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THE EFFECTS OF MORPHINE, NALOXONE, AND κ OPIOID MANIPULATION ON ENDOCRINE FUNCTIONING AND SOCIAL BEHAVIOR IN MONOGAMOUS TITI MONKEYS (*CALLICEBUS CUPREUS*)

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Abstract—The μ opioid receptor (MOR) and κ opioid receptor (KOR) have been implicated in pair-bond formation and maintenance in socially monogamous species. Utilizing monogamous titi monkeys (*Callicebus cupreus*), the present study examined the potential role opioids play in modulating the response to separation, a potent challenge to the pair-bond. In Experiment 1, paired male titi monkeys were separated from their pair-mate for 30-min and then received saline, naloxone (1.0 mg/kg), morphine (0.25 mg/kg), or the KOR agonist, U50,488 (0.01, 0.03, or 0.1 mg/kg) in a counter-balanced fashion, immediately prior to a 30-min reunion with their mate. Blood samples were collected immediately prior to and after the reunion. Males receiving morphine approached females less, initiated contact less, and females broke contact with the males less. The increase in cortisol in response to naloxone was greater compared to vehicle, and the increase in cortisol in response to the high dose of U50,488 compared to vehicle approached significance. In Experiment 2, paired males were treated with the KOR antagonist, GNTI (0.1, 0.3, or 1.0 mg/kg), or saline 24 h prior to a 60-min separation from their mate. Blood samples were collected at the time of injection and immediately before and after separation. Administration of the low dose of GNTI decreased the locomotor component of the separation response compared to vehicle. The present study found that the opioid system is involved in both the affiliative and separation distress components of a pair-bond, and these components are regulated by different opioid receptors.

Key words: titi monkey, mu opioid, pair-bonding, cortisol, monogamy, kappa opioid.

INTRODUCTION

Socially monogamous species form long-term associations between two adults. In some species, these relationships have been shown to be classic attachment bonds (Hazan and Shaver, 1987) and result in pair-mates spending considerable time in physical contact with one another, providing social buffering, and exhibiting substantial behavioral and physiological agitation upon involuntary separation (Mason and Mendoza, 1998). Due to the rarity of monogamy in mammals (Kleiman, 1977) there is a paucity of data on the neurobiological underpinnings of adult attachment. Research on infant–mother attachments and monogamous prairie voles (*Microtus ochrogaster*) suggests that the opioid system may play a role. The monogamous titi monkey (*Callicebus cupreus*) is an animal model that we can use to further our understanding of the relationship between opioids and adult attachment. The overarching premise of this paper is that different components of the opioid system play distinct and, potentially, opposing roles in regulating the behavioral and physiological determinants of the emotional bond that characterize adult attachment relationships.

The opioid system regulates infant affiliation toward an adult attachment figure and the response to involuntary separation. μ opioid receptor (MOR) agonists, such as morphine, decrease physical contact between social partners; whereas, opioid antagonists, such as naloxone, increase physical contact (Keverne et al., 1989; Schino and Troisi, 1992; Kalin et al., 1995; Martel et al., 1995). Furthermore, MOR agonists reduce infant separation vocalizations in monkeys, dogs, guinea pigs, and rat pups (Herman and Panksepp, 1978; Panksepp et al., 1980; Kalin et al., 1988; Nelson and Panksepp, 1998). More generally, activation of the MOR system produces euphoria in humans and conditioned place preferences in rodents (Bardo et al., 1995; Boecker et al., 2008).

In contrast, the κ opioid system promotes attachment-like responses and seems to do so by regulating negative affect. κ opioid receptor (KOR) agonists increase ultrasonic vocalizations in rat pups during maternal separation, and they can induce ultrasonic vocalizations in situations where they do not usually occur (Carden

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Abbreviations: AVP, vasopressin; BBB, blood–brain barrier; CV, coefficients of variation; GLMM, generalized linear mixed model; GNTI, 5′-Guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7-2′,3′-indolomorphinan dihydrochloride; HPA, hypothalamic–pituitary–adrenal; ID, identification; IM, intramuscular; KOR, κ opioid receptor; MOR, μ opioid receptor; U50,488, (±)-trans-U50,488.

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et al., 1991, 1994). The KOR system is involved in producing unpleasant affective responses to stressors (McLaughlin et al., 2006; Land et al., 2008). KOR agonists produce conditioned place aversions (Land et al., 2008) and dysphoria in humans (Pfeiffer et al., 1986). Mice deficient in dynorphin (the endogenous ligand of this system) or animals administered a KOR antagonist prior to a forced swim stress test do not develop the expected conditioned aversions (Land et al., 2008).

The function of opioids in adult attachment is not well studied. Shapiro and colleagues (1989) found that morphine reduced side-by-side contact in monogamous prairie voles, however the antagonist naloxone had no effect on social behavior. MOR blockade prevents pair-bond formation in prairie voles possibly by blocking the rewarding components of initial social interactions such as sexual behavior. Peripheral administration of the opioid antagonist, naltrexone, or central administration of the MOR antagonist, CTAP, in the dorsal striatum or dorsomedial shell of the nucleus accumbens blocks partner preference formation in prairie voles without affecting physical contact (Burkett et al., 2011; Resendez et al., 2013). A recent study discovered that prairie voles have greater MOR binding in the brain in general compared to polygamous meadow voles (Inoue et al., 2013). There is also evidence that the KOR plays a role in pair-bond maintenance. A facet of an established pair-bond in prairie voles is mate-guarding, when individuals are aggressive toward a stranger conspecific (Aragona and Wang, 2004). When pair-bonded male prairie voles are administered the KOR antagonist, nor-BNI, peripherally or locally in the nucleus accumbens shell, there is a decrease in attacks toward stranger males (Resendez et al., 2012). This finding suggests that the KOR is needed to maintain a pair-bond. It is possible that the KOR is driving the negative affective reaction to a stranger intruder (Resendez and Aragona, 2013).

The MOR and KOR also affect the physiological components of stress, particularly the hypothalamic–pituitary–adrenal (HPA) axis. In primates, including humans, and sheep MOR activation results in a decrease in cortisol concentrations (Zis et al., 1984; Parrott and Thornton, 1989; Broadbear et al., 2004), and opioid blockade with either naloxone or naltrexone increases cortisol concentrations (Williams et al., 2003; Wand et al., 2012; Ragen et al., 2013). KOR activation, in contrast, activates the HPA axis in humans, non-human primates, and rodents leading to increased corticotropin-releasing hormone, adrenocorticotropic hormone, and glucocorticoids (Calogero et al., 1996; Pascoe et al., 2008; Ranganathan et al., 2012).

