nor-BNI does not reduce selective aggression through changes in corticosterone signaling.

DISCUSSION

While sexually naïve prairie voles are initially highly affiliative toward novel conspecifics, once pair bonded, they show selective aggression toward unfamiliar conspecifics and this is indicative of mate guarding behavior necessary for pair bond maintenance. Mate guarding is especially adaptive for males as it helps to ensure paternity as well as prevent pregnancy termination (Stehn, 1981; Heske, 1984; Wolff & Dunlap, 2002). In the laboratory, mate guarding is studied using resident-intruder tests of selective aggression (Carter & Getz, 1993) and this behavior represents a circumstance in which social stimulation from a novel conspecific generates negatively valenced motivational behavior, aggressive rejection, that is herein regarded as 'aversive'. Here, we demonstrate that activation of kappa-opioid receptors (known to mediate aversion) (Mucha & Herz, 1985; Pfeiffer *et al.*, 1986), but not mu-opioid receptors (known to mediate reward and positive hedonics), regulate selective aggression. These effects are specific to the NAc shell, a component of brain motivational circuitry that is critical for neural processing of both social bonding (Li & Fleming, 2003; Champagne *et al.*, 2004; Aragona *et al.*, 2006; Aragona & Wang, 2007) as well as unconditioned incentives, including those of an aversive nature (Kalivas & Duffy, 1995; Ikemoto & Panksepp, 1999; Kelley & Berridge, 2002; Everitt & Robbins, 2005; Becker, 2009). As such, the current data suggest that kappa-opioid receptors within this region may facilitate the tagging of social stimuli as aversive and cause novel conspecifics to be aggressively rejected.

Kappa-opioid receptors mediate pair bond maintenance

Peripheral blockade of kappa- (but not mu) opioid receptors prevented aversive social motivation in pair bonded male prairie voles as indicated by a decrease in selective aggression. It is not surprising that peripheral blockade of mu-opioid receptors failed to inhibit selective aggression since previous studies have demonstrated that blockade of these receptors is aversive (van Ree et al., 1999; Skoubis et al., 2001; Le Merrer et al., 2009). In contrast to negative affective states induced by mu-opioid-receptor blockade, activation of these receptors is associated with positive hedonics and mediates the rewarding properties of positive social incentives (Pecina & Berridge, 2000) such as play and contact comfort (Panksepp *et al.*, 1980; Vanderschuren *et al.*, 1995; Trezza *et al.*, 2011). Moreover, activation of mu-opioid receptors is important for the early stages of pair bond formation as blockade of these receptors within the striatum inhibits the formation of a partner preference (Burkett *et al.*, 2011). This is especially interesting given the relationship between mu-opioid receptors and D_2-like receptors, which also facilitate pair bond formation (Gingrich *et al.*, 2000; Aragona *et al.*, 2006). Enkephalin, an endogenous ligand for mu-opioid receptors, is found in D_2-expressing medium spiny neurons and stimulation of D_2-like receptors decreases enkephalin (Gerfen *et al.*, 1990). Thus, it is possible that these systems interact to mediate naturally rewarding pro-social behaviors that are necessary for pair bond formation (Aragona *et al.*, 2009), while those that mediate negative affect and stress are important for aversive social encounters that are important for maintaining the bond.

Unlike mu-opioid receptors, kappa-opioid receptors antagonize reward (Shippenberg *et al.*, 1996; Carlezon & Miczek, 2010; Wee & Koob, 2010), and are thus a candidate to mediate aversive motivational social interactions, such as aggressive interactions. Indeed, peripheral blockade of kappa-opioid receptors decreased selective aggression in male prairie voles identifying the importance of this system in aversive social motivation. Consistent with previous studies, males were more aggressive than females (Gavish *et al.*, 1981) and higher levels of aggression in males may be associated with uncertain paternity (Werren *et al.*, 1980). Indeed, if males do not engage in mate guarding, some paired female prairie voles will mate with novel males (Wolff *et al.*, 2002). Thus, males risk devoting time and energy into offspring that are not their own if they allow another male to enter their territory. Consistent with this, we found that pregnancy was positively correlated with selective aggression, indicating that motivation to guard females increases if the reproductive potential is known to be high (Curtis, 2010).

Pair bond maintenance is mediated by kappa-opioid receptors in the NAc shell

The maintenance of a pair bond requires mate guarding which is associated with novel social stimuli to be processed as aversive. Previous studies have shown that aversive social motivation in prairie voles is mediated by activation of D_1-like receptors within the NAc shell as blockade of these receptors prevents selective aggression (Aragona *et al.*, 2006). Here we show that kappa-opioid receptors within this region are also critical for this behavior as blockade of kappa- but not mu-opioid receptors specifically within the NAc shell mediate this

behavior in both sexes. Given that kappa-opioid receptors within the NAc shell are important for processing aversion, the present study suggests that these receptors are important for signaling negative social incentives and may be key to generating the aversive motivation toward an unfamiliar conspecifics expressed by pair bonded prairie voles.

While the NAc shell is well known to play a critical role in the processing of unconditioned rewarding and aversive stimuli (Ikemoto & Panksepp, 1999; Ito *et al.*, 2000; Everitt & Robbins, 2005; Aragona *et al.*, 2008; Aragona *et al.*, 2009), the neural mechanisms within this brain region that a promote approach vs avoidance behavior are not well understood. For example, both unconditioned rewarding and aversive stimuli increase DA in the NAc shell (Kalivas & Duffy, 1995; Ikemoto, 2007) and this increase is associated with aspects of both approach and avoidance behaviors (Ikemoto & Panksepp, 1999; DiChiara *et al.*, 2004; Oleson *et al.*, 2012). Thus, while aversive chemical stimuli have been shown to decrease DA transmission in the NAc shell (Roitman *et al.*, 2010; Wheeler *et al.*, 2011), there are many conditions in which DA release in the NAc shell is critical for the attribution of motivational salience to unconditioned salient stimuli regardless of the valence (Kelley & Berridge, 2002). While recent data have demonstrated that different subsets of DA containing neurons within the VTA are activated by rewarding vs. aversive stimuli (Brischoux *et al.*, 2009; Matsumoto & Hikosaka, 2009; Bromberg-Martin *et al.*, 2010; Lammel *et al.*, 2011), it has yet to be determined how this may be associated with social incentives.

The present study suggests that for aversive motivation, kappa-opioid receptor activation is necessary to tag a conspecific with aversive motivational salience. Indeed, in addition to enhancing DA release within the NAc shell, exposure to stress is known to increase prodynorphin signaling (Chartoff *et al.*, 2009) and activation of kappa-opioid receptors within this region (Land *et al.*, 2008). Together, data from the current study and previously published work suggest that novel conspecific exposure to pair bonded voles increases DA transmission within the NAc shell which then activates D1-like receptors which may promote dynorphin release (Carlezon *et al.*, 1998). Subsequent kappa-opioid receptor activation may then directly facilitate the perception of the stimulus as aversive (Heijna et al 1990; Spanagel et al 1992).

While blockade of kappa-opioid receptors within the NAc shell decreased selective aggression in both sexes, blockade of these receptors within the NAc core had sex specific effects. Blockade of kappa-opioid receptors within the NAc core increased aggression in females, but had no effect on the behavior of males. Previous studies suggest that DA transmission within this region is not important for pair bonding (Aragona *et al.*, 2006). However, females were not studied in these previous experiments (Aragona et al 2006) and since the NAc core receives direct input from the NAc shell (van Dongen *et al.*, 2005), it is possible that increased DA transmission in the NAc shell drives core-mediated behavior important for pair bonding in females. Studies using rats have indeed implicated the NAc core in sex differences in other motivated behavior (van Haaren & Meyer, 1991; Li *et al.*, 2004; Wissman *et al.*, 2011). However,

additional experiments in voles are needed to determine if the NAc core is important for female pair bonding.

Conclusion

Although negative affective states induced by kappa-opioid receptor activation are usually associated with maladaptive conditions such as depression (Shirayama *et al.*, 2004; Carlezon *et al.*, 2006; Chartoff *et al.*, 2011), anxiety (Knoll *et al.*, 2007), or drug-related behaviors (Bruchas *et al.*, 2010; Schindler *et al.*, 2010; Walker *et al.*, 2011), acute activation of this system evolved to signal avoidance of potentially harmful stimuli (Amit & Galina, 1988; Yamada & Nabeshima, 1995; Bruchas *et al.*, 2007; Land *et al.*, 2008). This suggests that kappa-opioid receptor activation plays a critical role in encoding aversive properties of environmental stimuli. A similar phenomenon may also occur in pair bonded voles in that activation of kappa-opioid receptors may signal when a social stimulus should be avoided or prevented from entering a home territory. Thus, activation of aversive processing systems has adaptive properties and the neurobiology of aversive social motivation can be reliably studied using prairie vole pair bonding. Such studies are important because neural mechanisms that evolved to invigorate adaptive behavioral responses to aversive stimuli can also negatively impact mental health under conditions of chronic or abnormal activation.

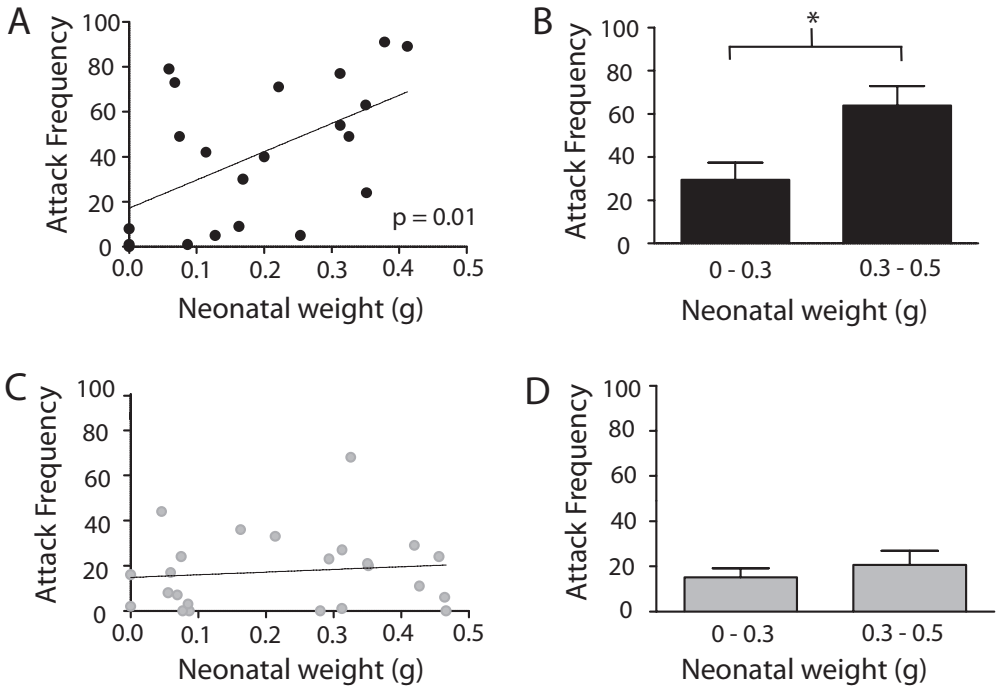

Figure 6

Selective aggression in males is associated with the pregnancy status of the female. **A, B**, Aggression in males is related to neonatal weight at the time of testing (n = 21; **A**), and males whose females are farther along in gestation are more aggressive than males (n = 14) whose females have more recently become pregnant (n = 7;**B**). C,D, Conversely, aggression levels in females has no relation to pregnancy status (n = 24; C) as females who are farther along in pregnancy (n = 14) do not become more aggressive than females who have more recently become pregnant (n = 7; D). *p < 0.05. Error bars indicate mean ± SEM.

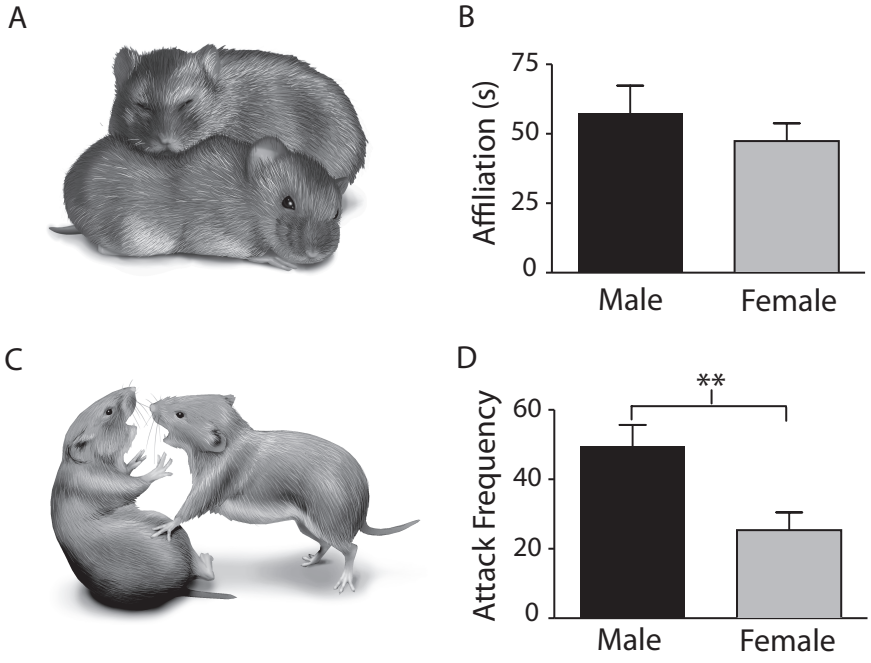

Figure 7

Selective affiliation and selective aggression in male and female prairie voles. **A**, Diagram of prairie vole engaging in one type of affiliatve behavior measured in the present study. **B**, Pair-bonded male (n = 18) and female (n = 21) prairie voles do not differ in the amount of social affiliation with novel individuals. **C**, Pair-bonded prairie vole (right) lunging at a resident intruder (left) who responds by displaying a characteristic submissive posture. **D**, Following pair bond formation, both sexes of the breeding pair show selective aggression to novel conspecifics. However, males of the breeding pair become significantly more aggressive than females. **$p < 0.005$. Error bars indicate mean ± SEM.

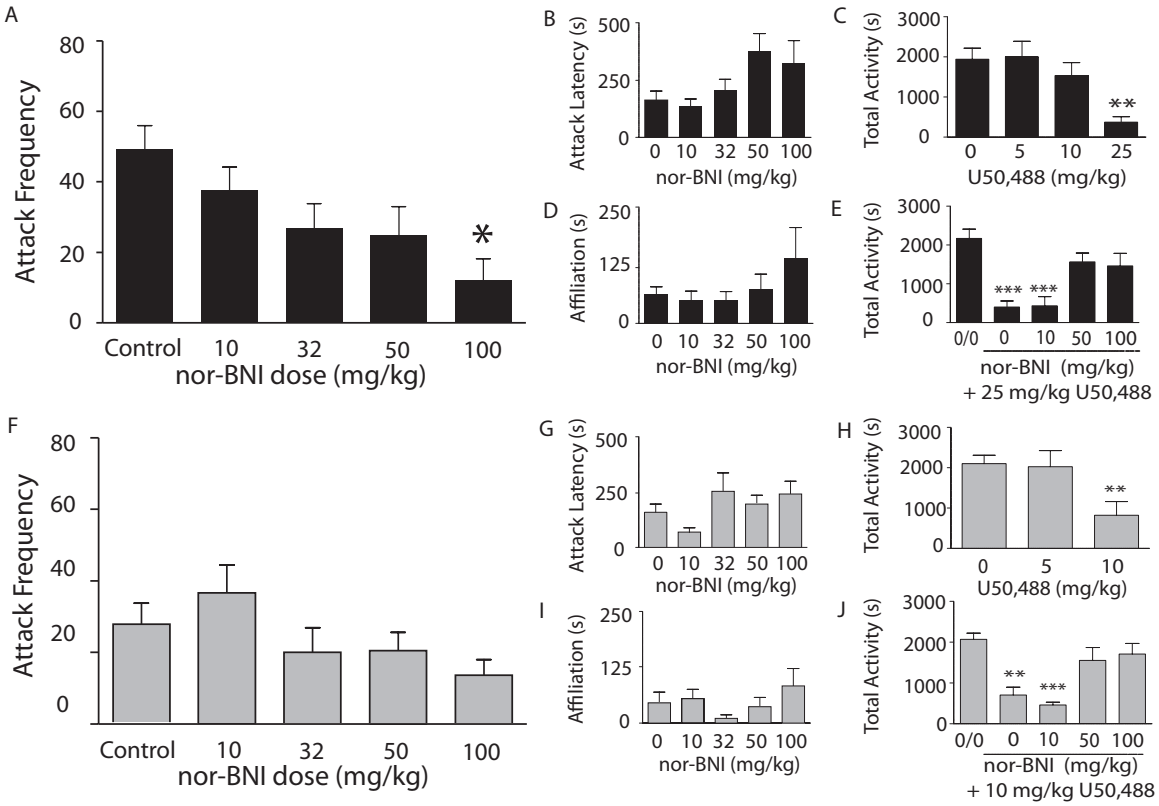

Figure 8

Peripheral administration of a kappa-opioid receptor antagonist has sex specific effects on selective aggression. **A,B,D**, The highest dose of nor-BNI significantly decreased aggression in pair-bonded males without affecting attack latency (**B**) or affiliation levels (n = 7 to 11; **D**). **C**, In males, 25 mg/kg of the kappa-opioid receptor agonist U50,488 significantly decreased motor activity during an open-field test (n = 5 to 7). **E**, The motor inhibitory effects of U50, 488 were reversed by 50 and 100 mg/kg nor-BNi, but not 10 mg/kg nor-BNI (n = 6 to 11). **F,G,I**, Peripheral doses of nor-BNI failed to decrease aggression in pair-bonded females (**F**) and had no significant effect on attack latency (**G**) or affiliation (n = 10 to 11; **I**). H,J, 10 mg/kg U50,488 decreased motor activity in females (n = 6 to 7; **H**) and this effect was reversed by 50 and 100 mg/kg nor-BNI (n = 6/group; **J**). **p < 0.005. Error bars indicate mean ± SEM.

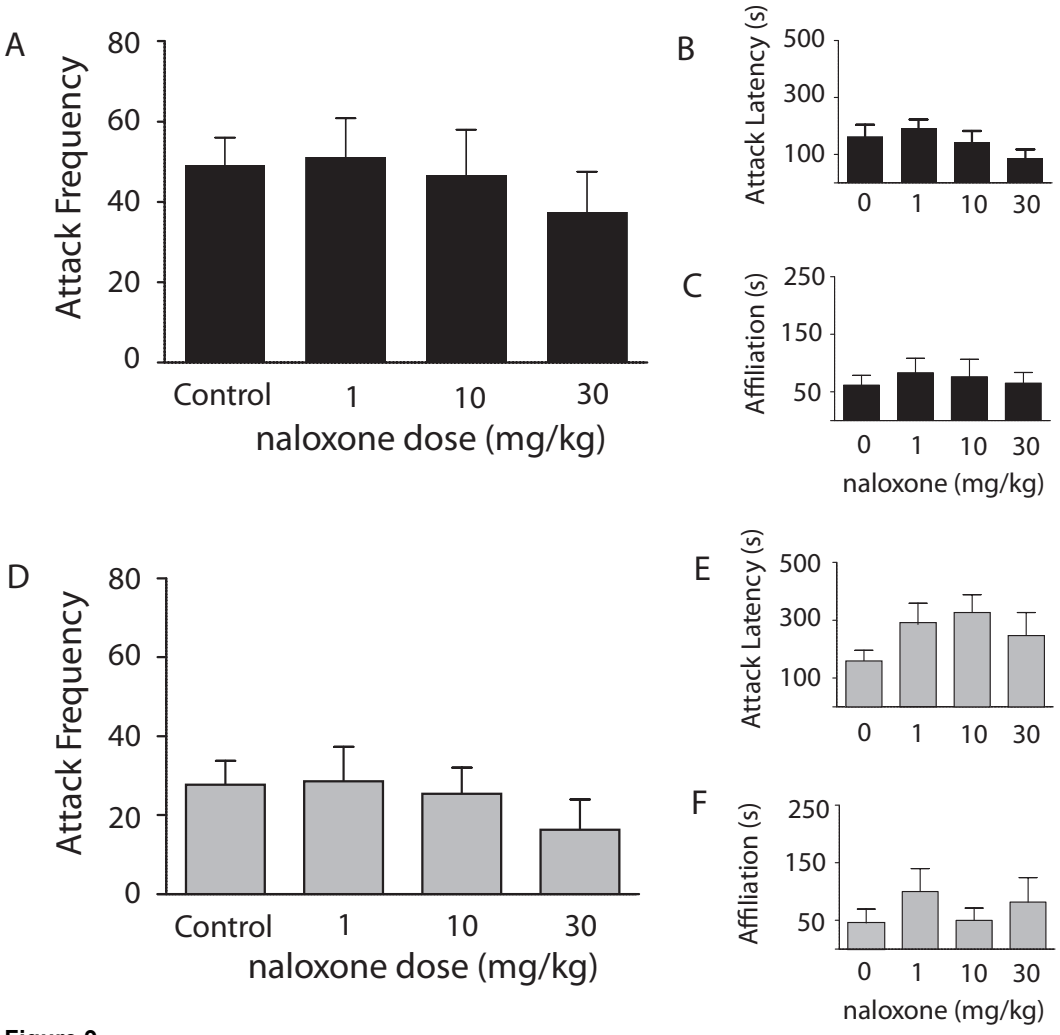

Figure 9

Mu-opioid receptors do not regulate selective aggression in prairie voles. **A-C**, Peripheral administration of the mu-opioid receptor antagonist, naloxone, had no effect on selective aggression, attack latency (**B**), or affiliative behavior (n = 10 to 11; **C**). D-F, Similarly, peripheral administration of naloxone failed to block aggression in pair-bonded females and had no effect on attack latency (**E**) or affiliaition levels (n = 10 to 12; **F**).

