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Peripherally Administered Non-peptide Oxytocin Antagonist, L368,899®, Accumulates in Limbic Brain Areas: A New Pharmacological Tool for the Study of Social Motivation in Non-Human Primates

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Abstract

Central administration of oxytocin (OT) antagonists inhibits maternal and sexual behavior in non-primates, providing the strongest experimental evidence that endogenous OT facilitates these behaviors. While there have been a few reports that ICV administration of OT increases social behaviors in monkeys, no studies to date have assessed the effects of OT antagonists. Therefore, we studied in rhesus monkeys whether L368,899®, a non-peptide antagonist produced by Merck that selectively blocks the human uterine OT receptor, penetrates the CNS after peripheral administration and alters female maternal and sexual behavior. In two studies in four male monkeys, L368,899 was injected iv (1 mg/kg) after which (1) CSF samples were collected at intervals over 4 h and (2) brains were collected at 60 min. Assay of samples confirmed that iv-administered L368,899 entered CSF and accumulated in the hypothalamus, septum, orbitofrontal cortex, amygdala and hippocampus, but not other areas. An adult female monkey was tested for interest in either an infant or sexual behavior, receiving a different iv treatment prior to each test (1 or 3 mg/kg of L368,899 or saline) OT antagonist treatment reduced or eliminated interest in the infant and sexual behavior. These results, although preliminary, are the first to directly implicate endogenous OT in activation of primate maternal interest and sexual behavior. While it remains to be empirically demonstrated that peripherally administered L368,899 blocks central OT receptors, our behavioral findings suggest that this non-peptide antagonist may facilitate testing OT involvement in a variety of social and other behaviors in primates.

Keywords

oxytocin; maternal behavior; sexual behavior; rhesus monkey; macaques

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Introduction

Oxytocin (OT) is a nonapeptide synthesized principally in the supraoptic and paraventricular nuclei. Most OT neurons project to the posterior pituitary but some also project within the central nervous system. Central OT has been implicated in activation of social attachments, such as maternal behavior in rats and sheep (Pedersen, 1997; Kendrick, 2000) and pair-bonding in monogamous prairie voles (Insel et al 1997; Young and Wang, 2004). Oxytocin also stimulates sexual behavior in rats (Argiolis, 1999; Argiolis and Melis, 2004; Witt, 1995). Among the most convincing evidence that endogenous central OT plays a physiologically significant role in rodent social motivation has been repeated demonstrations that intracerebroventricular (ICV) administration of OT receptor antagonists blocks or diminishes maternal and sexual behavior (Caldwell et al, 1994).

Social motivations are critical factors in human development and strongly affect mental and physical health throughout life (Bifulco and Moran, 1998; Brown et al, 2005; Carter et al, 2005, Dube et al 2003; Kaler and Freeman, 1994). A few studies hint that OT may also play a role in these areas in primates. ICV injection of OT increased aggression and sexual behavior in dominant male squirrel monkeys but increased social grooming and marking behaviors in males of lower rank (Winslow and Insel, 1991). Nulliparous rhesus monkeys looked at, touched, maintained proximity to and directed lipsmacks toward unfamiliar infants more after ICV infusion of OT than saline (Holman and Goy, 1995). In humans, intranasal administration of OT, which may increase OT concentrations in the CNS (Born et al., 2002 found that vasopressin, a structurally similar peptide, penetrated CNS), enhanced the suppressing effects of social support on cortisol and anxiety responses to psychological stress (Heinrichs et al., 2003), increased trust in bargaining games (Kosfeld et al., 2005) and decreased amygdala activation in response to viewing fearful and threatening facial expressions (Kirsch et al., 2005). In addition, Fries et al (2005) found that children with experience of early social deprivation have lower levels of urinary OT and fail to respond to contact with a caregiver with a rise in OT as do children without such deprivation.