Currently, there is only one study examining the effects of opioid manipulation in a monogamous non-human primate. Administration of morphine or naloxone to monogamous titi monkey males in established pair-bonds has no effect on affiliative behavior or separation distress behavior (Ragen et al., 2013). However, the temporary absence of the pair-mate resulted in an increase in naloxone's stress-related effects, indicated by exaggerated increases in locomotion, cortisol, and vasopressin (AVP) suggesting that the presence of a pair-mate aids in homeostatic regulation of the opioid system in titi monkeys (Ragen et al., 2013).

The finding that MOR manipulation had no effect on affiliative behavior was surprising due to the several studies in nonhuman primates and prairie voles that found that MOR activation decreases physical contact (Keverne et al., 1989; Shapiro et al., 1989; Kalin et al., 1995) and opioid blockade increases physical contact (Keverne et al., 1989; Schino and Troisi, 1992; Martel et al., 1995). One possible reason for the null findings was due to methodology. A study by Kalin et al. (1995) found that morphine decreased physical contact and naltrexone increased physical contact between mother and infant macaques when they were administered the drug immediately prior to a reunion after a 30-min separation. In the study by Ragen et al. (2013) titi monkeys were removed from their home cage, administered morphine or naloxone, and put immediately back. It is possible that a disruption of MOR via a separation was needed for MOR opioid manipulation to have an effect on social behavior in titi monkeys. In addition to affiliative behaviors, MOR manipulation had no effect on separation distress behaviors, particularly vocalizations. This is surprising due to the wealth of literature finding that MOR agonism decreases separation distress vocalizations in other attachment relations, specifically that between offspring and mother (Herman and Panksepp, 1978; Panksepp et al., 1978; Kalin et al., 1988; Nelson and Panksepp, 1998).

The present study sought to further explore the opioid system's role in affiliative and separation distress behavior in titi monkey males with established pair-bonds, and attempt to discover if and how the opioid system regulates social behavior and separation distress. Our first goal was to examine how MOR manipulation could affect affiliative behavior with a methodology different from Ragen et al. (2013) since no effect was observed. We predicted that MOR activation in paired titi monkeys with morphine would decrease affiliative behavior while opioid antagonism with naloxone would increase affiliative behavior after reunited with their pair-mate, which mirrors the paradigm used by Kalin et al. (1995). Our second goal was to explore whether the KOR was involved in separation distress in titi monkeys since it does not appear that the MOR is involved. We hypothesized that the KOR system may regulate the separation distress response because of its involvement in the induction of dysphoria (Land et al., 2008), its ability to induce ultrasonic vocalizations in rat pups (Carden et al., 1994), and its involvement in maintaining prairie vole pair-bonds (Resendez et al., 2012). Therefore we predicted that KOR activation via the KOR agonist, U50,488, would induce a separation distress response and that the KOR antagonist, GNTI (5′-Guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7-2′,3′-indolomorphinan dihydrochloride), would attenuate the separation distress response.

Finally, we measured how these pharmacological manipulations affected HPA activity and AVP. We predicted that morphine would decrease cortisol

concentrations, while naloxone and U50,488 would increase cortisol, and U50,488 would decrease AVP concentrations.

EXPERIMENTAL PROCEDURES

Subjects

Eight titi monkey males living in established pairs were used as subjects for each experiment. See Table 1 for subject details. For Experiment 1, one male was living with two offspring and four were living with one offspring. For Experiment 2, two males were living with two offspring and three were living with one offspring. Seven of the eight subjects were the same in Experiment 2 as in Experiment 1. A subject from Experiment 1 had to be replaced due to the health of its offspring. At the beginning of Experiment 1 the mean (±SEM) age of the subjects was 116.3 ± 15.4 months, and the mean (±SEM) time after pairing was 48.8 ± 12.1 months. At the beginning of Experiment 2 the mean (±SEM) age of the subjects was 112.8 ± 15.3 months, and the mean (±SEM) time after pairing was 50.4 ± 12.2 months. Adult pairs and any offspring were housed in cages (1.2 m × 1.2 m × 1.8 m) at the California National Primate Research Center. Rooms were set on a 12:12-h light:dark cycle with lights on at 0600 and lights off at 1800. The temperature was maintained at 21 °C. Housing conditions were similar to those found in Mendoza (1999). Subjects were fed daily at 0800 and 1300 h on a diet of New World monkey chow, apples, carrots, rice cereal, and bananas. Water was available *ad libitum*. All procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee.

Drugs

Dissolved morphine sulfate (Cardinal Health; Dublin, OH, USA) was obtained in 10-ml vials at a concentration of 10 mg/ml, and any dilutions were made using physiological saline. Naloxone hydrochloride (Fisher Scientific, Pittsburg, PA, USA) was dissolved in physiological saline and mixed fresh before test sessions. (±)-trans-U50,488 (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in saline and aliquots of each dose were stored at −20 °C and defrosted the day of testing. U50,488 has been shown to cross the blood–brain barrier (BBB) via indirect evidence from experiments that block its analgesic effects (Aldrich et al., 2009). GNTI (Tocris Bioscience, Minneapolis, MN, USA) was dissolved in saline, stored in aliquots at −20 °C and defrosted the day of testing. Since GNTI has not been used frequently to alter behavior, at least in rodents it is currently unclear whether GNTI crosses the BBB (Munro et al., 2012). However, it is important to note that peripheral administration of GNTI to macaques blocks the behavioral effects of peripheral administration of U50,488, which does cross the BBB (Negus et al., 2002). Naloxone, U50,488, and GNTI were all filtered through a 20-μm filter. Drugs and vehicle were administered by intramuscular (IM) injection in a volume of 0.1 ml/kg.