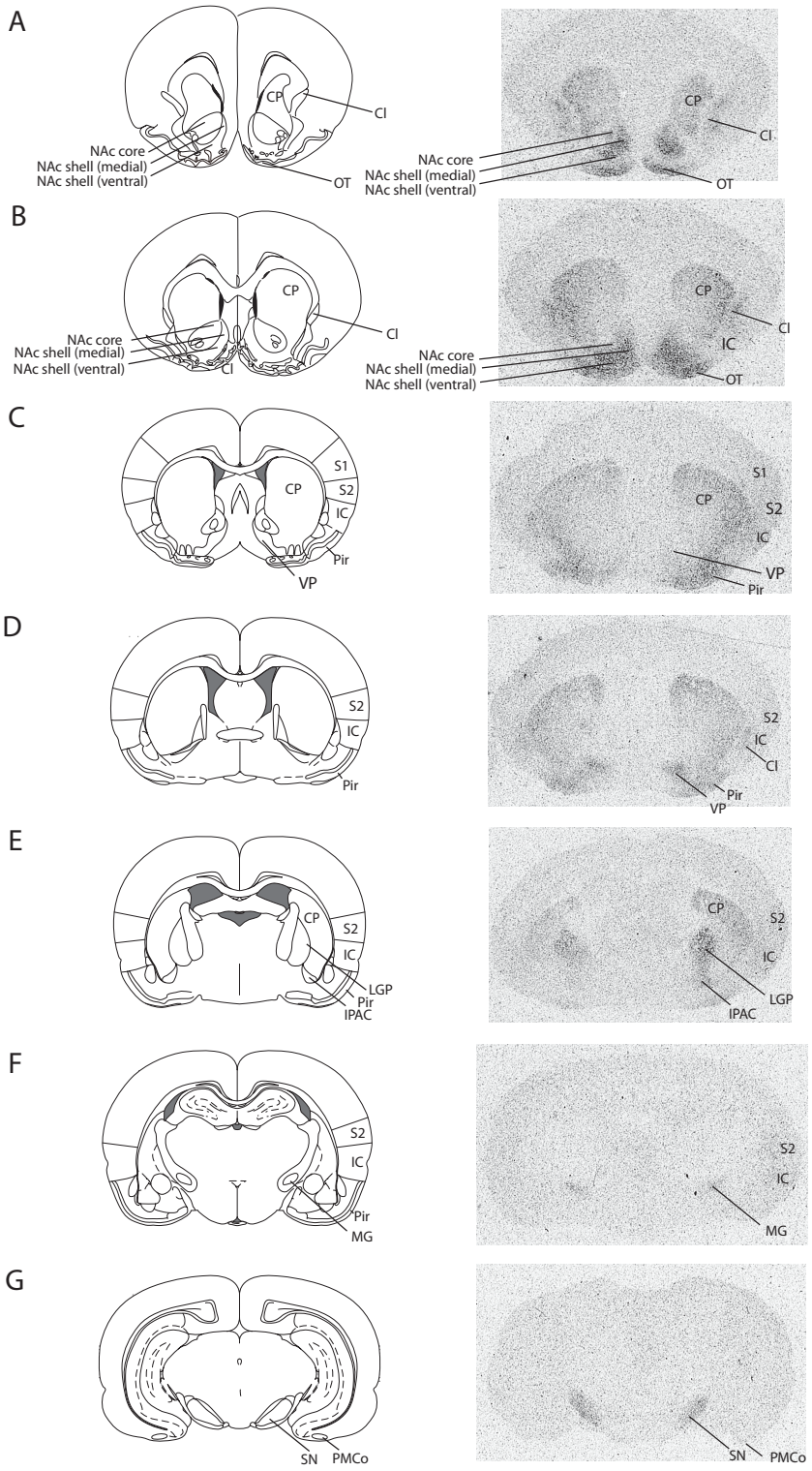

Figure 10

A-G, Distribution of kappa-opioid receptors in prairie voles. Cl, Claustrum; CP, caudate-putamen; IC, Insular cortex; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; LGP, lateral globus pallidus; MG, medial geniculate nucleus; PMCo posterior medial cortical amygdala; Pir, piriform cortex; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; SNC, substantia nigra compacta; SNR, substantia nigra reticulate.

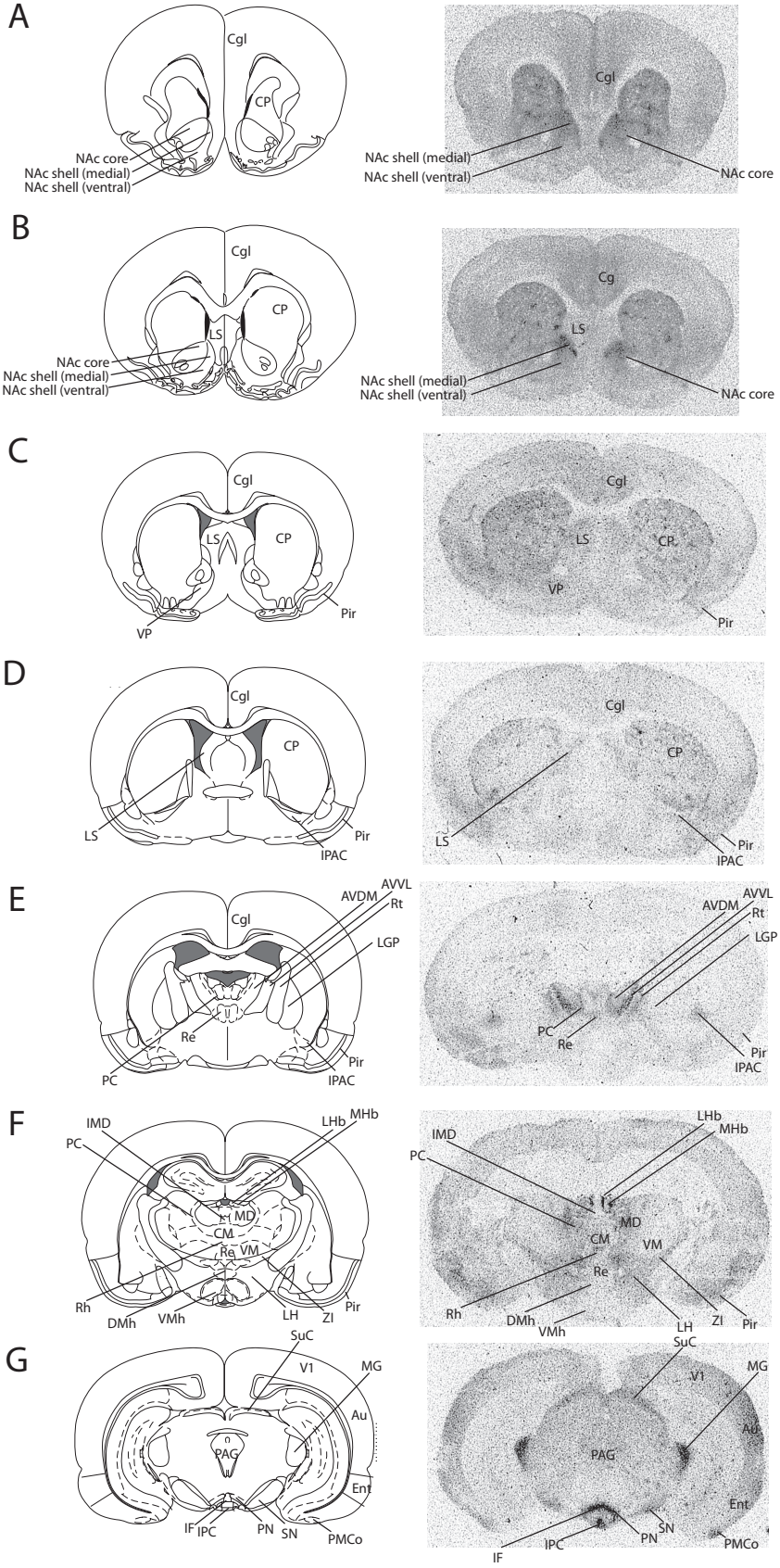
A
Cgl
CP
NAc core
NAc shell (medial)
NAc shell (ventral)
B
LS
C
Pir
VP
D
IPAC
E
AVVL
AVDM
Rt
LGP
Re
PC
F
IMD
LHb
MHb
MD
CM
VM
Rh
DMh
VMh
LH
ZI
G
SuC
V1
MG
Au
PAG
Ent
IF
IPC
PN
SN
PMCo

Figure 11

A-G, Distribution of mu-opioid receptors in prairie voles. Au, Auditory cortex; AVVL, anteroventral thalamic nucleus; AVDM, anteroventral thalamic nucleus; Cgl, cingulate cortex; CM, central medial thealamic nucleus; CP, caudate-putamen; DMh, dorsomedial hypothalamic nucleus; Ent, entorhinal cortex; IF, interfascicular nucleus; IMD, intermediodorsal thalamic nucleus; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; IPC, interpeduncular nucleus, caudal subnucleus; LGP, lateral globus pallidus; LH, lateral hypothalamic area; LHb, lateral habenular nucleus; LS, lateral septum; MD, medial dorsal nucleus; MG, medial geniculate nucleus; MHb, medial habenular nucleus; NAc, nucleus accumbens; PAG, periaqueductal gray; PMCo, posterior medial cortical amygdala; PC, paracentral thalamic nucleus; Pir, piriform cortex; PN, paranigral nucleus of the VTA; Re, Reuniens thalamic nucleus; Rh, Rhomboid thalamic nucleus; SN, substantia nigra; SuC, suprachiasmatic nucleus; V1, primary visual cortex; VM, ventromedial thalamic nucleus; ZI, zona incerta.

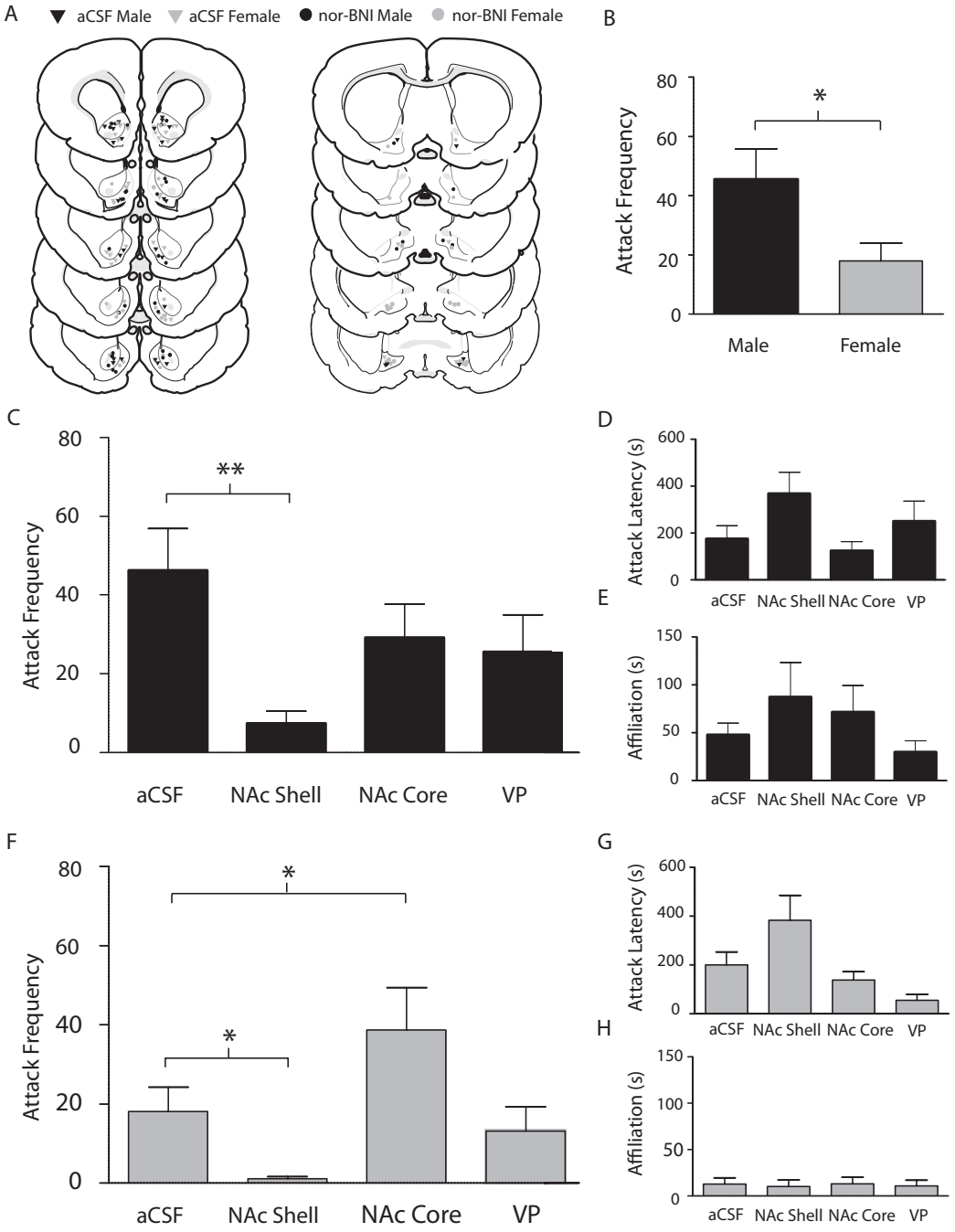

Figure 12

Kappa-opioid receptors within the NAc shell mediate selective aggression. **A**, Sites of injections of ACSF and the kappa-opioid receptor antagonist nor-BNI. **B**, Consistent with data from peripheral experiments, aggression in males is significantly higher in pair-bonded males (n = 16) than females (n = 16). **C,** Injections of nor-BNI into the NAc shell significantly decreased aggression compared to control injections and injections of nor-BNI in the NAc core. **D,E**, Additionally, nor-BNI in the NAc shell trended toward increasing attack latency and had no effect on affiliation levels in males (n = 6 to 8; **E)**. **F**, Blockade of kappa-opioid receptors in the NAc shell and core had opposing effects on aggression levels in females. Injections of nor-BNI in the NAc shell significantly decreased aggression levels, while injections of nor-BNI in the NAc core increased aggression levels compared to controls. **G,H**, No effects on attack latency, (**G**) or affiliation levels (H) occurred in females (n = 6 to 8). *$p < 0.05$; **$p < 0.005$. Error bars indicate mean ± SEM

Number of cage crosses in home cage one hour following peripheral injections of nor-BNI

nor-BNI	Control	10 mg/kg	32 mg/kg	50 mg/kg	100 mg/kg
Male	23.9 ± 7.4	34.0 ± 12.9	28.6 ± 7.2	24.1 ± 5.0	12.9 ± 8.8
Female	30.7 ± 4.1	39.8 ± 6.2	18.2 ± 5.3	24.6 ± 5.8	26.5 ± 11.6
Naloxone	**Control**	**1 mg/kg**	**10 mg/kg**	**30 mg/kg**	
Male	23.9 ± 7.4	21.1 ± 5.2	27.3 ± 12.1	24.7 ± 4.6	—
Female	30.7 ± 4.6	28.5 ± 6.9	16.0 ± 4.3*	13.9 ± 2.1*	—

Note. * $p < 0.05$

Table 3

During the habituation period, peripheral administration of nor-BNI had no effect on locomotor activity of male or female prairie voles at any dose tested. Peripheral administration of naloxone had no effect on locomotor activity of males, but caused a significant decrease in locomotor activity at the two highest doses tested.

Mean latency to withdraw tail following peripheral injections of U50, 488

nor-BNI	Saline	5 mg/kg	10 mg/kg	25 mg/kg
male prairie vole	4.1 ± 0.7	3.4 ± 0.6	10.9 ± 1.4**	8.6 ± 2.6
female prairie vole	9.3 ± 2.1	5.3 ± 1.7	10.5 ± 2.0	—
male C57Bl6 mouse	2.2 ± 0.1	—	—	—
female C57Bl6 mouse	2.1 ± 0.2	—	—	—

Note. ** $p < 0.05$

Table 4

Peripheral administration of 10 mg/kg of the kappa-opioid receptor agonist U50,488 significantly increase mean tail withdraw latency in male prairie voles, but did not have a significant effect at higher doses tested. In females, U50,488 did not increase tail withdraw latency at any of the doses tested. However, females had significantly higher baseline analgesia compared to male prairie voles and female C57Nl6 mice.

Mean latency to withdraw tail following pre-treatment with nor-BNI and peripheral injections of U50, 488 (Male: 25 mg/kg; Female 10 mg/kg)

	nor-BNI				
nor-BNI	**saline/saline**	**0 mg/kg**	**10 mg/kg**	**50 mg/kg**	**100 mg/kg**
male prairie vole	6.3 ± 1.2	9.0 ± 1.9	10.7 ± 1.5	7.7 ± 1.9	5.3 ± 1.7
female prairie vole	10.9 ± 1.9	7.8 ± 1.5	5.8 ± 0.7	4.7 ± 2.1	6.0 ± 1.4

Table 5

There was no significant differences on tail withdrawal latency between control male or female subjects (saline/saline) and those treated with various doses of nor-BNI as well as the highest dose of the kappa-opioid receptor agonist U50,488 administered to each sex.

Number of cage crosses in home cage one hour following site specific injection

	aCSF	nor-BNI			CTAP
	saline/saline	0 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg
Male	15.7 ± 2.2	13.0 ± 4.1	21.4 ± 6.9	12.0 ± 4.1	14.2 ± 5.3
Female	26.9 ± 4.2	16.2 ± 5.9	22.0 ± 10.8	16.3 ± 4.1	13.6 ± 7.7

Table 6

Site-specific administration of nor-BNI or CTAP had no significant effect on the locomotor activity of male or female prairie voles.

Mean concentration of plasma corticosteroid [ng/ml] in pair bonded prairie voles treated with either saline or nor-BNI one hour prior to a 10 minute resident-intrude test

nor-BNI	Saline	32 mg/kg	100 mg/kg
Male	1794.0 ± 163.4	2093.0 ± 170.5	1572.0 ± 204.6
Female	2106.0 ± 213.2	1986.0 ± 209.1	2821.0 ± 257.1

Table 7

Peripheral administration of nor-BNI had no significant effect on plasma corticosterone levels in male or female prairie voles.

Mean concentration of plasma corticosteroid [ng/ml] in pair bonded prairie voles receiving site-specific injections of either aCSF or nor-BNI into the NAc one hour prior to the resident-intruder test

	aCSF	nor-BNI	
	NAc	NAc shell	NAc core
Male	1636.0 ± 171.1	1556.0 ± 188.6	1842.0 ± 251.6
Female	2178.0 ± 263.3	2332.0 ± 191.5	1605.0 ± 334.0

Table 8

Site-specific administration of nor-BNI into the NAC shell or core had no significant effect on plasma corticosterone levels in male or female prairie voles.