Despite these promising findings, no studies to date have tested the effects of blocking central OT receptors in primates. Therefore, the significance of endogenous OT in primate social behavior remains uncertain. Unfortunately peptide OT antagonists used successfully in rodent studies have two major disadvantages in primates: they are much less selective for OT than for vasopressin V1a receptors (Toloczko et al., 1997) and they must be administered intracerebrally because their penetration through the blood brain barrier is quite limited.

Pharmaceutical companies have developed selective antagonists for the human uterine OT receptor as potential treatments for premature labor. If such an antagonist crossed the blood-brain barrier, studies of the central action of an OT antagonist following peripheral administration would be possible. One of these antagonists is L368,899, a high affinity, non-peptide molecule synthesized by Merck Research Laboratories, West Point PA (Pettibone and Freidinger, 1997). We report here a series of studies with Rhesus macaques (*Macaca mulatta*) demonstrating that L368,899 (a) crosses the blood-brain barrier, (b) accumulates in brain areas implicated in social behavior and (c) may diminish social motivation, as reflected in interest in infants and sexual behavior in females.

Study I - Peripherally-Administered L368,899 Accumulates in the Cerebrospinal Fluid

The first study was conducted to establish whether L368,899 crosses the blood/brain barrier and enters the cerebrospinal fluid (CSF) after intravenous (iv) administration and to determine the time course of central penetration of this compound. Subjects included four male rhesus monkeys, ranging in age from 14 to 20 years. Females could not be used in this or the second, terminal, study described below because all females were needed to maintain the breeding

colony. Each monkey was initially anesthetized with 5 mg/kg ketamine and maintained with 1.5% isoflurane throughout the experiment. Both administration of anesthesia and collection of CSF samples were performed by a veterinarian in the large animal operating suite in the Department of Laboratory Animal Medicine at the University of North Carolina at Chapel Hill. One mg/kg L368,899 was administered iv and CSF samples collected via the cisterna magna. Approximately 0.5 ml of CSF was collected at each sample time. Two CSF samples were collected from each monkey, with at least 100 min between each sample. Samples were collected at 0 and 130 min for the first monkey, at 80 and 180 min for the second monkey, at 45 and 160 min for the third monkey, and at 110 and 255 min for the last monkey, resulting in CSF sampling at 0, 45, 80, 110, 130, 160, 180 and 255 min after iv injection. The first sample (0 min), obtained just before iv injection, was to confirm the absence of detectable compound in the assay. In addition, 3 ml of blood was collected from each animal at 15 min after drug administration and at the time of each CSF sample collection. Samples were frozen at -70°C and shipped overnight on dry ice to be assayed at the Merck Research Laboratories, West Point, PA. Sample concentrations were determined by liquid chromatography/mass spectrometry (LC/MS) using a Sciex API 2000 Mass Spectrometer with a Heated Nebulizer. CSF samples required no pretreatment before analysis and were injected directly into the mass spectrometer. For each animal, concentration of L368,899 in each CSF sample was expressed as a percentage of the plasma concentration at the 15 min time point to standardize values among animals.

Results

Plasma concentrations of L368,899 and its metabolites from the 15 min samples varied from 165 to 346 ng/ml across the four animals, declined steadily over time, and was undetectable in the final sample taken 255 min after L368,899 iv injection, and at the initial 0 min baseline time-point. Figure 1 shows that L368,899 was already present in the CSF 40 min after L368,899 iv injection, reached peak concentration in the 110 min sample and was still present (at 1.5 % of the 15-min plasma level) in the 255 min CSF sample when its presence in the plasma was no longer detectable.