Blood sample collection

All animals were previously trained to enter a transport box (0.3 m × 0.3 m × 0.3 m). Subjects were hand captured from the transport box, the subject was manually restrained, and 1 ml of blood was collected from the femoral vein with a 1-cc syringe pretreated with heparin. The mean (±SEM) time from initial disturbance of the cage to completion of sample collection was

Table 1. Demographics of subjects in Experiment 1 and Experiment 2, and the treatments they received

Subject ID	Pair-mate ID	Subject age (mo)	Pair-mate age (mo)	Time paired (mo)	Offspring
Experiment 1[*]					
29775	37133	193	75	54	
35383	30557	100	172	54	
31716	29852	158	190	123	1M
32878	34866	136	110	27	1F
34531	36163	114	88	62	1M,1F
36988	37929	79	63	17	
36187	35292	88	107	30	1F
38,064	30486	63	174	25	1F
Experiment 2[^]					
29775	37133	195	77	56	
35383	30557	102	174	56	
31716	29852	160	192	125	1M, 1M
34531	36163	116	90	64	1M,1F
36988	37929	81	65	19	
36187[&]	35292	90	109	32	1F
38064	30486	65	176	27	1F
36150	34631	94	114	26	1M

[*] All animals received vehicle, 1.0 mg/kg naloxone, 0.25 mg/kg morphine, 0.01 mg/kg U50,488, 0.03 mg/kg U50,488 and 0.1 mg/kg U50,488.
[^] All animals received vehicle, 0.1 mg/kg GNTI, 0.5 mg/kg GNTI, and 1.0 mg/kg GNTI.
[&] Did not receive 0.1 mg/kg GNTI because of birth of an infant.

177.2 ± 5.0 s. Once collected, blood samples were stored on ice until all procedures for the day were completed; samples were then centrifuged for 15 min at 4 °C, the plasma fraction extracted and stored frozen (−70 °C) until assay. For Experiment 1, plasma was later assayed for cortisol and AVP, and plasma from Experiment 2 was assayed for cortisol. Plasma AVP concentrations were estimated in duplicate using commercial enzyme immunoassay kits (Enzo Life Sciences, Farmingdale, NY, USA) that were validated for titi monkeys (Bales et al., 2005). Intra- and inter-assay coefficients of variation (CV) for AVP were 6.46 and 6.80, respectively. CVs for all samples did not exceed 10%. Plasma cortisol concentrations were estimated in duplicate using commercial radioimmunoassay kits (Siemens Healthcare, Malvern, PA, USA). Prior to assay, samples were diluted 1:4 in PBS gel buffer. Assay procedures were modified with the addition of 0.5 and 2.5 μg/dl concentrations of standards along with the provided range of 1.0–49 μg/dl. Assay sensitivity has been determined to be 0.26069 μg/dl. This assay procedure has been validated and used with titi monkeys (Bales et al., 2007; Jarcho et al., 2011; Ragen et al., 2013). Intra- and inter-assay CVs for cortisol were 5.01 and 3.67, respectively. All data points had CVs lower than 10%.

Experiment 1

The first goal of Experiment 1 was to determine if opioid manipulation via morphine or naloxone would affect affiliative behavior in males upon reunion with their pair-mate after a brief separation. The second goal was to observe whether administration of the KOR agonist, U50,488, would sustain the separation distress response in males after they have been reunited with their pair-mate after a brief separation. To achieve this, the female pair-mate and any offspring were removed from the home cage for 30 min and remained out of visual and auditory range. Separation of the mate is likely to result in an increase in isolation peeps, locomotion, and HPA activity; separation from the offspring however, has previously been found to neither elicit distress behavior nor activate the HPA system (Mendoza and Mason, 1986b). We removed the pair-mate and offspring from the home cage and not the male because we only wanted to observe the effects of separation from a pair-mate and not the effects of a combined stressor of separation from a pair-mate and exposure to a novel environment. The 30-min session was filmed with the lone male being the focal animal, and an observer blinded to the drug condition later scored separation behaviors such as isolation peeps and locomotion. After the 30-min separation, the male was captured, blood was sampled, and an injection of a saline vehicle, morphine (0.25 mg/kg), naloxone (1.0 mg/kg), or U50,488 (0.01, 0.03, and 0.1 mg/kg) was administered IM. The male and his pair-mate were then returned to their home cage for 30 min. At the end of the 30-min session, the male was recaptured and underwent a second blood draw. The second 30-min session was filmed with the male being the focal animal. The timing of separation, drug administration, and reunion was based on Kalin et al. (1995) which found changes in physical contact between infant and mother macaques in response to opioid manipulation. Filmed sessions were later scored for both separation-related and affiliative behaviors (see Table 2 for ethogram). At the end of the entire 60-min session any offspring were returned to their home cage. Subjects received all five drug treatments in a pseudo-counterbalanced fashion. Animals were tested once per week. It is unlikely that the drugs used had long-lasting effects on behavior or HPA activity because the drugs used have a short duration of action, they were used acutely, and there was a seven day washout period between test sessions (Berkowitz, 1976; Pascoe et al., 2008; Wand et al., 2011).

The dose of morphine was based on the finding that it had no effect on motor behavior in a previous study of titi monkeys (Ragen et al., 2013). The dose of naloxone was chosen due to its ability to increase affiliative behavior in juvenile macaques (Schino and Troisi, 1992) as well as elicit an increase in cortisol concentrations in titi monkeys (Ragen et al., 2013). U50,488 was chosen as the KOR agonist due to its high specificity for KOR (Emmerson et al., 1994) as well as its ability to increase ultrasonic vocalizations in rat pups and even induce ultrasonic vocalizations when they are with their littermates, a situation where ultrasonic vocalizations do not occur (Carden et al., 1991, 1994). Additionally, U50,488 has consistently been shown to produce conditioned place aversions (McLaughlin et al., 2006; Land et al., 2008, 2009). The doses of U50,488 were chosen to avoid severe sedation, emesis and vomiting, which were based on observations in squirrel monkeys and rhesus macaques (Dykstra et al., 1987; Cox et al., 2007).

Experiment 2

The goal of Experiment 2 was to attenuate the separation distress response in male titi monkeys via administration of the KOR antagonist, GNTI. Behaviors of interest were locomotion and isolation peeps since both of these behaviors increase upon separation from a pair-mate (Mendoza and Mason, 1986a; Ragen et al., 2012). Male subjects were caught, underwent a blood draw, and administered GNTI (0.1, 0.5, or 1.0 mg/kg) or saline vehicle. Twenty-four hours after treatment administration, the male, female pair-mate, and offspring were caught and removed from the home cage. The male underwent a second blood draw and returned to his home cage for a 60-min separation while the female and offspring remained outside of visual and auditory range from the male. After separation, the male was caught and an additional blood sample collected. The 60-min separation was filmed and later scored for separation behaviors. After testing, the pair-mate and any offspring were returned to the home cage. A 60-min separation was chosen since it has been effective in producing a strong separation response (Mendoza and Mason, 1986a; Ragen et al., 2013).