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CHAPTER 4

INTERACTIONS BETWEEN DOPAMINE AND KAPPA-OPIOID RECEPTORS REGULATES THE MAINTEANCE OF PAIR BONDS IN THE SOCIALLY MONOGAMOUS PRAIRIE VOLES

ABSTRACT

The socially monogamous prairie vole is an excellent animal model for studying the neurobiology of social attachment. Prairie voles form enduring pair bonds and the maintenance of these bonds is characterized by the aggressive rejection of novel conspecifics. This 'selective aggression' serves as a behavioral proxy of pair bond maintenance and, here, we show that this behavior (dependent on fecundity in males, but not females), is mediated by interactions between D1-like and kappa-opioid receptors (KORs) within the nucleus accumbens (NAc) shell. Importantly, prairie voles only become aggressive toward novel individuals following the establishment of a pair bond and we show that the transition to this behavior is regulated by both an enhancement in dopamine (DA) release potential along with increased D1-like mRNA within the NAc. Together, these data indicate that motivational and valence-processing systems interact to mediate pair bond maintenance.

INTRODUCTION

A major predictor of overall mental well-being is the presence of stable social attachments (Baumeister & Leary, 1995). Thus, understanding the neural mechanisms that mediate the maintenance of social bonds is of critical importance to human health. An ideal animal model for studying the neural mechanisms of such behavior is the socially monogamous prairie vole (Carter *et al.*, 1995; Wang & Young, 1997). Unlike most mammalian species (Kleiman, 1977; Getz *et al.*, 1981), prairie voles form enduring attachments to their mating partners that can be characterized in the laboratory by two distinct phases, pair bond formation and pair bond maintenance (Aragona & Wang, 2009). Importantly, these two stages are associated with distinctly different types of social behaviors; the formation a pair bond is associated with affiliative social interactions that eventually lead to the development of a preference for a mating partner (i.e., a partner preference) (Williams *et al.*, 1992), while pair bond maintenance is associated with aversive social encounters, such as the aggressive rejection of a novel social stimulus (i.e., selective aggression) (Winslow *et al.*, 1993). Thus, prior to pair bond formation, prairie voles are generally affiliative and only after pair bond formation does a selective aggressive social strategy emerge (Insel *et al.*, 1995; Aragona *et al.*, 2006).

Previous studies on pair bond maintenance have identified that this stage of pair bonding is mediated by neural systems that regulate motivated behavior as well as valence processing, such as the dopaminergic and opioid systems, respectively (Resendez & Aragona, 2013). For example, D1-like dopamine (DA)

receptors become up-regulated within the nucleus accumbens (NAc) following pair bond formation and blockade of these receptors attenuates the aggressive rejection of novel social stimuli (Aragona *et al.*, 2006). Interestingly, activation of D1-like receptors results in an increase in the expression of dynorphin (DYN) (Gerfen *et al.*, 1990), the endogenous ligand for kappa-opioid receptors (KORs) (Chavkin *et al.*, 1982) and a critical mediator of aversive processing (Mucha & Herz, 1985). An interaction between D1-like receptors, that are important for the generation of motivational responses, and KORs, that are important for the encoding of aversion, is of particular interest to pair bond maintenance because blockade of KORs within the NAc shell also attenuates selective aggression (Resendez *et al.*, 2012). Therefore, these systems may interact to mediate aversive motivated social responses that are necessary for the maintenance of a pair bond.

To test the hypothesis that KORs within the NAc shell mediate bond maintenance by encoding social stimuli besides the partner as aversive, we utilize a social conditioning paradigm to show that direct activation of these receptors induces social aversion. Further, given that the establishment of a pair bond is associated with neuroanatomical reorganization of D1-like receptors within reward circuitry (Smeltzer *et al.*, 2006), we next employed real time PCR to determine if alterations of mRNA within dopaminergic and/or DYN/KOR systems is associated with the behavioral transition to pair bond maintenance. Next, to examine if the neurochemistry of this system is similarly altered, we employed fast scan cyclic voltammetry (FSCV) in striatal brain slices to examine DA

transmission dynamics in pair bonded and non-pair bonded voles. Finally, to test the hypothesis that interactions between D1-like and KORs directly mediate pair bond maintenance, we utilized site-specific behavioral pharmacology to simultaneously activate or inhibit both systems within the NAc shell in combination with tests of selective aggression—the key behavioral indicator in the establishment of a pair bond. Together, our results demonstrate how these systems interact to generate a negatively valenced motivational state (i.e., aversive) that is necessary for monogamous mate guarding and shed light on how these interactions may be involved in other motivational behaviors.

METHODS

Subjects: Adult male and female prairie voles were housed as previously described (Resendez *et al.*, 2012). For experiments that required pair bonded prairie voles, adult subjects were paired with an opposite sex partner for 14 days in a large cage that subsequently became the pair's 'home cage' cage. This cohabitation time allows for mating, impregnation, and nest sharing (Aragona *et al.*, 2006). To check for pregnancy, embryos were extracted from pregnant females, measured, and categorized as previously described (Resendez *et al.*, 2012). All procedures were conducted in accordance with the animal care guidelines at the University of Michigan.

Stereotactic surgery: Subjects received stereotactic surgery to implant a 26-gauge bilateral guide cannula (Plastics One) into the NAc shell (+1.7 mm rostral/caudal; ±1mm medial/lateral; -4.5 mm dorso/ventral). Subjects were allowed to recover in their home cage with either their cage mate or mating partner for 3 days prior to behavioral testing.

Partner Preference: Immediately prior to pairing with an opposite-sex conspecific, male subjects received site-specific injections (described above) of either aCSF or U50,488 (KOR agonist). Following injections, subjects cohabitated with a female partner for 1-hr. Following cohabitation, test subjects were placed in a three-chambered modified partner preference apparatus with their partner restricted to one chamber and a novel opposite-sex individual (stranger) restricted to the opposite chamber. Test subjects were free to move throughout the apparatus. The 3-hr test was recorded and later scored for duration of time spent in side-by-side contact with either the partner or stranger by an experimenter blind to treatment groups.

FSCV: Following rapid, live decapitation, brains were quickly extracted and immediately submerged in cold, pre-oxygenated high sucrose aCSF consisting of 180 mM sucrose, 30 mM NaCl, 4.5 mM KCl, 1mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 NaH_2PO_4, and 10 mM D-Glucose in deionized H_2O (pH 7.4). A vibratome (Leica VT1200S) was used to section the brain into coronal slices (400 μm) containing the dorsal striatum, the NAc core, and the NAc shell. Following sectioning, slices

were transferred to room temperature aCSF buffer solution consisting of 176.13 mM ascorbate, 180.16 mM glucose, 84.01 mM sodium bicarbonate, 58.44 mM NaCl, 156 mM NaH_2PO_4, 74.56 mM KCl, 147.01 mM $CaCl_2$, and 203.30 mM $MgCl_2$ in deionized H_2O (pH 7.4) and incubated for 1-hr. A buffer solution of this same composition (minus ascorbate) was used to perfuse the slices during recordings (1 ml/min). Both buffer solutions were continuously bubbled with 5% CO_2 and 95% O_2.

FSCV was conducted with recording electrodes fabricated from 1.2 mm pulled glass capillary tubes, with the carbon fiber cut to approximately 150 μm from the capillary glass seal. Using Tarheel CV (University of North Carolina, Chapel Hill) software written in LABVIEW (National Instruments, Austin, TX), a triangular ramp sweeping from -0.4V to +1.2 V versus a Ag/AgCl reference was applied to the carbon-fiber electrode at a rate of 10Hz. The characteristic oxidation current, seen at +0.6V during the upward ramp, and reduction current, at -0.2V during the downward ramp, of DA were identified using a background-subtracted cyclic voltammogram. The peak currents for DA were converted to concentration by calibrating each electrode to a known concentration of DA (3 μM).

DA release was evoked by a single, 5, or 20-pulse stimulation (350 μA) delivered in 5 min increments at 20 Hz with a bipolar stimulating electrode placed on the surface of the striatal slice approximately 150 μm from the recording electrode (Zhang *et al.*, 2009). A single pulse was chosen to mimic low levels of synchronous DA firing, while 5- and 20-pulse stimulations at 20 Hz were chosen

to mimic burst- (or phasic-) like firing patterns. Although a single-spike in DA neuron firing is consistent with tonic firing patterns, this type of firing pattern also requires an irregular firing frequency (Grace & Bunney, 1984). Given that a 1-pulse stimulation of a slice results in a global depolarization of terminals that would not occur at tonic levels, we cannot accurately mimic the asynchronous firing pattern associated with tonic DA. Each recording was 15s in duration and DA release was evoked at 5s. A total of 3 recordings at each pulse were made within each region and peak DA release was averaged for each subject. Slice stimulations occurred at regular 5-min intervals and readings were only recorded for experimental purposes once DA release was consistently stable

Measuring mRNA by reverse transcriptase PCR: Tissue punches from the ventral (NAc shell and core) were processed for mRNA quantification. Total RNA was extracted using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. mRNA was reverse transcribed using the iScript RT-PCR kit (Bio-Rad). Specific intron-spanning primers were used to amplify cDNA regions for transcripts of interest (*Avpr1a*, *Drd1*, *Drd2*, *Oxtr*, and *Pdyn*). q-PCR amplifications were performed in triplicate using an CFX96 real-time PCR system (Bio-Rad) at 95°C for 5-min, followed by 40 cycles of 95°C for 10s and 58°C for 30s, followed by real-time melt analysis to verify product specificity. *Nadh* was used as an internal control for normalization using the ΔΔCt method.

Statistics: To determine whether the data were normally distributed and equivalent in variance, we examined boxplots for each group. In cases where boxplots revealed that the data were not normally distributed or there was a lack of equal variance among groups, nonparametric tests were used. A preference for the partner or a novel social stimulus was determined with a Wilcoxon signed rank sum test for non-parametric data. A t-test was used to compare differences in total contact time or cage time. An alpha level was set at $p < 0.05$ for all statistical analysis. A one-way ANOVA followed by a Tukeys *post hoc* test was used to compare differences in stimulated dopamine following a 1, 5, or 20 pulse depolarizing stimulation within the dorsal striatum, NAc core, or NAc shell of male and female prairie voles. A two-way ANOVA followed by Bonferroni *post hoc* tests was used to determine sex differences in stimulated dopamine release. Differences in stimulated dopamine release among sexually naïve and pair bonded prairie voles within the dorsal striatum, NAc core, or NAc shell was determined with a t-test and a prior comparisons. A Mann-Whitney U test was used to compare differences in attack frequency between sexually naïve and pair bonded prairie voles. A t-test was used to compare differences in attack frequency between groups that were sub-optimally or optimally pregnant. A linear regression was used to identify a relationship between peak DA release and attack frequency as well as peak DA release and neonatal weight. A one-way ANOVA followed by Dunnets *post hoc* to control for multiple comparisons was used to identify differences in peak DA release among sexually naive subjects and pair bonded subjects grouped by stage of pregnancy. A series of

planned contrast was used to determine treatment differences in attack frequency as well as latency. All analysis were performed in SPSS version 21 for Windows.

Resident-intruder tests: Subjects received site-specific infusions of one of the following treatment groups 1-hr prior to resident-intruder testing: aCSF, 10 ng SCH 23390 (D1 receptor antagonist), 10 ng SCH 23390 and U50,488, or SKF 38393 (D1 receptor agonist) and 500 ng norBNI (KOR antagonist). 1-hr after drug infusion, the subject was placed in its home cage (in isolation) and its behavior was recorded for 10-min, allowing time for acclimation to the testing environment and the assessment of locomotor activity. Next, a randomly selected same-sex stimulus animal was introduced to the subject's home cage and behavioral interactions were recorded for 10-min. Locomotor activity during the habituation period was analyzed for the number of cage crossings and resident-intruder tests were scored for the frequency of aggressive behaviors (offensive rears, lunges, bites, and chase frequency). If aggression was never observed, an attack latency of 10-min was applied.

RESULTS

Activation of KORs within the NAc shell encode social aversion

Following the establishment of a pair bond, the valence of social cues associated with a novel social stimulus transitions from rewarding to aversive.

This transition in social valence is inferred from the dramatic shift in social behavior elicited by the presence of a novel conspecific: specifically, behavior directed at conspecifics shifts from social affiliation to aggressive rejection. We have previously shown that the aggressive component of this social aversion requires the activation of KORs specifically within the NAc shell (Resendez *et al.*, 2012). Importantly, activation of these receptors is well known to encode aversion, as determined by behavioral assays of conditioned avoidance (Mucha & Herz, 1985). We therefore posited from these data that NAc shell KORS act to mediate pair bond maintenance through the encoding of novel social stimuli as aversive (Resendez & Aragona, 2013). However, a direct assay of KOR mediated social aversion has yet to be conducted in this species.

To determine if NAc shell KORs encode social aversion, we utilized the partner preference paradigm in combination with social conditioning procedures that are insufficient to induce a preference for either the familiar partner or a novel social stimulus (i.e., the stranger). Specifically, following 1-hr of cohabitation with an opposite-sex conspecific, sexually naïve prairie voles will spend equal amounts of time in contact with the familiar partner compared to the stranger indicating that both social stimuli are of equivalent salience (DeVries *et al.*, 1995). In other words, there is no aversion or preference to either social stimulus under these conditioning procedures. Indeed, consistent with previous data, control males treated with artificial cerebrospinal fluid (aCSF) prior to a 1-hr cohabitation with a female partner did not prefer to spend more time in contact with either stimulus animal ($W_{(5)}$ = 33, z = -0.968, p = 0.333) (Figure 13B). In

contrast, activation of KORs within the NAc shell of sexually naïve males immediately prior to pairing with the female partner resulted in these males spending significantly more time in contact with the stranger compared to the partner ($W_{(6)}$ = 32.5, z = -2.561, p = 0.010) (Figure 13B). Moreover, in addition to avoiding contact with the partner paired with KOR activation, male subjects treated with a KOR agonist spent significantly less time in the chamber containing the partner ($t_{(12)}$ = 2.598, p = 0.0233) and more time in the chamber containing the stranger ($t_{(12)}$ = 3.210, p = 0.0075) (Figure 13C). The combination of the avoidance of social contact with the partner as well as avoidance of the chamber containing the partner suggests that activation of KORs within the NAc shell prior to a 1-hr cohabitation results in a previously benign social stimulus to be encoded as aversive. Moreover, this encoding is not the result of a general decrease in motivation for contact as there was no difference in total contact time (time spent with partner + time spent with stranger) among treatment groups ($t_{(11)}$ = 0.3535, p = 0.7304) (Figure 13D). Together, these data provide the first evidence that activation of KORs within the NAc shell are important for the encoding of social stimuli as aversive and therefore, the maintenance of monogamous pair bonds.

Reorganization of motivational and valence processing systems

Socially motivated behaviors related to reproduction differ greatly between sexually naïve prairie voles and those in an established pair bond. Specifically, the reproductive needs of sexually naive prairie voles revolve around finding a mating partner, which requires a willingness to approach and engage with novel

social conspecifics. In contrast, the reproductive goals of prairie voles already in an established pair bond are associated with protecting the mate, which requires that novel social stimuli are aggressively rejected (Resendez & Aragona, 2013). This shift in social motivation is mediated by a reorganization of neural mechanisms that regulate social behaviors specific to pair bond maintenance. For example, prairie voles in an established pair bond have significantly higher levels of D1-like receptors within the NAc compared to sexually naïve prairie voles. However, there is no difference in the density of D2-like receptors (important for pair bond formation) between sexually naïve and pair bond prairie voles (Aragona *et al.*, 2006). Thus, the reorganization of neural mechanisms associated with pair bonding is specific to those that mediate pair bond maintenance. To determine if pair bonding is also associated with changes in mRNA for the dopaminergic as well as the DYN/KOR system, we used reverse transcription and quantitative PCR (RT-qPCR) to investigate changes in the expression of these genes within the striatum.

Consistent with previous findings on DA receptor protein binding, we show that there is an increase in the expression of mRNA coding for D1- ($t_{(28)}$ = 2.809, p = 0.0090), but not D2-like ($t_{(28)}$ = 1.175, p = 0.2499), receptors within the ventral striatum of pair bonded males. Additionally, there was an up-regulation of mRNA coding for prodynorphin (Pdyn) within the ventral striatum ($t_{(28)}$ = 2.468, p = 0.020) (Figure 14A). Notably, a regression analysis revealed that changes in D1-like receptor and Pdyn expression were tightly correlated with each other suggesting that these systems may interact to mediate pair bond maintenance

(R^2 = 0.6419, $F_{(1,6)}$ = 10.75, p = 0.0168) (Figure 14B). Finally, there was no change in the expression of mRNA coding for genes that have previously been identified as important for pair bond formation (Figure 15A) indicating that the reorganization of the NAc shell is specific to neural systems that mediate pair bond maintenance.

DA transmission within the striatum and pair bond maintenance

Compared to sexually naïve prairie voles, pair bonded prairie voles show high levels of aggression toward novel individuals (Resendez *et al.*, 2012), which requires the activation of D1-like receptors specifically within the NAc shell (Aragona *et al.*, 2006). Importantly, these receptors are the low affinity type receptor and require high levels of DA release to be activated (Richfield *et al.*, 1989). To determine if changes in DA transmission that would facilitate the activation of low-affinity D1-like receptors (i.e., an enhanced release potential) occurs after pair bond formation, we used FSCV to measure stimulated DA release within the striatum of sexually naïve and pair bonded prairie voles. However, because this is the first time stimulated DA release has been measured within the striatum of prairie voles, we first characterized striatal DA release patterns within this species. Overall, striatal patterns of DA release were comparable to that of other species (Figure 16, 17, and 18) (Zhang *et al.*, 2009).

Stimulated DA release was enhanced within the NAc shell by an average of 134% for pair bonded males ($t_{(17)}$ = 2.443, p = 0.0258) (Figure 19C) and 199% for pair bonded females ($t_{(13)}$ = 2.475, p = 0.0279) (Figure 20F). Importantly, this effect was specific to the NAc shell as stimulated DA release within the dorsal

striatum (Male: $t_{(21)}$ = 0.0942, p = 1.753; Female: $t_{(17)}$ = 1.264, p = 0.2232) or the NAc core (Male: $t_{(18)}$ = 8654, p = 0.3982; Female $t_{(15)}$ = 0.7334, p = 0.4746) (Figure 20 and 21) was not significantly different between sexually naïve and pair bonded prairie voles of either sex. The enhancement in DA release potential specifically within the NAc shell may act to facilitate the activation of low-affinity D1-like receptors and, consequently, the aggressive rejection of novel conspecifics. To determine if the level of aggression toward intruders was related to the release potential of DA within the NAc shell, we ran resident-intruder tests in a separate group of animals prior to FSCV recordings. Indeed, attack frequency (a behavioral index of pair bond maintenance) was positively correlated with peak DA release within the NAc shell (R^2 = 0.4126, $F_{(1,9)}$ = 6.323, p = 0.0331) (Figure 19D). Importantly, there was no relationship between attack frequency and stimulated DA release within the dorsal striatum (R^2 = 0.938, $F_{(1,12)}$ = 2.885, p = 0.1152) or the NAc core (R^2 = 0.938, $F_{(1,12)}$ = 2.885, p = 0.1152) (Figure 22) further indicating that relationship between aggression and the release potential for DA is specific to the NAc shell.

As shown above, the average percent increase in DA release in pair bonded voles was lower in males compared to females. Initially, this finding was surprising considering D1-like receptor activation is important for selective aggression and pair bonded males are generally more aggressive than females (Figure 23) (Resendez *et al.*, 2012). However, although overall aggression levels are greater in males than females, there is a large amount of variation in the level of aggression paired males display toward an intruder. Recently, we have

identified that this variation is related to the fecundity of the pair. Specifically, in males, the strength of the pair bond is dependent on the rapid and successful establishment of pregnancy (i.e., optimally pregnant). As such, males whose females become optimally pregnant following 2-weeks of cohabitation are significantly more aggressive than males paired with females in which there was a delay in the establishment of pregnancy (i.e., sub-optimally pregnant) (Figure 23). In other words, the behavioral transition from social to aggressive is not an absolute and only occurs in males belonging to pairs that have demonstrated a potential for reproductive success. However, in females, the strength of the pair bond is unrelated to fecundity as selective aggression toward intruders is equivalent between sub-optimally and optimally pregnant females (Figure 23). These data suggest that there are underlying sex differences in the motivation to maintain the initial bond through mate guarding. We therefore tested the possibility that this sex difference is mediated by fecundity dependent modifications in DA transmission within male, but not female, prairie voles. Because there were no differences in stimulated DA release between subjects that had or had not undergone behavioral testing prior to FSCV recordings, these groups were combined for such analysis (Figure 24).