Most of the subjects in Experiment 1 were used in Experiment 2 (See Table 1). To prevent any possible long-lasting effects of drug or behavioral manipulation we began Experiment 2 three weeks after the end of Experiment 1. This time between experiments likely

Table 2. Ethogram listing measured behaviors

Behavior*	Description
Social behaviors	
Contact (sec)	Passive physical contact between subjects that does not involve tail-twining
Female Approach	Female moves within < 6 inches of the male
Female Breaks Contact	Female moves body and breaks contact with male
Female Initiates Contact	Female moves body and initiates contact with male
Female Leave	Female moves away from the male to a distance > 6 inches
Grooming (sec)	Male combs through the fur of the female with his hands and/or mouth
Grooming Solicitations	Male presents body part in front of the female to be groomed
Male Approach	Male moves within < 6 inches of the female
Male Breaks Contact	Male moves body and breaks contact with female
Male Initiates Contact	Male moves body and initiates contact with female
Male Leave	Male moves away from the female to a distance > 6 inches
Proximity (sec)	Subject is within arm's reach (~6 inches) of female
Receive Grooming (sec)	The focal animal is being groomed by the attachment figure
Tail-Twine (sec)	Sitting side-by-side with tails wrapped around each other for at least one rotation
Total Passive Contact (sec)	Combined time spent in contact and tail-twining
Separation behaviors	
Isolation Peeps	Short, high pitched vocalizations which usually occur in rapid succession
Neutral behaviors	
Locomotion (sec)§	Male displaces his body by at least one body length
Scratch (sec)	Rapid and repeated raking of the fur or skin with own hand or foot

* Behaviors followed by (sec) are durations measured in seconds. All other behaviors are frequencies.
§ Also an indicator of separation distress and behavioral arousal.

eliminated issues related to reuse of certain subjects. Doses of GNTI were chosen based on their ability to block the KOR agonist, U50,488, in rhesus macaques (Negus et al., 2002). The separation test was conducted 24 h after drug administration since that is when GNTI reaches its peak activity in the rhesus macaques (Negus et al., 2002). Testing occurred every other week since it takes two weeks for GNTI to be ineffective at blocking U50,488 (Negus et al., 2002). Doses were administered in a pseudo-counterbalanced order.

Statistics

Data were analyzed using generalized linear mixed models (GLMM) (Littell et al., 1996) in SAS 9.2 (SAS Institute, Cary, NC, USA). The goal of Experiment 1 was to examine how MOR manipulation influences affiliative (i.e. contact, proximity, etc.) and locomotor behavior and how KOR activation affects separation behavior and locomotor behaviors (i.e. isolation peeps). Therefore for Experiment 1 we performed two different comparisons. The first comparison was the comparison between morphine, naloxone, and vehicle. The second comparison was between the three doses of U50,588 and vehicle. The model for behavior for both of the comparisons included Treatment with animal ID (identification) as a random variable to account for repeated measures. The model for testing opioid manipulation on hormone concentrations included Treatment, Time, and a Treatment × Time interaction with the animal's ID as a random variable. For Experiment 1, we also made comparisons between treatments on the change in hormone concentration from the first blood sample to second blood sample. This model only included Treatment with animal ID as a random variable. If data were not normally distributed, a square root or quad root transformation was performed to normalize the data. The behaviors Grooming and Receive Grooming were unable to be normalized, and the GLMM was still performed because an F test is still considered to be robust for non-normally distributed data (Feir-Walsh and Toothaker, 1974). Post-hoc comparisons were performed with Least Squares Means. Post-hoc tests only compared drug treatment to vehicle. This allows for clarity of interpretation of results as well as reducing the number of comparisons. To correct for multiple comparisons a Hochberg–Benjamini false discovery rate was used (Benjamini and Hochberg, 1995). Significant P-values were set at 0.05, and all post-hoc tests were two-tailed.

RESULTS

Experiment 1

Behavior. Values and statistics for all MOR-related behaviors can be viewed in Table 3. There was a significant effect of Treatment on Locomotion ($F(2,14) = 5.98$, $P = 0.01$). In spite of a significant P-value for the omnibus test, post-hoc tests revealed no significant effect of morphine compared to vehicle ($F(1,14) = 4.28$; $P = 0.114$) or naloxone compared to vehicle ($F(1,14) = 1.36$; $P = 0.195$). Since there was a significant effect of Treatment on Locomotion and previous research has found opioid manipulation affects locomotion (Ragen et al., 2013) we included Locomotion as a covariate when analyzing the effects of drugs on variables such as Male Approach, Male Leave, Male Initiates Contact and Male Breaks Contact because these variables could be influenced by alterations in locomotor behavior. Opioid manipulation affected Male Approach ($F(2,13) = 4.00$, $P = 0.04$), Male Initiates Contact ($F(2,13) = 4.98$, $P = 0.025$, Fig. 1A), and Female Breaks Contact ($F(2,14) = 17.38$, $P = 0.0002$, Fig. 1B). There was a trend for morphine to reduce the number of Male Approaches compared to vehicle ($F(1,13) = 5.76$; $P = 0.064$). Morphine resulted in significantly fewer Male Initiates Contact compared to vehicle ($F(1,13) = 6.40$; $P = 0.05$). When males were administered morphine there was a significant decrease in Female Breaks

Table 3. Values (Mean ± SEM) for behaviors measured when subjects were given vehicle, 1.0 mg/kg of naloxone, and 0.25 mg/kg of morphine. Post-hoc test only compare drug treatment to vehicle

Behavior	Condition			*F*-statistic	*P*-value
	Vehicle	1.0 mg/kg Naloxone	0.25 mg/kg Morphine		
Contact	621.4 ± 88.8	543.4 ± 152.7	541.9 ± 171.7	0.18	0.83
Female Approach	4.5 ± 1.8	3.6 ± 1.3	1.6 ± 0.6	1.93	0.18
Female Breaks Contact	6.9 ± 1.3	9.8 ± 1.6*	2.3 ± 0.7**	**17.38**	**<0.001**
Female Initiates Contact	4.0 ± 1.6	5.3 ± 1.3	1.9 ± 0.5	2.97	0.08
Female Leave	6.5 ± 1.5	8.4 ± 2.3	8.1 ± 5.6	0.96	0.41
Grooming	1.4 ± 1.4	1.4 ± 1.4	1.9 ± 1.9	0.03	0.97
Grooming Solicitations	0.1 ± 0.1	0 ± 0	0.4 ± 0.2	2.88	0.09
Locomotion	41.9 ± 11.8	84.9 ± 43.6	17.3 ± 7.7	**5.98**	**0.01**^
Male Approach	6.4 ± 1.7	11.3 ± 4.3	3.1 ± 1.2	**5.32**	**0.04**^
Male Breaks Contact	4.6 ± 2.5	6.5 ± 2.0	2.2 ± 1.2	2.86	0.09
Male Initiates Contact	8.0 ± 2.1	11.5 ± 3.1	3.4 ± 1.6*	**5.62**	**0.03**
Male Leave	3.8 ± 1.8	5.9 ± 3.1	1.5 ± 0.8	2.00	0.17
Proximity	131.4 ± 53.5	167.0 ± 40.4	100.4 ± 49.4	1.25	0.32
Receive Grooming	10.9 ± 10.3	11.6 ± 10.7	56.0 ± 53.2	0.16	0.86
Scratch	40.8 ± 14.8	17.8 ± 6.6	64.4 ± 35.4	2.00	0.17
Tail-Twine	458.1 ± 138.5	450.4 ± 136.3	635.0 ± 230.0	0.47	0.63
Total Passive Contact	1079 ± 190.6	993.8 ± 171.5	1176.88 ± 220.1	0.55	0.59

Bold significant omnibus comparison.
* ⩽0.05 compared to vehicle.
** <0.01 compared to vehicle.
^ No significant difference between drug and vehicle.

Contact compared to vehicle ($F(1,14) = 12.96$; $P = 0.006$). Furthermore, when males were administered naloxone there was a significant increase in Female Breaks Contact compared to vehicle ($F(1,14) = 5.02$; $P < 0.042$). Since Male Initiates Contact followed a similar pattern to that of Female Breaks Contact we wanted to explore whether the effects of drug administration on the males were correlated to those found in females. Male Initiates Contact was positively correlated with Female Breaks Contact ($r = 0.73$; $P < 0.0001$) (Pearson's product-moment correlation) (Fig. 2). This correlation included data points from the vehicle, morphine, and naloxone conditions in all eight subjects. MOR manipulation had no effect on Contact, Female Approach, Female Initiates Contact, Female Leave, Grooming, Grooming Solicitations, Male Breaks Contact, Male Leave, Proximity, Receive Grooming, Tail-Twine or Total Passive Contact ($P > 0.05$).

For KOR behavioral analyses, there were no significant effects of Treatment on separation-related behaviors ($P > 0.05$) (Table 4). Since KOR treatment did not affect separation behavior, we wanted to confirm that the subjects were actually exhibiting a separation response at all. Therefore we performed an analysis on locomotion and isolation peeps collapsed over all treatments comparing when subjects were separated from their pair-mate (first 30 min) and when

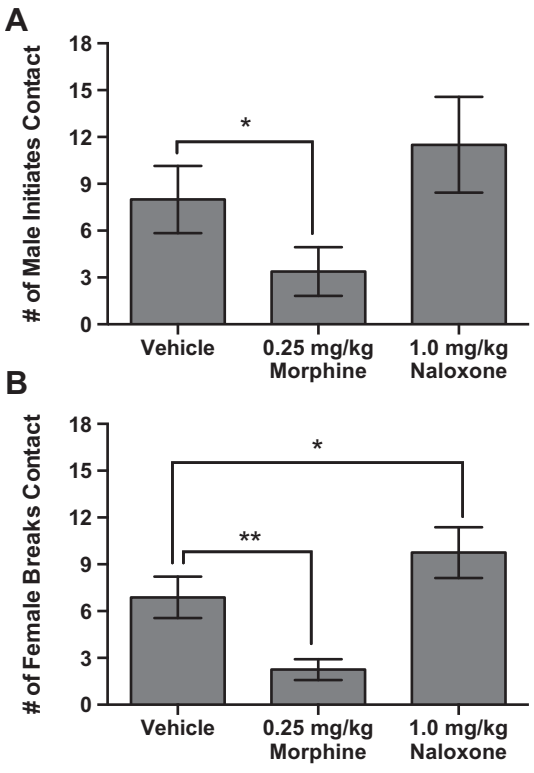

Fig. 1. Mean (±SEM) (A) number of Male Initiates Contact and (B) number of Female Breaks Contact comparing morphine and naloxone to vehicle. $^{*}P \leqslant 0.05$, $^{**}P < 0.01$,

Fig. 2. Correlation between Male Initiates Contact and Female Breaks Contact.

Table 4. Values (Mean ± SEM) for behaviors measured when subjects were given vehicle, 0.01 mg/kg U50,488, 0.03 mg/kg U50,488, and 0.1 mg/kg U50,488 mg/kg

Condition						
Behavior	Vehicle	0.01 mg/kg U50,488	0.03 mg/kg U50,488	0.1 mg/kg U50,488	*F*-statistic	*P*-value
Locomotion	41.9 ± 11.8	50.1 ± 14.9	33.9 ± 11.3	70.6 ± 25.0	1.96	0.15
Isolation Peeps	0.13 ± 0.13	0 ± 0	1.13 ± 1.13	0 ± 0	1.00	0.41

they were reunited with their pair-mate (second 30 min). We used a GLMM including Time and ID to analyze the data. As predicted, when males were separated from their pair-mate there was a significant increase in Locomotion (With: 49.13 ± 8.29 s, Without: 136.94 ± 8.29 s) ($F(1,55) = 14.26$; $P = 0.0004$) and Isolation Peeps (With: 0.32 ± 0.28, Without: 292.13 ± 50.84) ($F(1,55) = 47.41$; $P < 0.0001$). This indicates that the animals showed the predicted distress response upon separation, but that the chosen doses of U50,488 had no effect.