To determine if fecundity influences the propensity for enhanced DA transmission within pair bonded males, we directly compared peak DA release to the average neonatal weight of the offspring—an established indicator of gestational stage (Resendez *et al.*, 2012). Indeed, among pair bonded males, peak DA release specifically within the NAc shell was positively correlated with

neonatal weight (R^2 = 0.2897, $F_{(1,20)}$ = 8.156, p = 0.0098) (Figure 19E and 20H). Moreover, a one-way ANOVA revealed a significant difference in peak DA release within the NAc shell (but not other regions of the striatum) among sexually naïve and paired males separated by the female partner's stage of pregnancy (i.e., not pregnant, sub-optimally pregnant, optimally pregnant) (one-way ANOVA, $F_{(3,39)}$ = 0.2878, p = 0.049) (Figure 19F and 20I). Dunnet's post hoc test indicated that males whose females were optimally pregnant had significantly higher levels of peak DA release within the NAc shell compared to sexually naïve males (p = 0.042). However, there was no difference in stimulated DA release between sexually naïve males and males whose females were either not pregnant (p = 0.823) or were sub-optimally pregnant (p = 0.871) (Figure 19F). Importantly, a similar relationship between DA transmission dynamics and fecundity was not found in pair bonded females (Figure 20). Together, these data demonstrate that, for pair bonded males, DA release potential within the NAc shell only becomes enhanced if the female is optimally pregnant and therefore provides the first possible neural mechanism for fecundity dependent changes in male social behavior.

Interactions between D1-like and KORs mediate selective aggression

To determine if interactions between D1-like and KORs within the NAc shell regulate pair bond maintenance, we administered a combination of their perspective agonists and antagonists prior to resident-intruder testing (Aragona *et al.*, 2006; Resendez *et al.*, 2012). In paired bonded male subjects, one-way ANOVAs indicated an overall difference in attack frequency ($F_{(3,25)}$ = 5.554, p =

0.005) (Figure 25B) as well attack latency ($F_{(3,25)}$ = 5.539, p = 0.005) (Figure 25C). Consistent with previously published data (Aragona *et al.*, 2006), blockade of D1-like receptors within the NAc shell significantly attenuated attack frequency (p = 0.025) (Figure 25B) as well as significantly increased the latency to initiate an attack (p = 0.042) (Figure 25C).

To next determine if D1-mediated aggression is downstream from activation of the DYN/KOR system, a D1-like receptor antagonist was administered in the presence of a KOR agonist. Simultaneous blockade of D1-like receptors and activation of KORs restored aggression levels (p = 0.915) (Figure 25B) as well as attack latency (p= 0.543) (Figure 25C) to control levels. However, elimination of selective aggression by blockade of KORs was not reversed by co-infusion of a D1-like receptor agonist (attack frequency, p = 0.006; attack latency p = 0.001) (Figure 25B and C). Together, these data provide strong evidence that these systems interact to mediate pair bond maintenance by an initial increase in DA transmission, which then facilitates downstream activation of KORS. As, there were no differences in affiliative ($F_{(3,25)}$ = 1.951, p = 0.151) or locomotor behavior ($F_{(3,23)}$ = 0.750, p = 0.535) among treatment groups (data not shown), the date strongly suggest that drug manipulations are being exerted through their actions on selective aggression and are not secondary to effects on other behaviors. Moreover, a similar mechanism in the regulation of selective aggression in females was also identified in pair bonded females (Figure 26). Thus, interactions between D1-like

and KORs within the NAc shell are important for selective aggression, and therefore pair bond maintenance.

DISCUSSION

The present study demonstrates that selective aggression is mediated through D1-like receptor induced activation of KORs within the NAc shell, an important brain nucleus for aversive motivational processing (Ikemoto & Panksepp, 1999; Resendez *et al.*, 2012). Additionally, we demonstrate that pair bonding causes a dramatic reorganization of these systems, which likely mediates the behavioral transition characteristic of an established pair bond (Aragona *et al.*, 2006). Specifically, prior to pair bond formation, sexually naïve prairie voles are generally affiliative toward novel conspecifics; however, following bond formation, a switch occurs from an affiliative social strategy to one dominated by the aggressive rejection of novel conspecifics. This behavioral switch is important to the maintenance of monogamous bonds as it functions to maintain the initial bond by preventing affiliative social interactions with any individual besides a mate as well as guard the mate and the territory (Shapiro *et al.*, 1986).

NAc DA and aversive processing

The present study demonstrates that DA transmission within the NAc shell is important for the maintenance of pair bonding through the role it plays in

mediating the aggressive rejection of novel conspecific, a behavior which can be described as aversively motivated (Resendez & Aragona, 2013). As phasic DA transmission mediates appetitive goal-directed behaviors (Schultz, 1998; Tsai *et al.*, 2009), this system has long been implicated in the control of motivated behaviors directed at rewarding stimuli (Robbins & Everitt, 1996; Berridge & Robinson, 1998). However, the role of DA transmission in aversive motivated behaviors is controversial despite the fact that adaptive behavioral responses toward a noxious stimulus often require the excitation of behavior. This controversy is rooted in the seemingly opposing actions of DA neurons to noxious stimuli. Specifically, in response to a noxious stimulus, some researchers have reported increases in DA neurotransmission (Kalivas & Duffy, 1995; Pezze *et al.*, 2005; Anstrom *et al.*, 2009; Brischoux *et al.*, 2009), while others have reported decreases (Ungless *et al.*, 2004; Roitman *et al.*, 2008; Brischoux *et al.*, 2009; Mileykovskiy & Morales, 2011; Wheeler *et al.*, 2011). It has been suggested that these conflicting results may be due to differences in temporal coding of a noxious stimulus (Anstrom *et al.*, 2009) or due to diversity among DA neurons (Matsumoto & Hikosaka, 2009; Lammel *et al.*, 2011), but perhaps differences in dopaminergic responses to noxious stimuli are also related to the type of behavioral response that is adaptive for each stimulus. In other words, whether it is adaptive to pause behavior or if it is adaptive to actively avoid or defend against an aversive stimulus, such as an intruder into a breeding pairs home territory.

Under aversive conditions where behavioral excitation is adaptive, increases in DA transmission have been reported to occur. For example, a cue predicting a painful stimulus increases DA release specifically within the NAc shell of both rodents (Badrinaryan *et al.*, 2012) and humans (Baliki *et al.*, 2013). Given that the NAc shell plays an important role in generating behavioral excitation to salient environmental stimuli (Ikemoto & Panksepp, 1999), this increase in phasic DA release and the consequent activation of D1-like receptors may play an important role in preparing an animal to react to an aversive stimulus, perhaps through D1-mediated increases in locomotor activity (Kravitz *et al.*, 2010). Indeed, the display of species-specific defensive behavior under stressful environments requires the concurrent activation of both D1- and D2-like receptors throughout the NAc shell (Richard *et al.*, 2013). In the present study, we provide additional evidence that the release of DA into the NAc shell and the consequent activation of D1-like receptors are required for the appropriate behavioral response toward an aversive social stimulus. Importantly, these data further indicate that DA release within the NAc shell is important for both appetitive and aversive motivated behaviors.

Importantly, while there is evidence suggesting that under certain environmental conditions, both rewarding and aversive stimuli can lead to phasic activation of DA neurons, it does not mean that computation of the stimuli at the neuronal level and/or the consequent activation of motivational circuitry occurs in the same manner (Bromberg-Martin *et al.*, 2010). In other words, our data does not imply that the reward and aversion are represented or encoded as a single

dimension within motivational circuitry, but simply that both types of stimuli can require activation of the DA system. Indeed, a positively valenced social stimulus does not cause the same motivated response as a negatively valenced social stimulus (Resendez & Aragona, 2013) suggesting that there must be some other layer of stimulus processing that guides the direction of socially motivated behaviors. One system that has been implicated in valence processing and, therefore, guiding the direction of motivated behavior, is the endogenous opioid system (Le Merrer *et al.*, 2009).

Interactions between D1-like and KORs mediate pair bond maintenance

Results from the present study support the model that D1-like receptor regulation of selective aggression functions through downstream activation of the DYN/KOR system and the encoding of a novel social stimulus as aversive. Interactions between these systems are mediated by D1-induced activation of signaling cascades that increase DYN and consequently, KOR activation. Specifically, stimulation of D1-like receptors phosphorylates cAMP response binding protein (CREB) to induce the expression and release of DYN (Carlezon *et al.*, 1998). Interestingly, the over expression of CREB within the NAc facilitates the expression of DYN and can make low doses of cocaine, a psychostimulant that elevates DA within the NAc and is normally rewarding, aversive (Pliakas *et al.*, 2001). Thus, DA can signal both reward and aversion depending on the ability of the system to activate the DYN/KOR system. In the present study, we demonstrate that pair bonding is associated with an enhanced

release potential for DA within the NAc shell as well as an up-regulation of both D1-like receptors and DYN within the ventral striatum. This reorganization may sensitize the system to code elevations in DA elicited by a novel social stimulus as aversive instead of rewarding.

The encoding of aversion by KORs is theorized to occur by KOR-mediated decreases in DA concentration (McCutcheon *et al.*, 2012). Specifically, KORs are located on the terminals of DA neurons and stimulation of these receptors shuts off DA release. This reduction in DA release reduces stimulation of D2-like receptors located on GABAergic medium spiny neurons (MSNs) that project to the indirect pathway (Carlezon & Thomas, 2009). Importantly, D2-like receptors are coupled to the G-protein G_i (inhibitory) and reduced activation of these receptors increases firing of D2-expressing MSNs resulted in the inhibition downstream target structures, such as ventral pallidum (VP), that are important for reward processing. In contrast, conditions that promote a decrease in MSN firing, such as activation of D2-like or mu-opioid receptors, is associated with reward states, possibly through the disinhibition of downstream reward processing regions (Richard *et al.*, 2013). Thus, it may be that the initial excitement of D1-like receptors by burst-like DA release serves to activate the DYN/KOR system and subsequently shut down DA release and disinhibit reward processing brain nuclei. This mechanism is consistent with the hypothesis that the NAc encodes reward and aversion through respective decreases and increases in the firing of MSNs that project to the VP (Carlezon & Thomas, 2009).

Conclusion

In addition to pair bonding attenuating the reward value of novel social stimuli, it has recently been demonstrated to decrease the reward value of psychostimulants such as amphetamine. This decrease in reward value is mediated by the up-regulation of D1-like receptors within the NAc as blockade of these receptors reinstates the rewarding properties of amphetamine (Liu *et al.*, 2011). Given the close interaction between D1-like receptors and the DYN/KOR system as well as the known ability of KOR activation within the NAc shell to attenuate the rewarding properties of drugs of abuse (Shippenberg *et al.*, 1996), it is possible that the protective effect of pair bonding against the rewarding properties of drugs of abuse is also mediated by facilitated activation of the DYN/KOR system. Thus, this mechanism that evolved to maintain the pair bond by attenuating the reward value of other potential mating patterns may serve an additional adaptive benefit in protecting against drug reward and, importantly, provide insight into how selective social attachments protect against drug reward in our own species.

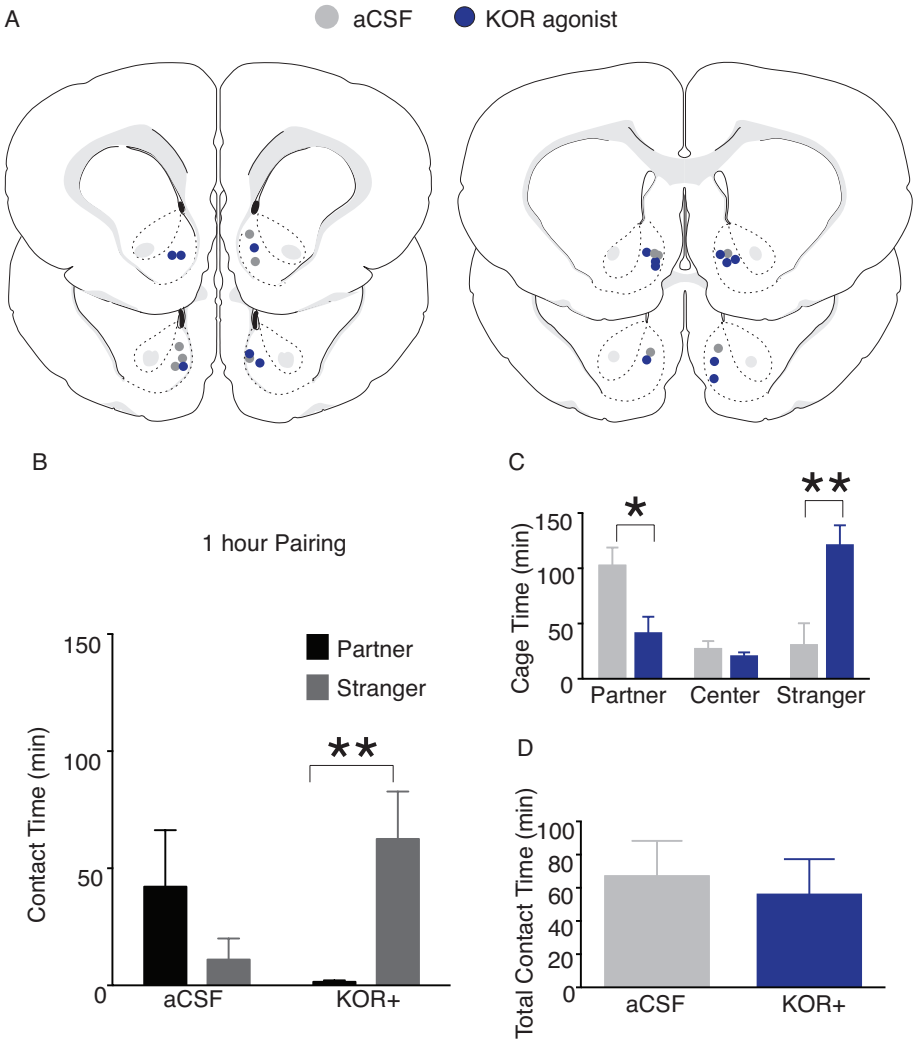

Figure 13

Activation of NAc shell KORs induces social aversion (A) Histological verification of injection sites. (B) Male subjects treated with aCSF and paired with a female partner for 1-hour did not prefer to spend more time in contact with either stimulus animal (n = 6). However, subjects treated with a KOR agonist spent significantly more time in contact with stranger (n = 7). (C) Additionally they also spent significantly less time in the partner's cage and more time in the chamber containing the stranger. There was no difference in total contact time among treatment groups. Summary data are presented as mean ± SEM. *P < 0.05, **P < 0.005.

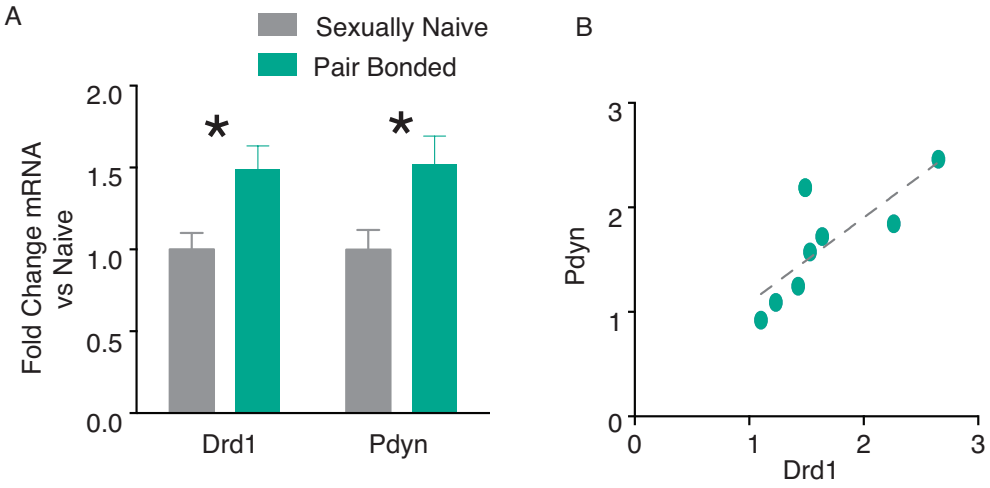

Figure 14

Pair bonding increases the expression of mRNA for Drd1 and Pdyn. (A) Pair bonding significantly increases the expression of mRNA coding for D1-like receptors as well as Pdyn. (B) The increase in Drd1 mRNA was positively correlated with the increase in expression of Pdyn.

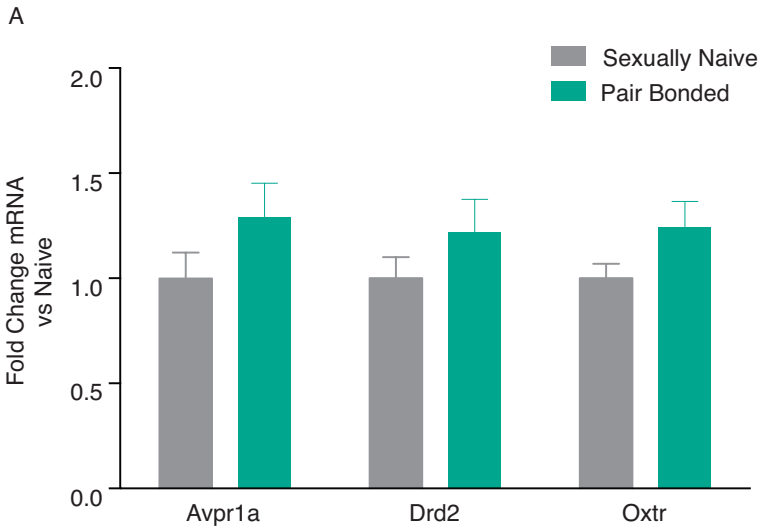

Figure 15

There was no change in mRNA coding for genes associated with pair bond formation. (A) There was no difference in mRNA for Avpr1a ($t_{(28)}$ = 1.149, p = 0.1670), Drd2 ($t_{(28)}$ = 1.175, p = 0.2499), or Oxtr ($t_{(28)}$ = 1.723, p = 0.0950) between sexually naïve and pair bonded prairie voles.

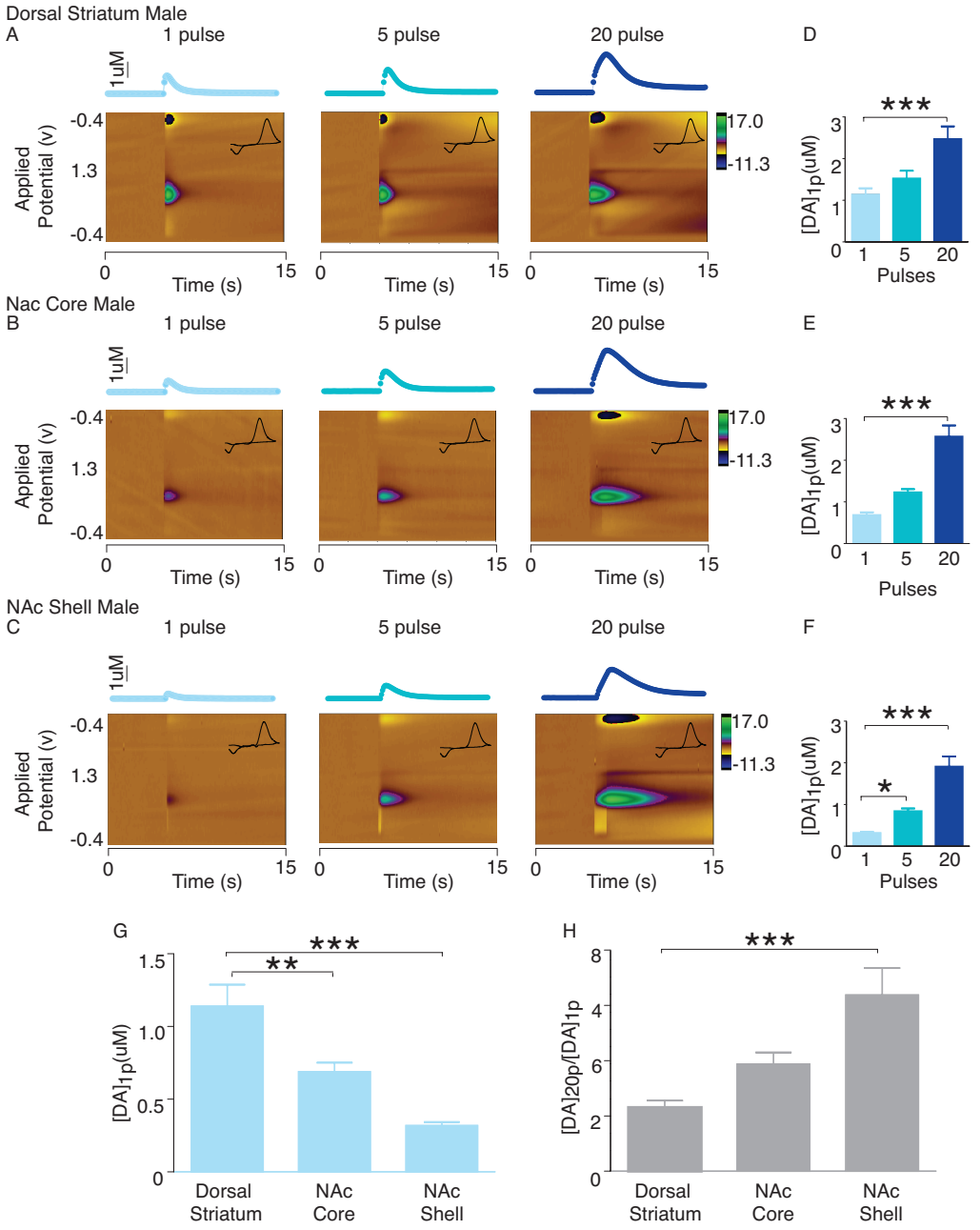

Figure 16

DA transmission in striatal slice preparations of male prairie voles.

Because this is the first time stimulated DA release has been measured in the striatum of this species, we first wanted to characterize striatal firing properties in prairie voles as DA release potentials are known to vary along a dorsal to ventral gradient (Jones *et al.*, 1995; Brown *et al.*, 2011). For example, a 1-pulse stimulation mimicking low-levels of synchronous DA release, results in the greatest magnitude of DA release within the dorsal striatum, intermediate levels within the NAc core, and the lowest levels within the NAc shell (Zhang *et al.*, 2009). A 1-pulse stimulation was therefore utilized to compare regional difference in stimulated DA release following a low, yet synchronous, firing pattern. Additionally, the contrast between a low 1-pulse stimulation and a burst-like (20 pulse) stimulation also differs regionally within the striatum; the dorsal striatum exhibits very little contrast between low firing patterns and burst-like stimulations, the NAc core exhibits an intermediate level of contrast, and the NAc shell exhibits the greatest level of contrast

between these firing patterns (Zhang *et al.*, 2009). We therefore applied higher stimulation parameters, such as 5 and 20-pulse stimulations at 20 Hz, to determine the relative contrast between low (1 pulse) and burst-like firing patterns throughout the striatum of prairie voles. Overall, the general pattern of DA release within the striatum of prairie voles was comparable to that of other species (Zhang *et al.*, 2009).

(A-C) Representative color plots and traces showing differences in DA transmission throughout the striatum of male prairie voles. (D) Consistent with known striatal firing patterns, a 5-pulse depolarizing stimulation in the dorsal striatum of male prairie voles did not result in a significant difference in peak DA release compared to a 1-pulse depolarizing stimulation (one-way ANOVA, $F_{(2,32)} = 9.5$, p = 0.001, Tukey *post hoc* test p = 0.45). However, a 20-pulse stimulation resulted in a significantly higher level of peak DA release compared to a 1-pulse stimulation (Tukey *post hoc* test p = 0.001) (n = 11). (E) In the NAc core, there was a trend for a significant difference in peak DA release between 1- and 5-pulses (one-way ANOVA, $F_{(2,29)} = 9.381$, p = 0.000, Tukey *post hoc* test p = 0.065) and a significant difference in peak DA release between 1- and 20-pulses (Tukey *post hoc* test p = 0.000) (n = 10). (F) In the NAc shell, both a 5-pulse (one-way ANOVA, $F_{(2,29)} = 6.607$, p = 0.000, Tukey *post hoc* test p = 0.043) and 20-pulse (Tukey *post hoc* test p = 0.000) stimulation resulted in a higher level of peak DA release compared to a 1-pulse stimulation (n = 10). Together, these data suggest an inverse relationship between the propensity for peak DA release at low firing. (G) A direct comparison of peak DA release following a one-pulse stimulation revealed an overall difference in stimulated DA release throughout the striatum that is consistent with the striatal release properties of other species (one-way ANOVA, $F_{(2,30)} = 17.275$, p = 0.000). A 1-pulse stimulation resulted in a significantly greater amount of peak DA in the dorsal striatum compared to the NAc core (p = 0.009) or the NAc shell (p = 0.000). (H) Also consistent with other species, there was no difference in the ratio between a 1 and 20-pulse stimulation between the NAc core and the dorsal striatum (Tukey *post hoc* test p = 0.185). However, the ratio between a 1- and 20-pulse stimulation was significantly greater in the NAc shell (one-way ANOVA, $F_{(2,30)} = 11.217$, p = 0.000, Tukey *post hoc* test p = 0.000). Together, these data indicate that while the dorsolateral striatum has the greatest release potential in response to a 1-pulse stimulation, the NAc shell has the greatest propensity for burst-like firing. Summary data are presented as mean ± SEM. $^{\#}P < 0.07$, $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

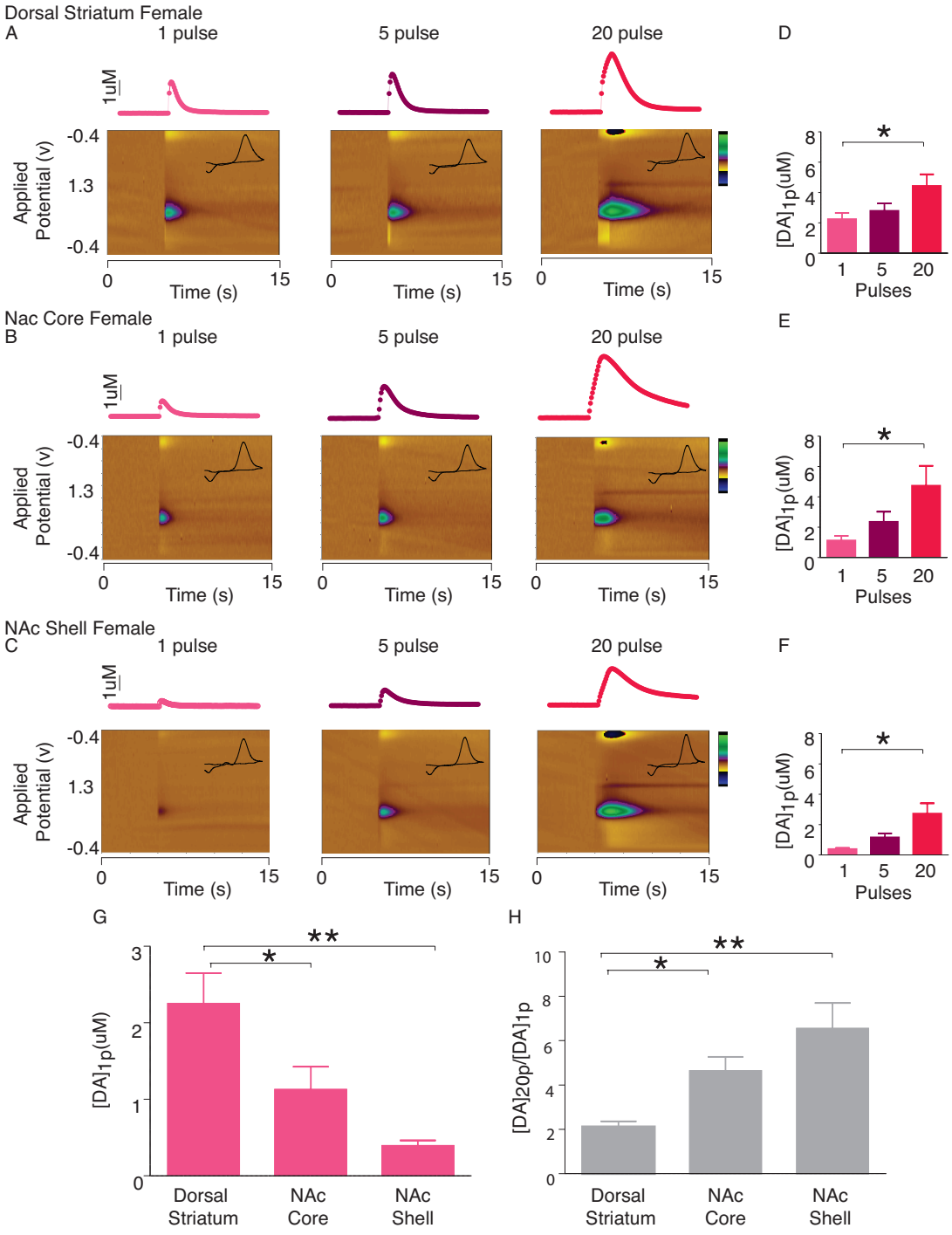

Figure 17

DA transmission within the striatum of female prairie voles. (A-C) Representative color plots and traces for sexually naïve females. (D-F) Within striatum of females, there was no significant difference in peak DA release between a 1- or 5-pulse stimulation in the dorsal striatum (one-way ANOVA, $F_{(2,32)}$ = 4.028, p = 0.028, Tukey *post hoc* test p = 0.778) (n = 11), NAc core (one-way ANOVA, $F_{(2,26)}$ = 4.531, p = 0.580, Tukey *post hoc* test p = 0.45) (n = 9), or NAc shell (one-way ANOVA, $F_{(2,23)}$ = 7.917, p = 0.003, Tukey *post hoc* test p = 0.416) (n = 8). There was, however, a significant difference in peak DA release between in 1- and 20-

pulse stimulation in the dorsal striatum (Tukey *post hoc* test p = 0.028), the NAc core (Tukey *post hoc* test p = 0.018), as well as the NAc shell (Tukey *post hoc* test p = 0.002). (G) Within the striatum of female prairie voles, a 1-pulse stimulation resulted in a significantly greater amount peak DA in the dorsal striatum compared to the NAc core (one-way ANOVA, $F_{(2,27)} = 8.569$, p = 0.001, Tukey *post hoc* test p = 0.046) and NAc shell (Tukey *post hoc* test p = 0.001). (H) However, the ratio between a 1- and 20-pulse stimulation was significantly greater in the NAc core of females (one-way ANOVA, $F_{(2,27)} = 10.601$, p = 0.000, Tukey *post hoc* test p = 0.034) and the NAc shell (Tukey *post hoc* test p = 0.000). Summary data are presented as mean ± SEM. $*P < 0.05$, $**P < 0.005$.

Thus, the overall patterns of DA release within the striatum of prairie voles are consistent with that of other species with the amplitude of peak DA release decreasing on a ventral gradient and the ratio between a 1- and 20-pulse stimulation increasing on a dorsal gradient through the striatum. A greater propensity to respond to burst-like DA firing within the NAc shell is significant as unexpected salient stimuli (either positively or negatively valenced) preferentially increase DA release within the NAc shell (Kalivas & Duffy, 1995) indicating that DA release within the NAc shell plays a general role in the excitation of behavior.

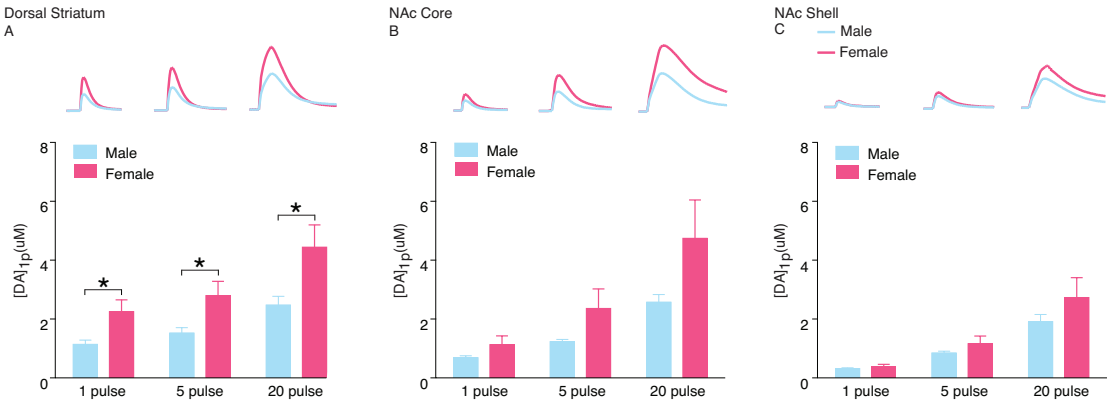

Figure 18

Sex differences in stimulated DA release. (A) There was a sex difference in stimulated DA release within the dorsal striatum (two-way ANOVA, $F_{(1,20)} = 6.798$, $p = 0.0169$) following a 1- ($p = 0.017$) , 5 ($p = 0.023$) , and 20-pulse ($p = 0.026$) stimulation (Bonferroni correction). In general, this finding is consistent with previous data in other species that has identified that both stimulation of the medial forebrain bundle in vivo as well as stimulation of the dorsal striatum in vitro evokes a greater amount of DA release in the dorsal striatum of females compared to males (Walker *et al.*, 2000). However, microdialysis studies, which are capable of measuring tonic levels of DA, show similar levels of extracellular DA within the striatum of both male and female rats (Xiao & Becker, 1994). Therefore, sex differences in DA neurotransmission within the dorsal striatum are related to differences in the release potential for DA, and not due to baseline differences in extracellular DA.

(B,C) There was no sex difference in stimulated DA release within the NAc core (two-way ANOVA, $F_{(1, 17)} = 3.091$, $p = 0.0967$) or NAc shell (two-way ANOVA, $F_{(1,16)} = 1.615$, $p = 0.2219$). Although there was not a significant difference in peak DA release within the NAc core, the difference in the core was intermediate to the dorsal striatum and NAc shell providing further evidence that this brain region behaves as a striatal transition zone (Resendez *et al.*, 2013).

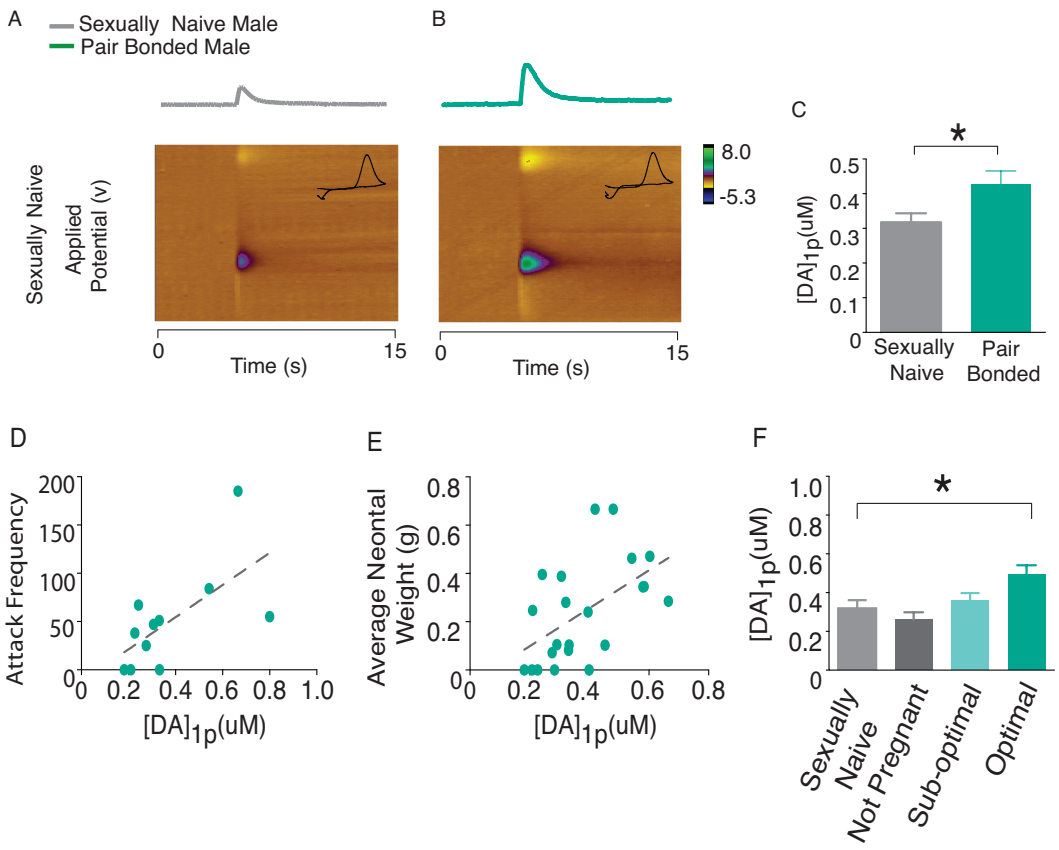

Figure 19

Stimulated DA release is enhanced within the NAc shell of pair bonded males. (A,B) Representative traces and color plots of stimulated DA release following a 1-pulse depolarizing stimulation in sexually naïve and paired males. (C) Within the NAc shell, a 1-pulse stimulation resulted in a greater level of peak DA release in pair bonded males compared to sexually naïve males (sexually naive: n =10, paired: n = 9). (D,E) Additionally, among pair bonded males, there was a positive correlation between peak DA release and attack frequency (n = 8) as well as a positive correlation between neonatal weight and peak DA release following a 1-pulse stimulation in the NAc shell (n = 23). (F) Moreover, an overall ANOVA revealed a significant difference in peak stimulated DA release among sexually naïve males and paired males separated by the female partner's stage of pregnancy (sexually naive: n = 18, not pregnant: n = 4; suboptimal: n = 10; optimal: n =7). Post hoc test revealed that males whose females were optimally pregnant had significantly higher levels of peak DA release within the NAc shell compared to sexually naïve males, but there was no difference in stimulated DA release between sexually naïve males and males whose females were not pregnant or males whose females were sub-optimally pregnant. Summary data are presented as mean ± SEM. *$P < 0.05$.