Hormones. A Pre morphine sample underwent a freeze–thaw cycle therefore it was removed from AVP analysis due to AVP breaking down during a freeze thaw cycle. Additionally a Pre Vehicle sample was unable to be collected from one subject so cortisol and AVP data could not be obtained. In the MOR manipulation model on AVP, there was a significant effect of Time ($F(1,31) = 5.51$; $P = 0.03$) but no effect of Treatment (Vehicle: 226.74 ± 16.9 pg/mL; Morphine: 205.62 ± 13.46 pg/mL; Naloxone: 257 ± 22.3 pg/mL) ($F(2,31) = 2.85$; $P = 0.07$) or Treatment × Time interaction ($F(2,31) = 2.23$; $P = 0.12$). Plasma AVP was significantly greater in the Post sample (249.45 ± 17.17 pg/mL) compared to the Pre sample (209.59 ± 11.12 pg/mL) ($F(1,31) = 5.51$; $P = 0.03$). In the MOR manipulation model on cortisol, there was a significant effect of Time ($F(1,32) = 34.72$; $P < 0.0001$ µg/dL), and Treatment ($F(2,32) = 20.43$; $P < 0.0001$), but not a Time × Treatment interaction ($F(2,32) = 2.61$; $P = 0.09$). Levels of plasma cortisol were significantly greater in the Post sample (49.46 ± 2.32 µg/dL) compared to the Pre sample (38.32 ± 2.29 µg/dL) ($F(1,32) = 34.72$; $P < 0.0001$). Naloxone treatment resulted in significantly higher plasma cortisol levels (50.31 ± 3.67 µg/dL) compared to vehicle (43.94 ± 2.15 µg/dL) ($F(1,32) = 7.13$; $P = 0.01$), and morphine (37.65 ± 3.67 µg/dL) treatment resulted in significantly lower plasma cortisol levels compared to vehicle ($F(1,32) = 13.72$; $P = 0.0008$).

We also analyzed the effects of Treatment on the change in hormone concentrations from the Pre to Post sample. There was a significant effect of Treatment on the change in cortisol levels ($F(2,12) = 7.66$; $P = 0.007$) (Fig. 3A). The change in cortisol in response to naloxone was significantly greater compared to vehicle ($F(1,12)$; $P = 0.016$). There was a statistical trend for an effect of Treatment on the change of AVP levels ($F(2,11) = 3.66$; $P = 0.06$) from Pre to Post (Fig. 3B).

For KOR manipulation on AVP, there was no significant effect of Time ($F(1,14) = 0.53$; $P = 0.47$) (Pre: 230.71 ± 14.06 pg/mL; Post: 231.86 ± 14.34 pg/mL), Treatment ($F(3,14) = 0.08$; $P = 0.97$) (Vehicle: 226.74 ± 16.9 pg/mL; Low: 225.19 ± 13.00 pg/mL; Medium: 230.19 ± 20.4 pg/mL; High: 234.52 ± 19.70 pg/mL), or Time × Treatment interaction ($F(3,14) = 0.20$; $P = 0.89$). For KOR manipulation on cortisol, there was a significant effect of Time ($F(1,14) = 12.25$; $P = 0.001$), but no effect of Treatment (Vehicle: 43.94 ± 2.15 µg/dL; Low: 41.64 ± 3.4 µg/dL; Medium: 42.61 ± 2.26 µg/dL; High: 45.76 ± 2.69 µg/dL) ($F(3,14) = 0.85$; $P = 0.47$) or Time × Treatment interaction ($F(3,14) = 0.57$, $P = 0.64$). Plasma cortisol levels were significantly greater in the Post (47.63 ± 1.63 µg/dL) sample compared to the Pre (39.21 ± 1.84 µg/dL) sample ($F(1,14) = 0.$ 12.25; $P = 0.001$). There was a significant effect of KOR manipulation on the change in cortisol from the Pre to Post sample ($F(3,19) = 4.19$; $P = 0.02$) (Fig. 3C). Despite this significant omnibus test there were no significant effects when comparing the low ($F(1,19) = 0.07$; $P = 0.798$), medium ($F(1,19) = 2.27$; $P = 0.149$), or high ($F(1,19) = 3.60$; $P = 0.073$) dose to vehicle. There was no significant effect of KOR manipulation on the change in AVP from the Pre to Post sample ($F(3,19) = 0.29$; $P = 0.83$) (Fig. 3D).

Experiment 2

Behavior. KOR blockade had a significant effect on Locomotion ($F(3,20) = 3.25$; $P = 0.04$) (Fig. 4B). Administration of the 0.1 mg/kg dose of GNTI resulted in significantly less Locomotion compared to vehicle ($F(1,20) = 8.01$; $P = 0.03$). KOR blockade with GNTI had no effect on Isolation Peeps ($F(3,20) = 2.06$; $P = 0.14$) (Fig. 4A).

Hormones. For KOR blockade on cortisol, there was a significant effect of Time ($F(1,71) = 71.98$; $P < 0.0001$; Fig. 4C), but no effect of Treatment (Vehicle: 29.20 ± 2.52 µg/dL; Low: 28.67 ± 2.20 µg/dL; Medium: 31.35 ± 2.5 µg/dL; High: 32.40 ± 2.58 µg/dL) ($F(3,71) = 1.48$; $P = 0.23$) or Treatment × Time interaction ($F(6,71) = 0.29$; $P = 0.94$). Plasma levels of cortisol were significantly greater in the Post sample compared to baseline ($F(1,71) = 118.37$; $P < 0.0001$) and the Pre sample ($F(1,71) = 93.90$; $P < 0.0001$). There was no difference between the baseline and the Pre sample ($F(3,71) = 1.68$; $P = 0.20$).

DISCUSSION

The present study had two aims. The first was to determine whether manipulation of the opioid system via morphine and naloxone would affect physical affiliative

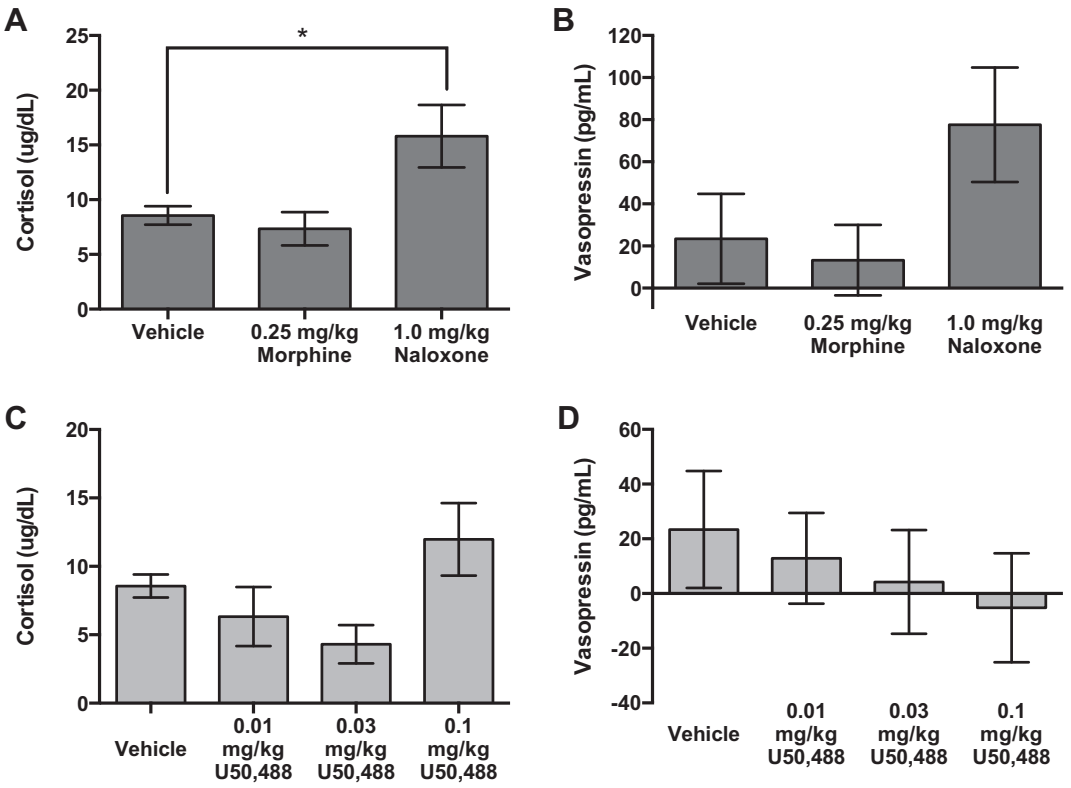

Fig. 3. Mean (±SEM) change in (A) cortisol and (B) vasopressin from pre to post samples comparing naloxone and morphine to vehicle. Mean (±SEM) change in (C) cortisol and (D) vasopressin from pre to post samples comparing all three doses of U50,488 to vehicle. $^{*}P < 0.05$, $^{**}P < 0.01$.

behavior in paired male titi monkeys; an effect observed in primate infant–mother relationships (Kalin et al., 1995) as well as adult, nonmonogamous primate relationships (Keverne et al., 1989; Martel et al., 1995). Unlike previous studies administering morphine and naloxone to prairie voles (Shapiro et al., 1989) and nonmonogamous monkeys (Keverne et al., 1989; Schino and Troisi, 1992; Kalin et al., 1995) we did not find a change in overall physical contact, which includes grooming and total passive contact. These findings do replicate our previous study in titi monkeys (Ragen et al., 2013). Morphine did, however, result in males initiating contact with their pair-mate less frequently. This effect is not due to morphine's effect on locomotion since it was included as a covariate in the model. It is unlikely that alterations in initiating physical contact in titi monkeys are related to changes in sexual motivation. Even though naltrexone is able to decrease mating bouts in prairie voles during pair-bond formation (Burkett et al., 2011), sexual behavior in established titi monkey pairs is infrequent (Ragen et al., 2012) and rarely observed in the present study (data not presented). The decrease in initiating contacting is also likely not an artifact of nausea since there were no indicators of emesis such as salivation, vomiting, or food aversion. It is important to note that high doses of morphine can bind to KOR, which is observed in macaque brain membranes (Emmerson et al., 1994). It is unknown whether KOR agonists can alter affiliative behavior, but there is evidence that KOR blockade in prairie voles has no effect on affiliative behavior (Resendez et al., 2012).

It is interesting that males administered morphine decreased the number of times they initiated contact with females but that there was no overall change in physical contact. This finding could be explained by the female pair-mate's behavior, since she would break physical contact less frequently when the male was administered morphine. Furthermore, there was a positive correlation between the number of times the male initiated contact with the female and the number of times she broke contact with him suggesting some coordination of behavior. It has been hypothesized that individuals maintain an optimal level of opioidergic tone via physical contact and disruption of the opioid system (ex. morphine administration) will alter physical contact (Nelson and Panksepp, 1998). The results of the present study support this hypothesis since the over activation of MOR by morphine may have been the reason for a reduction of physical contact being sought out by males and why the females broke contact less frequently. It is important to note that naloxone is relatively non-specific and that the use of more specific MOR antagonist could have different effects. It is possible that selective MOR blockade would be able to increase motivation to engage in physical contact, but the blockade of other opioid receptors prevents this effect. However, the choice of naloxone in the present study was due to its precedent of activating HPA activity and increasing social behavior in other species.

In addition to altering behavior, we found that the separation/reunion paradigm and opioid manipulation affected plasma AVP and cortisol. Levels of AVP and

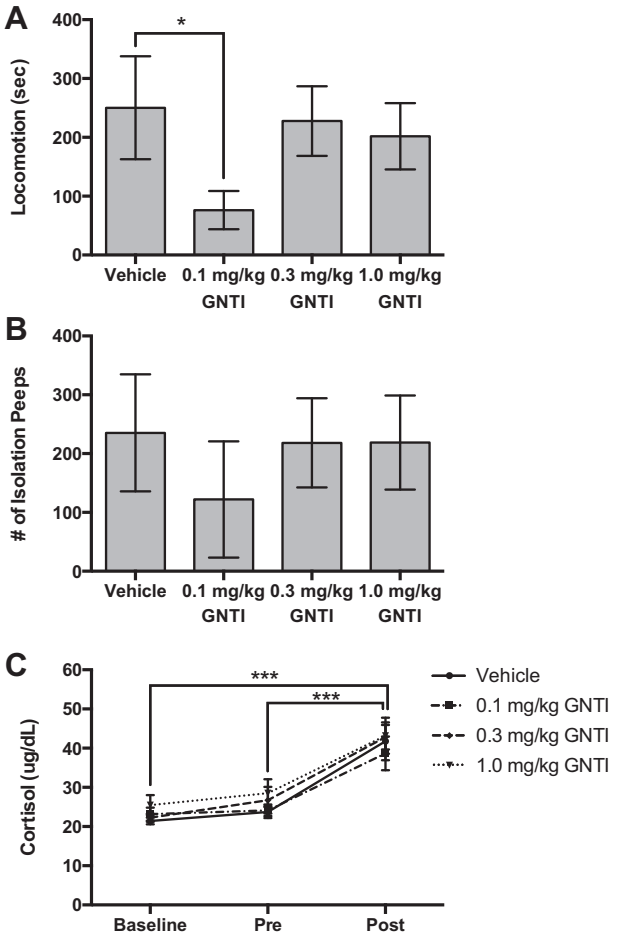

Fig. 4. The effects of GNTI on (A) Locomotion, (B) Isolation Peeps, and (C) cortisol when a male is separated from his pair-mate. $^{*}P < 0.05$, $^{***}P < 0.0001$.