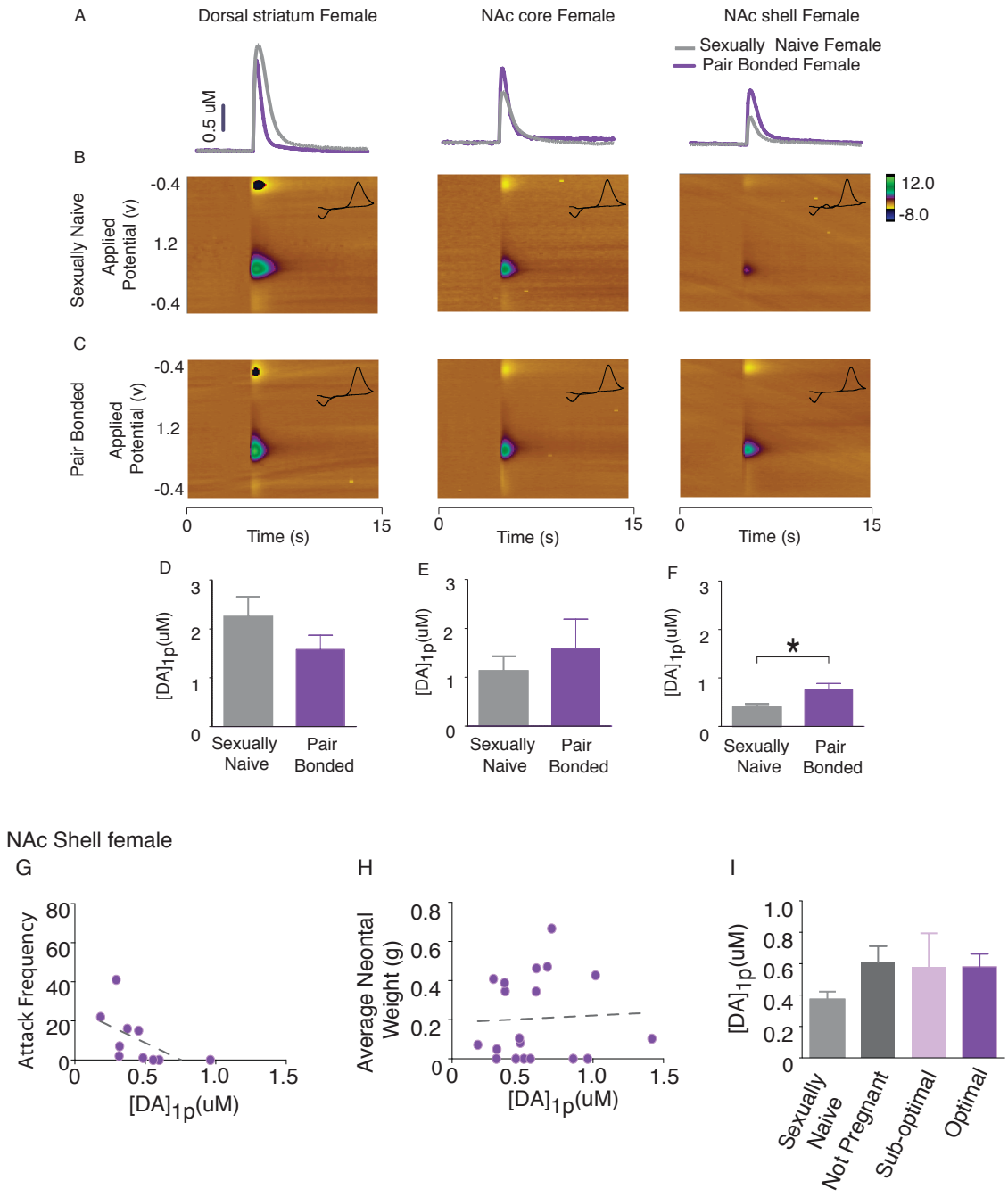

Figure 20

Stimulated DA release is enhanced within the NAc shell of pair bonded females, but not other regions of the striatum. Representative (A) traces and (B,C) color plots of stimulated DA release following a 1-pulse depolarizing stimulation in (B) sexually naïve and (C) paired females prairie voles. (D-F) There was no difference in peak DA release between sexually naïve and pair bonded females following a 1-pulse stimulation within the (D) dorsal striatum (sexually naive: n = 11, paired: n = 12) or (E) the NAc core (sexually naive: n = 11, paired: n = 12). (F) However, a one-pulse stimulation evoked significantly higher levels of dopamine release within of the NAc shell of pair bonded females (sexually naive: n = 8, paired: n = 7). (G) In contrast to pair bonded males, there was no significant relationship between attack frequency and stimulated DA release within the NAc shell of paired females (R^2 = 0.3202, $F_{(1,8)}$ = 3.768, p = 0.0882). (n = 10). This finding was surprising given that the activation of D1-like receptors within the NAc shell is required

for the display of selective aggression in pair bonded males as well as females (Figure S10). A possible explanation for this sex difference may lie within general sex differences in attack behavior. Specifically, although the establishment of a pair bond significantly increases aggression in both male and female (Figure S8) prairie voles, the relative increase is generally much more robust in males. Qualitatively, pair bonded males are about twice as aggressive as paired females suggesting that the propensity to attack is greater in males than females. Therefore, the lack of an effect in females may be related to lower baseline levels in aggression compared to males. Consistent with this hypothesis, data from pharmacological data (Figure S10) shows that while blockade of D1-like receptors attenuates attack frequency in both sexes, it only increases attack latency in males suggesting that activation of these receptors may be more important for initiating attack behavior in males than females. (H) Also in contrast, within the NAc shell of pair bonded females, there was no relationship between peak stimulated DA release and neonatal weight within the NAc shell (R^2 = 0.3202, $F_{(1,8)}$ = 3.768, p = 0.0882) (n = 19). (I) There was also no overall difference in peak DA release among sexually naïve females and paired females within the NAc shell when the pair bonded females were separated by stage of pregnancy ($F_{(3,31)}$ = 1.672, p = 0.196). Instead, there were moderate increases in peak DA release among all three groups (not pregnant, sub-optimally pregnant, or optimally pregnant) of paired females. This lack of a relationship between pregnancy and peak DA release among pair bonded females may account for the slightly more robust increase in peak DA in release in paired females compared to paired males (sexually naive: n = 13, not pregnant: n = 5; suboptimal: n = 5; optimal: n = 8). Summary data are presented as mean ± SEM. *P < 0.05.

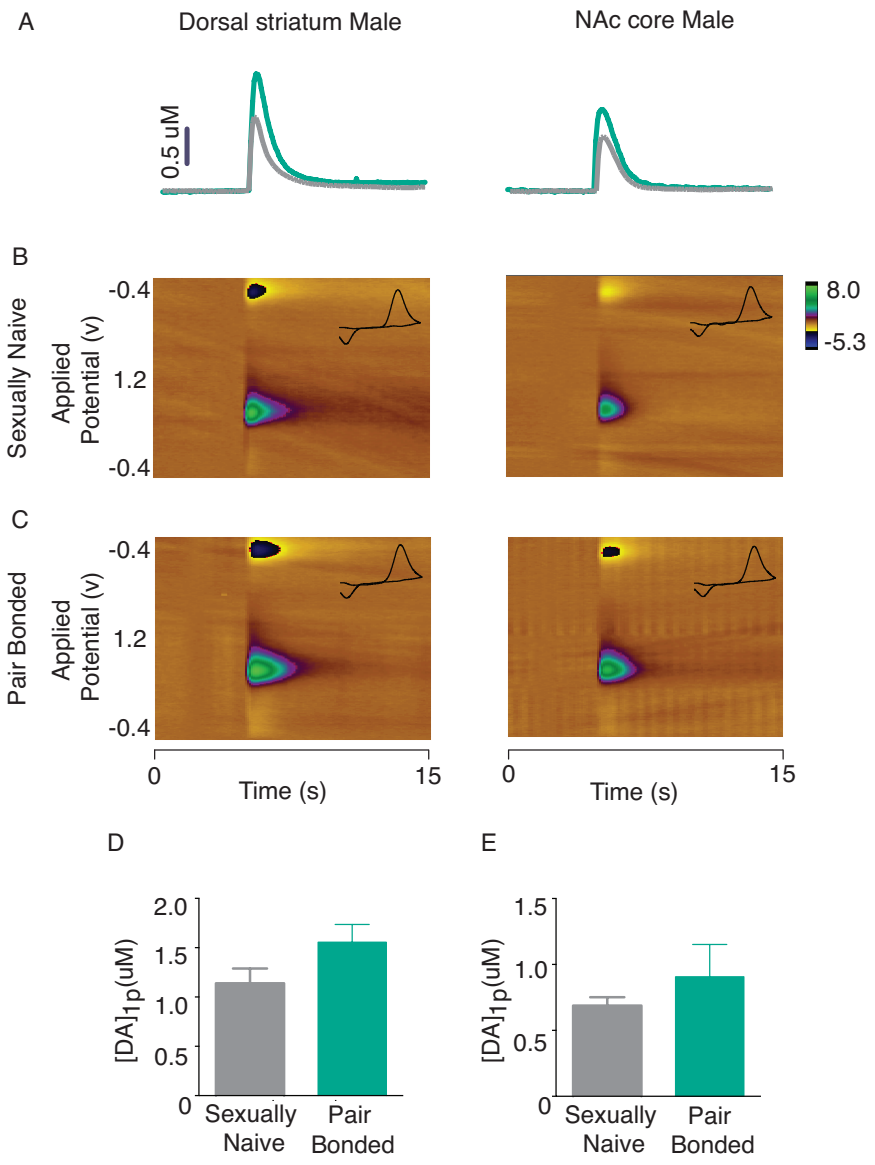

Figure 21

There is no change in stimulated DA release within the dorsal striatum of the NAc core of pair bonded males. (A-C) Representative (A) traces and (B,C) color plots of stimulated DA release following a 1-pulse depolarizing stimulation in (B) sexually naïve and (C) paired males. (D,E) There was no difference in stimulated DA release between sexually naïve or pair bonded prairie voles following a 1-pulse stimulation in the (D) dorsal striatum ($F_{(3,48)}$ = 1.936, p = 0.137) (sexually naive: n = 11, paired: n = 12) or (E) the NAc core ($F_{(3,42)}$ = 0.344, p = 0.794) (sexually naive: n = 10, paired: n = 10). Thus, enhanced DA release within the striatum of pair bonded males is specific to the NAc shell. Summary data are presented as mean ± SEM. *P < 0.05.

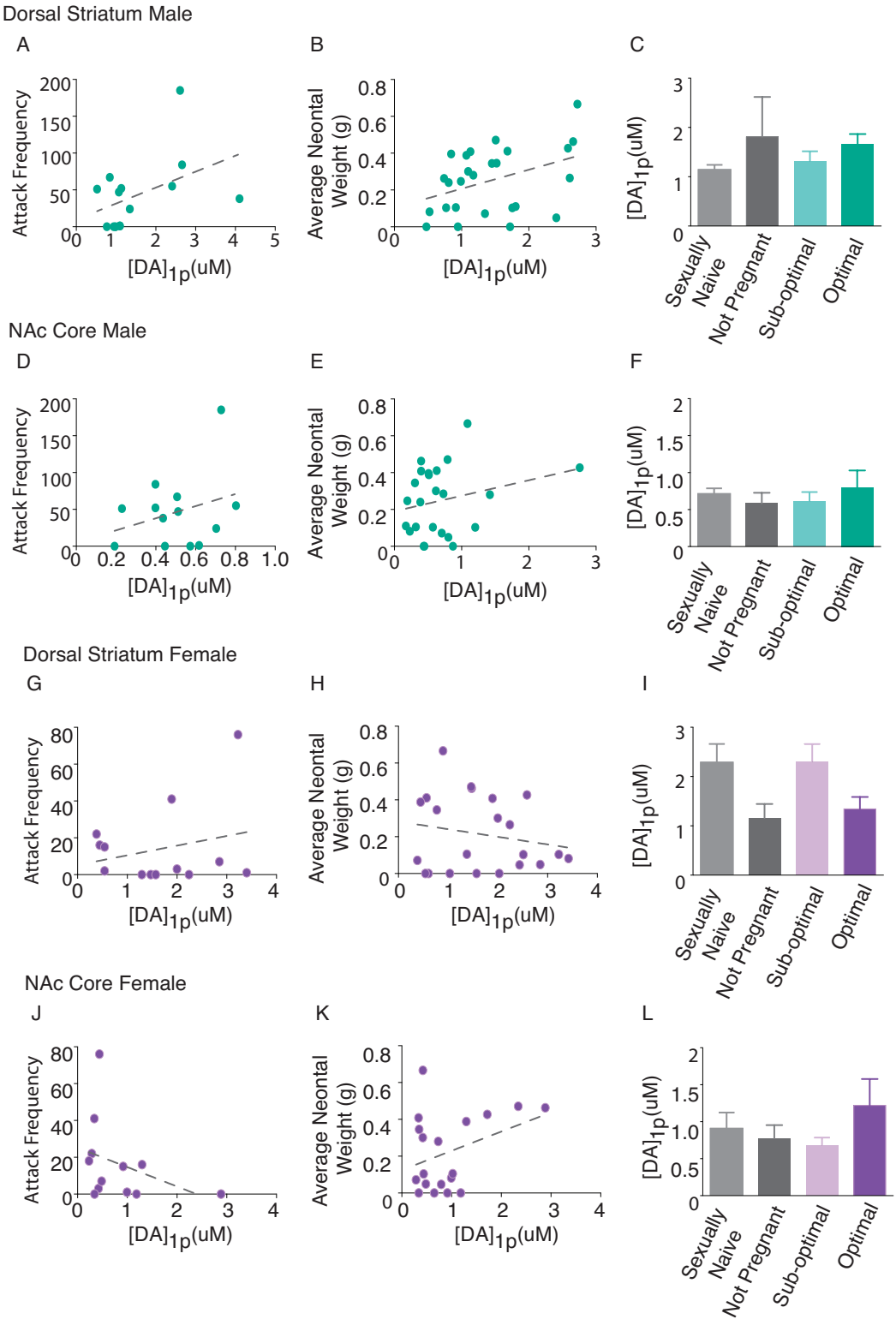

Figure 22

Relationship between stimulated DA release within the dorsal striatum as well as the NAc core to characteristics of pair bonding. (B) Although there was a positive correlation between peak DA release and

neonatal weight within the dorsal striatum of paired males (R^2 = 0.1592, $F_{(1,24)}$ = 4.544, p = 0.0435) (n = 26), (C) there was no significant difference in stimulated DA release within the dorsal striatum among sexually naïve and paired males (sexually naive: n = 22, not pregnant: n = 4; suboptimal: n = 12; optimal: n =11). (A) There was also no relationship between attack frequency and dopamine release within the dorsal striatum of paired males (R^2 = 0.938, $F_{(1,12)}$ = 2.885, p = 0.1152) (n = 14). (D,E) Within the NAc core of paired males, there was no relationship between peak DA release and attack frequency (R^2 = 0.938, $F_{(1,12)}$ = 2.885, p = 0.1152) (n = 14) or peak DA release and neonatal weight (R^2 = 0.0620, $F_{(1,22)}$ = 1.454, p = 0.2407) (n = 24). (F) There was also no difference in peak DA release among sexually naive and paired males (one-way ANOVA, $F_{(3,42)}$ = 0.344, p = 0.794) (sexually naive: n = 19, not pregnant: n = 3; suboptimal: n = 11; optimal: n = 10). (G,J) Among female subjects, there was no relationship between peak DA release and attack frequency within the dorsal striatum (R^2 = 0.0615, $F_{(1,11)}$ = 0.7209, p = 0.41140) (n = 13) or the NAc core (R^2 = 0.1247, $F_{(1,10)}$ = 1.424, p = 0.2603) (n = 12) (H,K) Additionally, there was no relationship between peak DA release and neonatal weight within the dorsal striatum (R^2 = 0.03629, $F_{(1,20)}$ = 0.7531, p = 0.3958) (n = 22) or the NAc core (R^2 = 0.1297, $F_{(1,17)}$ = 2.534, p = 0.1298) (n = 19). (I,L) Finally, there was no difference in peak DA release among sexually naïve females and paired females within the dorsal striatum ($F_{(3,38)}$ = 2.094, p = 0.119) (sexually naive: n = 17, not pregnant: n = 5; suboptimal: n = 8; optimal: n = 9) or the NAc core $F_{(3,32)}$ = 0.711, p = 0.553) (sexually naive: n = 14, not pregnant: n = 4; suboptimal: n = 7; optimal: n = 8).

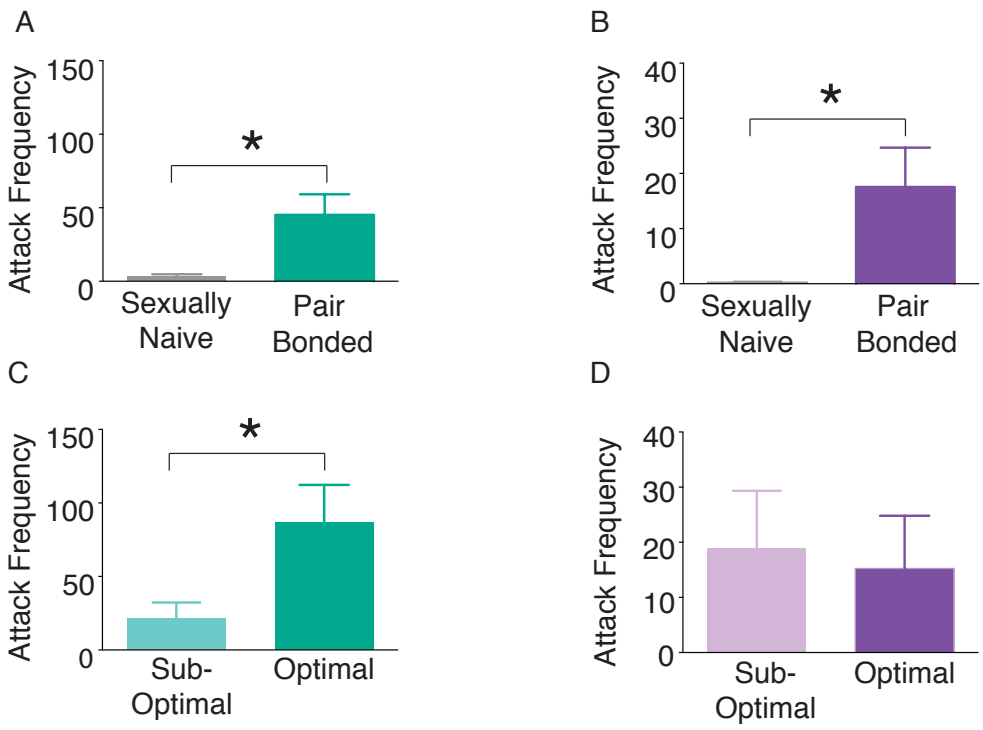

Figure 23

Sex differences in selective aggression. (A,B) Following pair bond formation, there is a significant increase in attack frequency in both male $U_{(1,22)} = 33$, $z = -2.261$, $p = 0.026$ (naïve: n = 11; paired: n = 13) and female $U_{(1, 15)} = 11$, $z = -2.336$, $p = 0.027$ (naïve: n = 6; paired: n = 11) prairie voles. (C,D) However, among pair bonded prairie voles, a comparison of optimal vs. sub-optimal pregnancy resulted in a significant difference in attack frequency in (C) male ($t_{(9)} = 2.541$, $p = 0.0316$) (sub-optimal: n = 6; optimal: n = 5), but not (D) female prairie voles ($t_{(9)} = 2.541$, $p = 0.0316$) (sub-optimal: n = 7; optimal: n = 4).

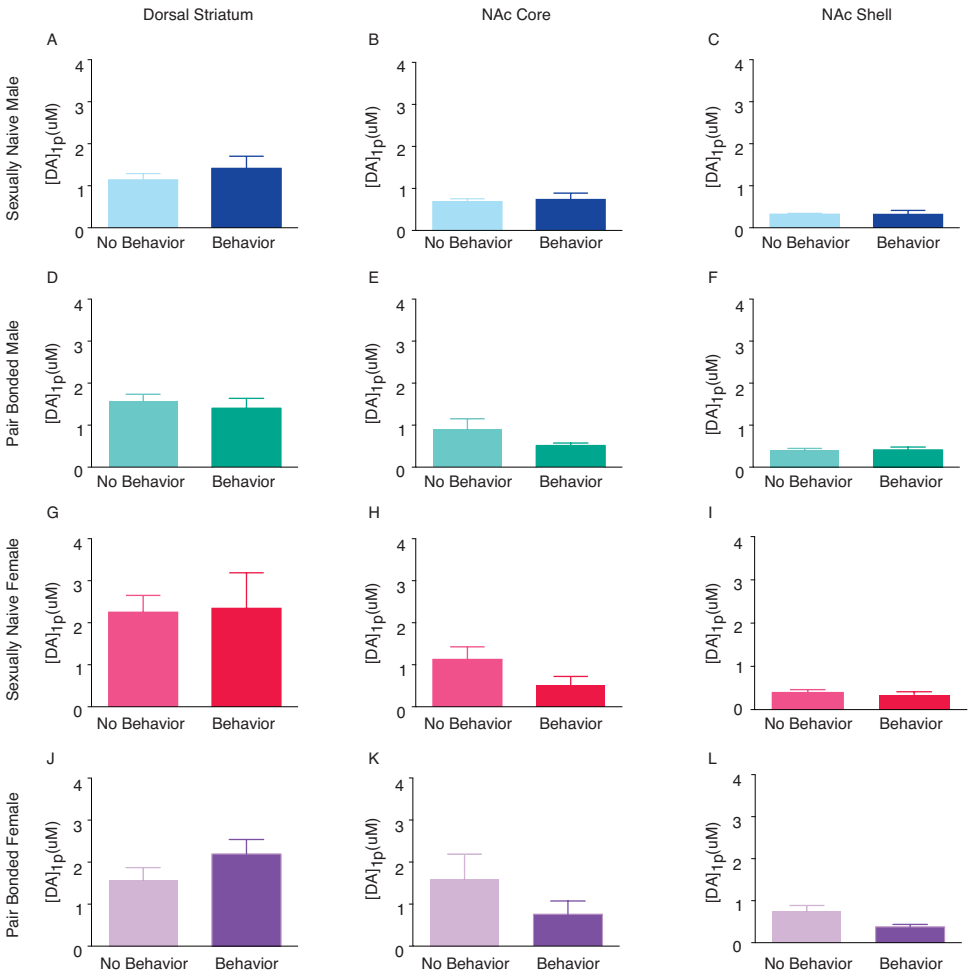

Figure 24

Differences in stimulated DA release between non-behavioral and behavioral tested groups. (A-C) Among sexually naive males, there was no difference in stimulated DA release within the dorsal striatum ($t_{(20)}$ = 0.821, p > 0.05) (no behavior n = 11, behavior n = 11), NAc core ($t_{(17)}$ = 0.7669, p > 0.05) (no behavior n = 10, behavior n = 9), or NAc shell ($t_{(16)}$ = 0.01048, p > 0.05) (no behavior n = 10, behavior n = 18) between males that had or had not undergone behavioral testing. (D-F) Among pair bonded males, there was also no difference in stimulated DA release between males that had or had not undergone behavioral testing within the dorsal striatum ($t_{(21)}$ = 0.5159, p > 0.05) (no behavior n = 12, behavior n = 11), NAc core ($t_{(19)}$ = 1.624, p > 0.05) (no behavior n = 10, behavior n = 11), or NAc shell ($t_{(15)}$ = 0.03571, p > 0.05) (no behavior n = 8, behavior n = 9). (G-I) Among control females, there was no difference in stimulated DA release between males that had or had not undergone behavioral testing within the dorsal striatum ($t_{(15)}$ = 0.119, p > 0.05) (no behavior n = 11, behavior n = 6), NAc core ($t_{(12)}$ = 0.1851, p > 0.05) (no behavior n = 9, behavior n = 5), or NAc shell ($t_{(11)}$ = 0.4888, p > 0.05) (no behavior n = 8, behavior n = 5). (J-L) Among pair bonded females, there was also no difference in stimulated DA release between males that had or had not undergone behavioral testing within the dorsal striatum ($t_{(14)}$ = 0.1.360 p > 0.05) (no behavior n = 8, behavior n = 8), NAc core ($t_{(14)}$ = 1.249, p > 0.05) (no behavior n = 8, behavior n = 8), or NAc shell ($t_{(11)}$ = 2.416, p > 0.05) (no behavior n = 7, behavior n = 6). Summary data are presented as mean ± SEM. t-test, Bonferroni corrections.