cortisol increased from the pre to post sample. These increases are likely due to the stressor of social separation, being captured, undergoing a blood draw, and receiving an injection. Similar effects have previously been found in titi monkeys (Ragen et al., 2013). Additionally, naloxone administration resulted in a greater increase in cortisol compared to vehicle and morphine. This is an effect previously shown in titi monkeys (Ragen et al., 2013), and the HPA activating effects of naloxone are well established (Parrott and Thornton, 1989; Wand et al., 2012). The increase in AVP in response to opioid manipulation only approached significance ($P = 0.06$). A previous study done in titi monkeys found that naloxone increased plasma AVP (Ragen et al., 2013). It is possible that the effect of naloxone on plasma AVP is less robust, and the presence of the pair-mate during reunion buffered the effects of naloxone on AVP release.

Our second goal for Experiment 1 was to activate the KOR and induce a separation distress response in males after they had been reunited with their female pair-mate following a brief separation. We also wanted to examine whether KOR manipulation resulted in changes in plasma AVP and cortisol. The KOR agonist, U50,488, did not sustain separation behaviors (i.e. isolation peeps) when males were reunited with their pair-mate after separation which is contrary to our prediction. This is in contrast to its effects in rat pups (Carden et al., 1996), in which KOR activation produces isolation calls in the presence of litter mates. There could be multiple explanations for why U50,488 had no effect in the current study. First, the effects of KOR agonists on separation distress behaviors could differ depending on species as well as the type of attachment. Another possibility is that administration of the drug during the reunion (and thus the female's presence) was able to fully buffer any aversive effect of a KOR agonist. If we had administered U50,488 when a male was separated from his pair-mate, there may have been a potentiation of separation distress behaviors as seen by Carden et al. (1994). Another, but not mutually exclusive explanation, is that the dose of U50,488 was not high enough to induce separation distress behaviors. The doses of U50,488 used in the present study were relatively low and were chosen to prevent emesis and extreme sedation, which could have confounded the separation distress response (Cox et al., 2007). It is also possible that if we had removed the male from the home cage in addition to the separation the stress level would have been heightened enough for U50,488 to have a greater effect and therefore sustain a separation distress response when reunited with his pair-mate in the home cage.

The effects of KOR activation increasing cortisol were not robust. The increase in cortisol in response to the high dose of U50,488 only approached significance compared to vehicle ($P = 0.07$). This is not similar to the findings in previous studies (Calogero et al., 1996; Pascoe et al., 2008; Ranganathan et al., 2012). It is likely that the cause of the null effect is due to low doses of U50,488 that were chosen.

The reason for Experiment 2 was to examine further if the KOR is involved in the separation distress response and we predicted that KOR blockade with GNTI would attenuate the separation distress response in male titi monkeys. KOR blockade has been found to attenuate aggressive behavior in paired prairie vole males when exposed to a stranger male, an indicator of an established pair-bond (Resendez et al., 2012). We found that GNTI was able to attenuate the locomotor component of the separation distress response, but it did not have a significant effect on isolation vocalizations. This effect is likely not due to GNTI impacting gross locomotion since only very high doses GNTI can have a sedative effect (Negus et al., 2002), and the present study found that only the lowest dose of GNTI had a significant effect. Higher doses of GNTI may not be affecting locomotion in titi monkeys because they may be blocking MOR (Metzger et al., 2001). Despite GNTI's great specificity for KOR, mutations in the MOR can result in GNTI having a greater affinity toward the MOR (Metzger et al., 2001). Therefore, at higher doses GNTI may be blocking MORs, which results in an increase in locomotion in titi monkeys separated from their pair-mate (Ragen et al., 2013). Even though GNTI was able to attenuate the behavioral component

of the separation distress response, it did not alter the effect of social separation on HPA activation. This finding is similar to previous research by McLaughlin and colleagues (2006) who found that KOR blockade was able to reduce the negative affective component of a stressor but not the endocrine response. The lack of effects could also be due to the repeated use of GNTI, since to our knowledge repeated use of GNTI and its effects on HPA activity has not been studied. The data from Experiment 2 suggest that KOR plays a role in the behavioral but not the physiological separation distress response potentially by reducing the negative affective component of separation. GNTI could have had a greater effect on brain areas responsible for the affective components of KOR activation, such as the nucleus accumbens, dorsal raphe, ventral tegmental area (Land et al., 2009; Chefer et al., 2013), compared to areas that more directly activate the HPA axis (i.e. hypothalamus).

This study provides additional support to the idea that the opioid system regulates different components of the pair-bond in titi monkeys. More specifically, the opioid system could be playing a role in maintaining the pair-bond. The MOR system may be acting as a positive reinforcer for pair-maintenance whereby MORs are activated upon reunion after a separation and produce a positive affective state. In support of the hypothesis that individuals aim to maintain a homeostatic level of MOR activity, over activation of MOR upon reunion with morphine would result in a decrease in motivation to initiate contact. In contrast to MOR, KOR may act as a negative reinforcer to promote proximity maintenance. Upon involuntary separation there may be KOR activation and an induction of a negative affective state which would result in distress behaviors. Therefore, if you were to block KOR with an antagonist, there would be an attenuation of separation behavior. The idea that KOR act as a negative reinforcer to promote pair-bond maintenance is supported by studies done in prairie voles (Resendez et al., 2012; Resendez and Aragona, 2013). Future research will map MOR and KOR binding in the titi monkey brain. Mapping the location of MOR and KOR binding in the titi monkey brain would provide insight into whether the role of MOR and KOR are due to differences in their neuroanatomical locations or just differences in how these receptors function. It would also provide information on where opiate drugs are acting to produce behavioral and physiological changes.

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