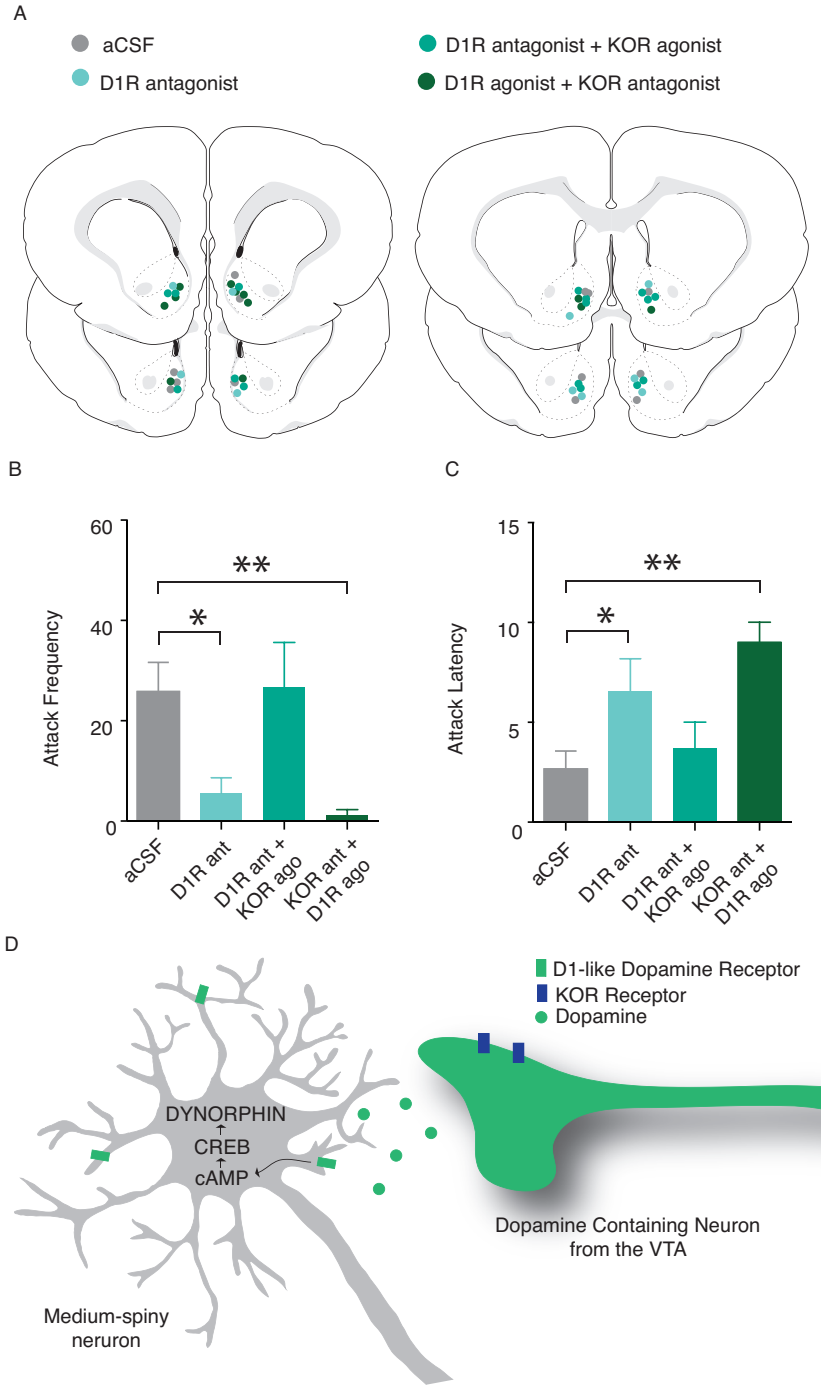

Figure 25

Interactions between D1-like and KORs mediate pair bond maintenance. (A) Histological verification of injection sites. (B,C) Compared to pair bonded males treated with aCSF (n = 6), blockade of D1-like receptors within the NAc shell attenuated selective aggression as well increased the latency to attack (n = 6). However, aggression levels and attack latencies were returned to normal when the D1-like receptor antagonist was administered in the presence of a KOR agonist (n = 7) suggesting that D1-mediated aggression results from downstream activation of KORs. This interaction was confirmed by the ability of a KOR antagonist to attenuate selective aggression despite the simultaneous activation of D1-like receptors (n = 7). (D) Mechanistic diagram demonstrating the interaction between D1-like receptors and the Dynorphin/KOR system. Summary data are presented as mean ± SEM. *P < 0.05, **P < 0.005.

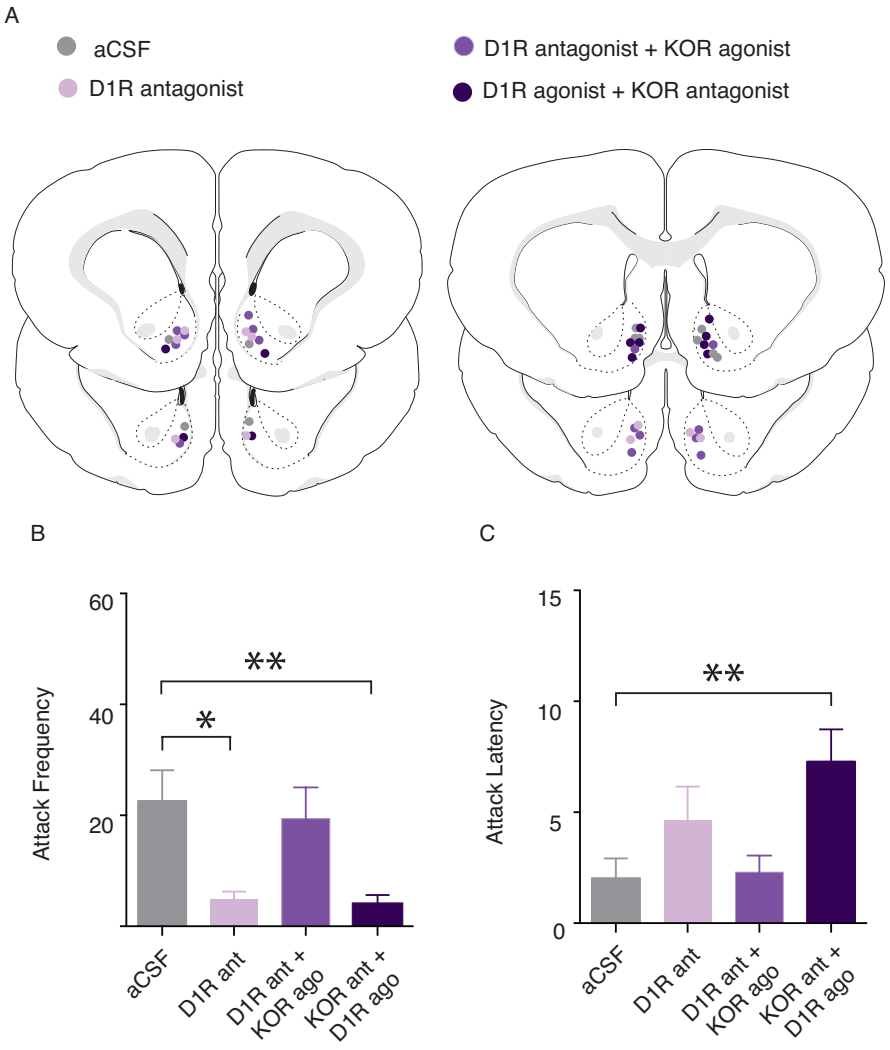

Figure 26

Interactions between D1-like and KORs mediate pair bond maintenance in male and female prairie voles. (A) Histological verification of injection sites. (B,C) A one-way ANOVA indicated an overall treatment effect in attack frequency ($F_{(3,23)}$ = 4.586, p = 0.013) as well as attack latency ($F_{(3,23)}$ = 4.774, p = 0.012) among pair bonded females. (C) Importantly, although activation of D1-like receptors was previously demonstrated to be important for selective aggression in males (Aragona *et al.*, 2006), it was unknown if these receptors also mediate this behavior in pair bonded females. Here, we show for the first time that activation of D1-like receptors within the NAc shell is also required for selective aggression in pair bonded females as attack behavior was significantly attenuated (p = 0.013) by blockade of these receptors. Similar to pair bonded males, blockade of these receptors while simultaneously activating KORs returned attack frequency to control levels (p = 0.620). (B,C) Interactions between these systems were further indicated by the ability a KOR antagonist to significantly attenuate (B) selective aggression (p = 0.008) as well as (C) increase attack latency (p = 0.005) despite simultaneous activation of D1-like receptors. There was no overall effect on affiliation ($F_{(3,23)}$ = 1.582, p = 0.225) or locomotor activity ($F_{(3,23)}$ = 0.688, p = 0.570) (Data not shown). (aCSF: n = 6; D1 antagonist: n = 6; D1 antagonist + KOR agonist: n = 7); D1 agonist + KOR antagonist: n = 6). Summary data are presented as mean ± SEM. *P < 0.05, **P < 0.005.

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CHAPTER 5
DISCUSSION

GENERAL SUMMARY

The ability of an animal to appropriately navigate its social environment, such as approaching and seeking positive social relationships while actively avoiding those that are potentially harmful, is critical to the evolutionary success of a species (Mateo, 1996; Kringelbach, 2010). This requires that positive social stimuli (i.e. a mating partner, parent offspring interactions, social play, social bonding, group cooperation, etc.) be processed as rewarding (Trezza *et al.*, 2010; Trezza *et al.*, 2011) while those that are harmful (i.e. an intruder, predator, or simply social isolation) be processed as aversive (Cacioppo *et al.*, 2011; Resendez *et al.*, 2012). Such processing is strongly influenced by endogenous opioid transmission (Cabanac, 2010; Dickinson & Balleine, 2010; Frijda, 2010b; Leknes & Tracey, 2010). Here, we have demonstrated that monogamous pair bonds are formed and maintained by a balance between mu- and kappa-opioid receptor activation (Burkett *et al.*, 2011; Resendez *et al.*, 2012). Activation of each of these systems plays a critical role in guiding motivated behavior (Cabanac, 1999) — that is promoting seeking and contact with positive social stimuli and avoiding negative social encounters (Resendez & Aragona, 2013).

Utilizing the socially monogamous prairie vole (Getz *et al.*, 1981; Getz *et al.*, 1993) as an animal model of selective social attachment (Carter *et al.*, 1995; Young *et al.*, 1998; Young *et al.*, 2001; Young *et al.*, 2008; Aragona & Wang, 2009), we first show that the formation of social bonds is mediated by activation of mu-opioid receptors throughout the striatum (Figure 27) (Resendez *et al.*, 2013). The initial formation of a pair bond is associated with positive social interactions with the mating partner and these interactions require sexually naïve prairie voles to be generally affiliative toward novel conspecifics and readily approach unknown individuals (Aragona *et al.*, 2006). Such affiliative social interactions have long been theorized to be mediated by activation of mu-opioid receptors (Panksepp *et al.*, 1980). Specifically the opioid hypothesis of social attachment posits that activation of mu-opioid receptors induces a positive hedonic state and this hedonic state reinforces positively valenced social behaviors (Panksepp *et al.*, 1980). Importantly, careful anatomical studies of the neurochemistry of positive hedonics associated with food reward have identified that mu-opioid receptors specifically within the dorso-medial shell of the nucleus accumbens mediates this psychological state (Pecina *et al.*, 2006). And, in the present body of work, we have demonstrated for the first time that activation of mu-opioid receptors within the dorso-medial nucleus shell is also required for pair bond formation, thus providing the first neural mechanism for positive hedonics associated with attachment formation (Resendez *et al.*, 2013). Additionally, we demonstrated that mu-opioid receptors within the dorsal striatum, that are involved in appettitive motivated behavior (Difeliceantonio *et al.*, 2012), also

mediate pair bond formation through their regulation of mating behavior (Resendez *et al.*, 2013). Together, these data demonstrate that activation of neural substrates of positive hedonics and motivation during affiliative and mating behavior is important for pair bond formation (Resendez & Aragona, 2013).

In contrast to pair bond formation, pair bond maintenance is mediated by the aversive processing of novel social stimuli (Figure 27). The neural processing of individuals, other than the mating partner as aversive involves the dynorphin/kappa-opioid receptor system (Resendez *et al.*, 2012) — a key neural generator of stress and aversion (Mucha & Herz, 1985; Mague *et al.*, 2003; Shirayama *et al.*, 2004; Bruchas *et al.*, 2007; Schindler *et al.*, 2010; Smith *et al.*, 2012). This system is activated during stressful or threatening situations (Yamada & Nabeshima, 1995) and may indicate that a social stimulus is threatening (Bruchas *et al.*, 2011). For example, when an intruder enters the home territory of an established breeding pair, it is important to the maintenance of the pair bond that the mate is guarded and prevented from mating with any novel individual (Getz & Carter, 1996). This prevention requires that an intruder is encoded as aversive and prevented from entering the home territory (Getz *et al.*, 1981; Carter & Getz, 1993; Ophir *et al.*, 2008; Solomon *et al.*, 2009). Thus, in order to maintain the pair bond, novel individuals must be perceived as aversive and prevented from entering the home territory through aggressive rejection (i.e. mate guarding) (Carter & Getz, 1993).

Importantly, we have demonstrated that kappa-opioid receptor regulation of pair bond maintenance is specific to the nucleus accumbens shell (Resendez

et al., 2012) — a key brain region for affect and motivation (Ikemoto & Panksepp, 1999; Kelley & Berridge, 2002; Roitman *et al.*, 2005; Taha & Fields, 2005; Aragona *et al.*, 2009). This brain region is activated in the presence of highly salient rewarding and aversive stimuli (Di Chiara *et al.*, 1999; Ito *et al.*, 2000; Robinson & Berridge, 2000; Kelley & Berridge, 2002; Carelli, 2004; Carlezon & Thomas, 2009; Umberg & Pothos, 2011) and is involved in the regulation of both approach and avoidance behaviors (Ikemoto & Panksepp, 1999). For example, both aversive and rewarding stimuli increase extracellular levels of dopamine in this region (Kalivas & Duffy, 1995; Ikemoto, 2007; Bromberg-Martin *et al.*, 2010) suggesting the general involvement of robust motivation in both positively and negatively valenced circumstances (Schultz, 2006; Berridge, 2007; Di Chiara & Bassareo, 2007; Becker, 2009; Tsai *et al.*, 2009; Koob & Volkow, 2010; Willuhn *et al.*, 2010; Alcaro & Panksepp, 2011; Hull, 2011; Volkow *et al.*, 2012). One possible mechanism for guiding the direction of motivated behavior is through interactions with brain affective systems that are important for valence coding.

Motivated behavior is strongly mediated by brain affective systems (Chen & Bargh, 1999; Cacioppo *et al.*, 2004; Winkielman *et al.*, 2005; Frijda, 2010b; Frijda, 2010a), such as the endogenous opioid system, with positive hedonics guiding approach behaviors and aversive signaling mediating avoidance behaviors (Vanderschuren *et al.*, 1995; Cacioppo *et al.*, 2004; Stein *et al.*, 2007; Le Merrer *et al.*, 2009; Kringelbach & Berridge, 2012). Interestingly, activation of motivational systems required for the display of selective aggressive and thus

pair bond maintenance, such as D1-like dopamine receptors (Aragona *et al.*, 2006), interact directly with aversion encoding opioid systems. Specifically, stimulation of low-affinity D1-like dopamine receptors that require high levels of dopamine release to be activated (Richfield *et al.*, 1989) increases expression of dynorphin (Gerfen *et al.*, 1990), the endogenous ligand of kappa-opioid receptors (Chavkin *et al.*, 1982). Given that D1-like receptors are important for motivated behavior (Sutton & Beninger, 1999; Aragona *et al.*, 2006; St Onge *et al.*, 2011) and kappa-opioid receptors signal aversion (Bals-Kubik *et al.*, 1989; Bals-Kubik *et al.*, 1993; McLaughlin *et al.*, 2003; McLaughlin *et al.*, 2006; Bruchas *et al.*, 2009; Bruchas *et al.*, 2010; Resendez *et al.*, 2012), it is possible, that in the case of an aversive stimulus, D1-like receptor activation may facilitate the release of dynorphin and subsequent activation of kappa-opioid receptors. This hypothesis was supported by behavioral pharmacology data presented in chapter three showing that D1-mediate aggression exerts its effects on pair bond maintenance through downstream activation of kappa-opioid receptors. Thus, interactions between these systems may be required to tag a social stimulus as aversive and promote motivated behavior in the direction of either avoiding or possibly attacking this stimulus.

The formation and maintenance of stable social bonds is a critical component of human health and happiness (House *et al.*, 1988; Sobal *et al.*, 1992; Umberson *et al.*, 2010; Cacioppo *et al.*, 2011). From an evolutionary perspective, these bonds serve a very important purpose and that is to facilitate mating and the successful rearing of offspring (Watson *et al.*, 2010). Here, we

have provided evidence that the formation and maintenance of such bonds requires the ability to appropriately code the valence of social cues (Resendez & Aragona, 2013). This evidence was primarily provided by the disruption of social behaviors associated with pair bond formation and maintenance following pharmacological blockade of either mu- or kappa-opioid receptors, respectively. Studies of behavioral pharmacology are useful because they can provide information in regards to whether or not a certain neurochemical system is involved in a behavior; however, they do not provide information on the specific release patterns of the associated neurochemicals during the actual social interactions. Thus, although our data show convincing evidence that activation of both mu- and kappa-opioid receptors is required for pair bonding, more work is necessary to figure out the specific release dynamics of dopamine and endogenous opioids that result in either affiliative or aversive social encounters.

OPIOID REGULATION OF PAIR BOND FORMATION

Pair bond formation is associated with positive social interactions such as affiliation, mating, and side-by-side contact (Getz & Carter, 1996). These types of social interactions require the seeking out of a mating partner and continued close proximity once a mating partner has been established (Resendez & Aragona, 2013). To achieve these types of interactions the mating partner must be processed as rewarding (Aragona & Wang, 2009) and here we have demonstrated that this in part mediated by the activation of mu-opioid receptors within the striatum. Specifically, mu-opioid receptors within the dorsal striatum

are important for mating behavior suggesting a role in motivational aspects of bond formation, while mu-opioid receptors within the dorso-medial nucleus accumbens shell inhibited pair bond formation without affecting mating suggesting the mu-opioid receptors within this region mediate hedonic aspects of pair bond formation (Resendez *et al.*, 2013). However, in order to more fully understand how mu-opioid receptors regulate pair bond formation, it is important to determine the temporal dynamics of opioid neurotransmission throughout the striatum during social interactions associated with pair bond formation.

Within the striatum, two endogenous opioids, enkephalin and beta-endorphin, both have high affinity for mu-opioid receptors (Mansour *et al.*, 1995), but differ in the neuron population that they are released from as well as the anatomical localization of their release (Bloom, 1983). Specifically, enkephalin is released locally throughout the dorsal and ventral striatum from medium spiny neurons containing the D2-like dopamine receptor (Gerfen *et al.*, 1990), while beta-endorphin is only released within the ventral striatum from beta-endorphin containing neurons from the arcuate nucleus of the hypothalamus (Finley *et al.*, 1981). Additionally, within the nucleus accumbens, these two opioids also differ in their regulation of social behavior as beta-endorphin, but not enkephalin, enhances social play in juvenile rodents (Trezza *et al.*, 2011). Thus, within the nucleus shell, it is also important to determine which endogenous opioid mediates partner preference formation.

One technique for measuring extracellular levels of opioid peptides in the brain is microdialysis. Importantly, this technique allows for multiple

neurochemicals, such as enkephalin and beta-endorphin, to be measured in the brain of freely moving animals (Marinelli *et al.*, 2004; Marinelli *et al.*, 2005). Additionally, recent technological advances utilizing smaller probes in combination with liquid chromatography-mass spectrometry allows for opioids to be detected at greater spatial and temporal resolution than previously possibly. Specifically, opioids can be detected at intervals of 20 minutes (and, possibly shorter, see below) within discrete regions of the striatum, such as the rostral-medial region of the dorsal striatum (Difeliceantonio *et al.*, 2012). Application of such a technique to studies of pair bonding in prairie voles would allow for the specific opioid ligand that is being released within each region of the striatum to be identified and, possibly, for the release of each ligand during mating and affiliation to be determined (Slaney *et al.*, 2012). Specifically, mating behavior occurs at highest levels during the first 6 hours of pairing and is followed by long periods of side by side contact (Burkett & Young, 2012). Therefore, measuring opioid release during periods of the cohabitation where the probability of each of these behaviors are at their highest may provide further insight into opioid regulation of pair bond formation.

INTERACTIONS BETWEEN THE DOPAMINE AND OPIOID SYSTEM IN PAIR BOND MAINTENANCE

Previous research on the neurobiology of pair bond maintenance has determined that activation of D1-like receptors within the nucleus accumbens shell is important for selective aggression (Aragona *et al.*, 2006). Given that

these receptors are the low affinity type receptor and require high levels of dopamine release to be activated (Richfield *et al.*, 1989), it was hypothesized that pair bonded prairie voles would have an enhancement of dopamine transmission within the NAc shell. Indeed, we show that both pair bonded male and female prairie voles have an enhancement of stimulated dopamine within the NAc shell, but not other regions of the striatum. This enhancement in dopamine release potential specifically within the nucleus accumbens shell may act to facilitate the activation of D1-like dopamine receptors that are important for selective aggression and therefore contribute to the transition from a generally affiliative social strategy to one dominated by aggressive rejection.

While both sexes showed an enhancement of dopamine release potential within the nucleues shell, this enhancement was dependent on the fecundity of the pair for male, but not female prairie voles. Fecundity of the pair is determined by measuring the neonatal weight (an indicator of gestational stage) at the time of testing and depending on the average weight of the pups, the female is either classified as optimally, sub-optimally, or not pregnant (Resendez *et al.*, 2012). A classification of optimal pregnancy means that the female was induced into estrus and became pregnant as soon as physiologically possible upon pairing with a male (indicated by an average neonatal weight of > 0.30g), while a sub-optimal pregnancy means that there was a delay in pregnancy (indicated by an average neonatal weight of < 0.30g) (Curtis, 2010). Importantly, male prairie voles were paired with females that were optimally pregnant were significantly more aggressive than males that were paired with females who were sub-

optimally pregnant. In contrast, pregnancy has no effect on aggression levels in pair bonded females as there is no difference in aggression directed at intruders between females that are sub-optimally or optimally pregnant (Resendez *et al.*, 2012). Together, these data suggest that males are more motivated to guard females in which they are known to have a high chance of reproductive success with, while the establishment of pregnancy has no effect on female pair bonding. The lack of an effect in females is hypothesized to be related to sex differences in reproductive needs (Resendez *et al.*, 2012). Specifically, females require extended periods of contact with a male to become sexually receptive (Cushing & Carter, 1999) and therefore may need to form bonds faster than males in order to keep the female in close contact with a male.

To determine if sex differences in selective aggression are related to sex differences in the relationship between pregnancy status and peak dopamine release potential within the nucleus accumbens shell, we separated the peak dopamine release values of pair bonded subjects by pregnancy (i.e., not pregnant, sub-optimal, or optimal) and compared these values to sexually naïve subjects. Among pair bonded males, an enhancement in stimulated dopamine release within the NAc shell only occurred in male prairie voles that were paired with a female who was optimally pregnant. However, among pair bonded females, there was a general increase in peak dopamine release regardless of the pregnancy status of the female. Given that selective aggression is mediated by dopaminergic activation of D1-like dopamine receptors (Aragona *et al.*, 2006), this sex difference in changes in dopamine neurotransmission provides a

mechanism as to why males paired with optimally pregnant females are more aggressive than males that are paired with sub-optimally pregnant females. Indeed, both neonatal weight as well as the level of aggression during the resident-intruder paradigm was positively correlated with peak dopamine release within the NAc shell of pair bonded males, but not females. Together, these data suggest that the farther along in pregnancy that the female partner is, the greater the propensity for dopamine release is, and the more aggressive the male is suggesting that males are more motivated to protect a female that they know they have a high reproductive potential with.

Although the above data provide convincing evidence that a greater propensity for dopamine release mediates selective aggression in pair bonded prairie voles, the dopamine measurements described above were made in *in vitro* slice preparations and therefore cannot speak directly to how dopamine transmission *in vivo* mediates selective aggression. As mentioned above, D1-like receptors are the low-affinity type receptor (Richfield *et al.*, 1989) and it is thus hypothesized that the presence of an intruder into the home cage (or territory) induces burst-like firing of dopamine neurons evoking high levels of dopamine release specifically within the NAc shell (Aragona & Wang, 2009). This hypothesis is based on multiple lines of existing evidence: (a) blockade of D1-like receptors within the NAc shell, but not the core attenuates selective aggression (Aragona *et al.*, 2006), (b) stimulated dopamine release is enhanced within the Nac shell, but not other regions of the striatum, in pair bonded prairie voles (Chapter 4), and (c) the fact that aversive stimuli preferentially enhance

stimulated dopamine release within the NAc shell (Kalivas & Duffy, 1995). Thus, a considerable amount of evidence exists to suggest that high levels of dopamine release within the NAc shell mediate selective aggression, but *in vivo* measurements of dopamine transmission during the selective aggression paradigm are lacking.

The resident-intruder paradigm is a 6-10 minute social interaction test in which an intruder animal is placed into the home cage of the test subject (Gobrogge & Wang, 2011). Within the first minute of the test, animals will usually approach each other and subsequently engage in olfactory investigation for one to two minutes (personal observation). In the case of pair bonded prairie voles, this individual is usually processed as aversive and aggressively rejected (Resendez & Aragona, 2013). These aggressive bouts last anywhere for 30 seconds to one minute and occur periodically throughout the testing period (personal observation). To determine if phasic dopamine transmission mediates these brief bouts of aggression would require the ability to measure dopamine transmission on a fast time scale.

One method for measuring dopamine transmission in awake behaving animals is microdialysis (Mermelstein & Becker, 1995). With traditional microdialysis methods, dopamine can be measured during behavior, but only at relatively long time scales (i.e., 10 minutes) that do not capture the dynamic nature of most behavioral interactions (Robinson *et al.*, 2003). In addition, traditional microdialysis probes tend to be relatively large (200-400 μm diameter and 1-4 mm length) (Slaney *et al.*, 2012) thus, offering poor spatial resolution that

is not optimal for determining differences in dopamine transmission between close brain nuclei, such as the nucleus accumbens core and shell (Robinson *et al.*, 2003). Given the poor spatial and temporal resolution of traditional microdialysis methods, this technique is not optimal for determining the precise dopamine transmission dynamics within the nucleus accumbens shell that contribute to selective aggression in pair bonded prairie voles.

One technique that offers both high spatial and temporal resolution of dopamine transmission is the application of fast-scan cyclic voltammetry (FSCV) to freely moving animals (Renec *et al.*, 1997; Phillips *et al.*, 2003). In contrast to traditional microdialysis methods, FSCV can measure dopamine transmission every 100 milliseconds, a time-scale that is on par with real-time dopamine release dynamics (Robinson *et al.*, 2003) and allows for dopamine transmission to be correlated to discrete behavioral events (Roitman *et al.*, 2008; Day *et al.*, 2010; Willuhn *et al.*, 2010; Wheeler *et al.*, 2011; Badrinaryan *et al.*, 2012; Oleson *et al.*, 2012), including those of a social nature (Robinson *et al.*, 2001; Champagne *et al.*, 2004; Robinson *et al.*, 2011). Additionally, to measure changes in dopamine transmission, FSCV utilizes small carbon-fiber microelectrodes that are 5-30 μm in diameter and 25-400 μm in length (Robinson *et al.*, 2003). These electrodes are considerably smaller than traditional microdialysis probes and allow for discrete spatial resolution, such as the ability to accurately distinguish between sub-compartments of the nucleus accumbens (Aragona *et al.*, 2009; Maina & Mathews, 2010). Thus, FSCV offers high temporal and spatial resolution (Robinson *et al.*, 2003) that is ideal for

determining the precise dopamine transmission properties that mediate selective aggression in pair bonded prairie voles.

In addition, FSCV can be combined with the administration of pharmacological agents that directly target neurochemical systems known to modulate dopamine transmission (Aragona *et al.*, 2008; Zhang *et al.*, 2009; Maina & Mathews, 2010), such as the endogenous opioid system (Ebner *et al.*, 2010). We have shown that activation of kappa-opioid receptors within the nucleus accumbens shell is important for selective aggression and activation of these receptors inhibits dopamine release (Shippenberg & Herz, 1986; Di Chiara & Imperato, 1988; Ebner *et al.*, 2010). However, it is unknown how kappa-opioid receptor activation during an aggressive encounter modulates dopamine dynamics and how this modulation effects the expression of aggression. To determine how kappa-opioid receptor modulation of dopamine transmission affects aggressive behavior, a kappa-opioid receptor antagonist could be administered prior to *in vivo* measurements of dopamine transmission during the resident-intruder paradigm. This would remove the ability of kappa-opioid receptor activation to modulate dopamine transmission thus, allowing for interactions between these systems during an aggressive social encounter to be determined.

Activation of kappa-opioid receptors is required for the display of selective aggression and these receptors are activated by the endogenous opioid, dynorphin (Chavkin *et al.*, 1982). Within the striatum, dynorphin is released locally from medium spiny neurons that express D1-like dopamine receptors

(Tejeda *et al.*, 2012) and stimulation of these receptors increases the expression of dynorphin (Gerfen *et al.*, 1990). Given the close interaction between D1-like dopamine receptors and the dynorphin/kappa-opioid receptor system, it has been hypothesized that D1-mediated aggression occurs through down-stream activation of kappa-opioid receptors (Resendez & Aragona, 2013). Testing this hypothesis directly would require *in vivo* measurements of dynorphin release during the resident-intruder paradigm in pair bonded subjects that have either been pretreated with peripheral administration of saline (control) or a D1-like receptor antagonist. If our hypothesized mechanism is correct, the presence of an intruder would result in increased levels of dynorphin and aggression in control subjects, but not in subjects pre-treated with a D1-like receptor antagonist.

Testing our hypothesized mechanism requires the ability to measure extracellular concentrations of dynorphin within the nucleus accumbens shell of freely moving prairie voles. Additionally, given the specificity of the D1- and kappa-mediated aggression to the nucleus accumbens shell and the dynamic nature of aggressive social encounters, testing of this hypothesis also requires an *in vivo* dynorphin measurement that has high spatial and temporal resolution. As mentioned above, microdialysis probes can now be manufactured small enough to accurately measure opioid peptides from discrete compartments within the striatum (Difeliceantonio *et al.*, 2012). Additionally, improvements in limits of detection allow for opioids, including dynorphin, to be measured at faster sampling rates (3.8 minutes compared to 30 minutes) (Zhou *et al.*, 2013) that can

be more accurately correlated to behavioral evens such as bouts of aggression. The combination of these improved microdialysis procedures (Slaney *et al.*, 2012) with behavioral pharmacology targeting D1-like dopamine receptors (Aragona *et al.*, 2006) will allow us to directly determine if interactions between D1-like dopamine receptors and the dynorphin/kappa-opioid receptor system within the nucleus accumbens indeed mediate selective aggression.

FUTURE DIRECTIONS

Social bonding requires the activation of reward circuitry and the appropriate valence processing of social cues within this system (Resendez & Aragona, 2013). However, reward circuitry is also subject to the influence of unnatural rewards, such as drugs of abuse that can cause permanent and dramatic alterations to this system (Panksepp *et al.*, 2002; Kelley, 2004; Robinson, 2004; Hyman *et al.*, 2006). Importantly, these alterations have been shown to cause severe disruptions in species-typical social behavior (Knight *et al.*, 2001; Gobrogge *et al.*, 2009; Liu *et al.*, 2010; Young *et al.*, 2011). Additionally, psycho-social stressors have also been shown to induce permanent alterations in reward circuitry that can disrupt the propensity for social bonding (Panksepp *et al.*, 1997; Tucker *et al.*, 2005; Stein *et al.*, 2007). Thus, both pharmacological and environmental insults to reward circuitry can interfere with naturally rewarding behaviors that are important for species survival, including those of a social nature.

Although both drugs of abuse and stress can be devastating to the appropriate development of species-typical social behaviors (Johns *et al.*, 1997; Panksepp, 2003; Slamberova *et al.*, 2005; Vanderschuren *et al.*, 2008), some individuals seem to be resilient to such insults to reward circuitry (Robinson & Berridge, 2003). Interestingly, one natural buffer appears to be the presence of stable social bonds (Ellickson *et al.*, 1999; Bell *et al.*, 2000). Therefore, while the absence of stable social relationships (a severe psychological stressor to highly social species) can leave an individual vulnerable to psychological disorders, such as addiction (Brennan & Shaver, 1995; Vungkhanching *et al.*, 2004; Caspers *et al.*, 2005), positive social relations can protect against the rewarding properties of drugs of abuse (Kosten *et al.*, 1987; Westenbroek *et al.*, 2013), perhaps due to socially induced alterations in reward circuitry (Insel, 2003). Importantly, the neural protective effects of social bonding against drugs of abuse can readily be studied in the social monogamous prairie vole.

Similar to other mammals, including humans, prairie voles find psychostimulants, such as amphetamine, to be rewarding (Aragona *et al.*, 2007b). However, this reward value is attenuated in pair bonded prairie voles (Liu *et al.*, 2010). Specifically, following pair bond formation, higher doses of amphetamine are required to induce a conditioned place preference than doses used in sexually naïve prairie voles (Aragona *et al.*, 2007a; Liu *et al.*, 2010). Moreover, this attenuation is the result of an up-regulation of D1-like receptors within the nucleus accumbens (Aragona *et al.*, 2006) as blockade of these receptors removes the protective effects of pair bonding (Liu *et al.*, 2010). Given,

that activation of D1-like receptors increases dynoprhin expression (Steiner & Gerfen, 1996) and that activation of the dynorphin/kappa-opioid receptor system attenuates reward (Shippenberg & Herz, 1986; Di Chiara & Imperato, 1988; Spanagel *et al.*, 1990; Todtenkopf *et al.*, 2004), it is possible that the protective effects of D1-like dopamine receptors are actually mediated by downstream activation of the dynorphin/kappa-opioid receptor system. Thus, it will be important to determine if alterations in the dynorphin/kappa-opioid receptor system that occur after pair bonding directly mediate the protective effects against drug reward.

CONCLUSION

The data presented in the present dissertation suggests that diverse neurochemical systems regulate the motivational control of social behaviors associated with pair bonding. Specifically, our results have demonstrated that activation of mu-opioid receptors that are associated with positive hedonics mediates partner preference formation while kappa-opioid receptors that are associated with aversion mediate pair bond maintenance (Figure 27). The importance of these receptor systems has primarily been demonstrated through pharmacological manipulations; therefore, *in vivo* measurements of dopamine and opioid neurotransmission during social interactions associated with pair bonding are required to confirm that these systems indeed mediate these behaviors. Additionally, *in vivo* measures of dopamine and opioid neurotransmission would allow for the timing of the release during social

interactions associated with pair bond formation and maintenance to be determined. This is especially important in regards to pair bond maintenance, as the release properties of dynorphin within the striatum (or other regions of the brain) during naturally occurring behaviors are currently unknown. Nonetheless, our data, in general, suggest that interactions between valence coding systems and motivational circuitry are critical for guiding the direction of socially motivated behaviors, such as the motivation to form and maintain pair bonds.

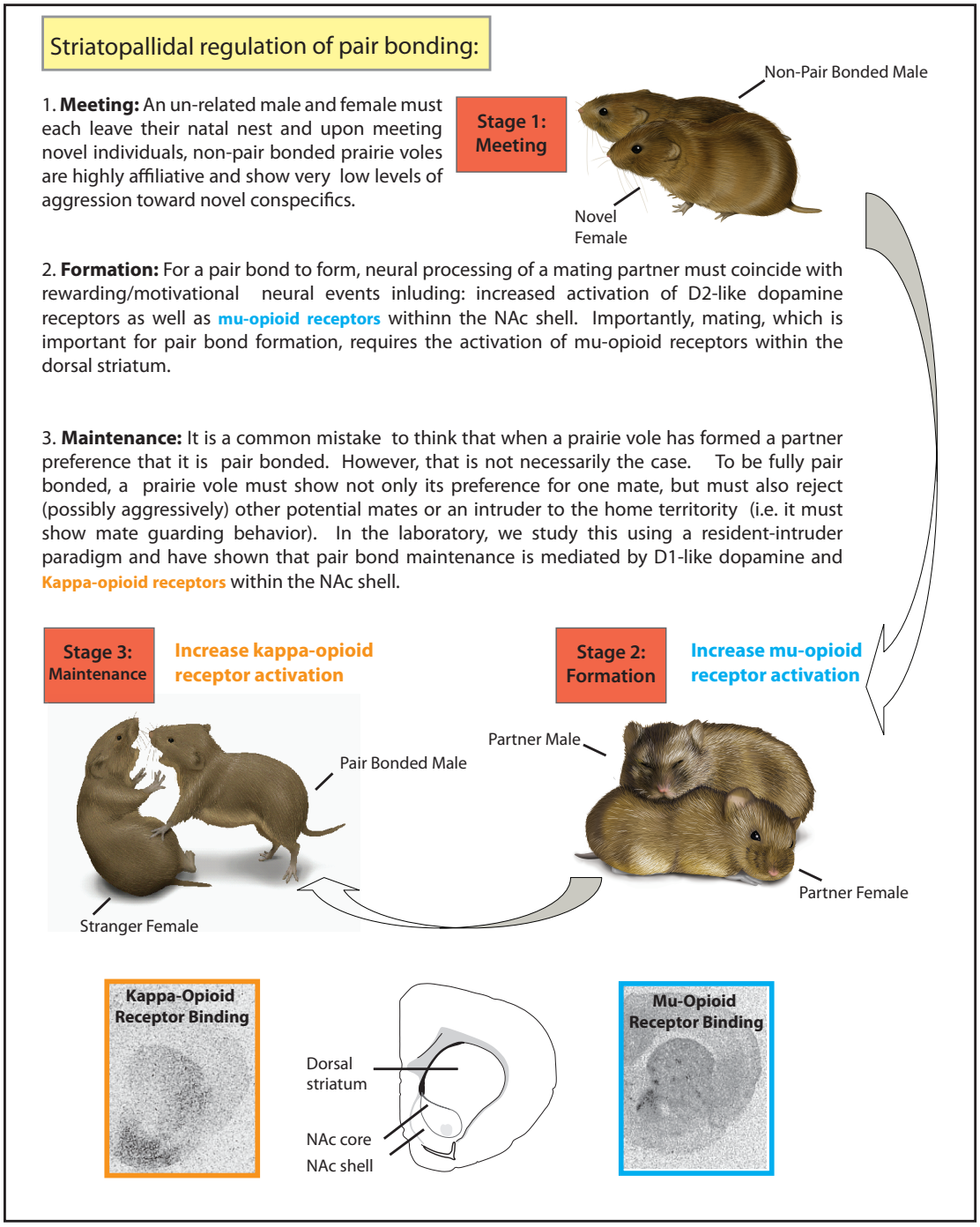

Figure 27

Model of opioid regulation of pair bonding in prairie voles.

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