Study II - Peripherally-Administered L368,899 Accumulates in the Hypothalamus, Limbic Structures and Orbitofrontal Cortex

The second study was conducted to establish whether L368,899 accumulates in brain areas of interest. One month after the first experiment, the same four monkeys were again immobilized with 5 mg/kg ketamine and maintained under deep anesthesia with 1.5% isoflurane. Approximately 60 min after iv infusion of 1mg/kg of L368,899 each animal was euthanized by iv administration of 90 mg/kg of pentobarbital immediately after a CSF sample was collected. Brains were harvested within 45 min after death. Several brain areas, including amygdala, parietal cortex, visual cortex, orbitofrontal cortex, caudate, cerebellum, hippocampus, hypothalamus, septum, and brainstem were dissected. Samples were collected at room temperature, and immediately frozen at -70°C until sent for assay to Merck Research Laboratories, West Point, PA. Brain samples were homogenized with a 5-fold volume (w/v) of HPLC mobile phase then prepared using protein precipitation before analysis. Sample concentrations of L368,899 were determined by LC/MS using a Sciex API 2000 Mass Spectrometer with a Heated Nebulizer.

Results

Figure 2 shows that L368,899 accumulated in the hypothalamus, orbitofrontal cortex, amygdala, hippocampus, and septum at concentrations well above the lowest level detectable by this assay (12 ng/mg of brain tissue). L368,899 was not detectable in other areas of the brain, including visual cortex, parietal cortex, caudate, cerebellum and brainstem. Most of the

brain regions in which L368,899 accumulated are known to express OT receptors in non-primate mammals (Gimpl and Fahrenholz, 2001). These results suggest that OT receptors may also be located in these regions of the primate brain, regions which have been implicated in the regulation of social behavior in non-primate mammals.

Study III - L368,899 Decreased Interest in an Infant

This study tested the hypothesis that iv administration of L368,899 would reduce interest in an infant in an adult female rhesus monkey. The subject was 4 years old, nulliparous and weighted 4.5 kg. At this age, nulliparous female rhesus monkeys usually exhibit high levels of interest in infants (Bard, 1995; Caine and Mitchell, 1980; Guerra, 1989; Lancaster, 1971; Quiatt, 1979). The female's menstrual cycle was tracked for 2 months prior to testing by observing perineal coloration and the presence of menstrual blood. Testing was conducted during the luteal phase of the cycle to avoid periovulatory sexual motivations from interfering in the test. A 9-month-old infant monkey served as the stimulus during each behavior trial.

Testing occurred in a large arena 3.2 × 1.8 × 2.1 m made of wire mesh except two ends which were Plexiglas®, (see Goursaud and Bachevalier (2007) for more detailed description). Both monkeys were habituated to the handling procedures and the test arena prior to testing, and a caregiver (second author) familiar to both the adult female and the infant was present to provide comfort and reassurance to the infant during the test.

Two experimental series of three tests were run one month apart. In the first series, the female monkey was administered saline (5 ml) on Day 1, 1 mg/kg L368,899 (i.e. 4.5 mg) on Day 3 and 3 mg/kg L368,899 (i.e. 13.5 mg) on Day 5. The drug was dissolved in buffered normal saline at a concentration of 1 mg/ml or 3 mg/ml so that equal volumes of vehicle were injected on all trials. In the second experimental series, the female was administered saline (5 ml) on days 1, 3, and 5. This series was conducted to characterize female behavioral responses towards the infant during repeated testing under no-drug conditions.

On each day of testing, the adult female was placed in chair-restraint and injected with antagonist or saline vehicle in the antecubital vein and returned to her home cage. Testing was conducted 60 min after injection. Immediately prior to testing, the infant was transported in a small, wire mesh holding box, from the nursery adjacent to the testing arena. The holding box was then secured to a wire mesh wall on the outside of the test arena. This arrangement permitted the adult female to see, touch (through the wire), sit near, and otherwise interact with the infant, while preventing full contact with and potential injury to the infant. The female monkey was then transported and released into the arena from the side opposite to the location of the stimulus infant. Female behavior was observed for 30 min and videotaped with a Sony digital camera. Images were captured to mpeg files on an IBM T30 laptop computer for subsequent analyses of behavioral responses.

Video files were transferred to CDs and assigned random identifying labels to ensure that the observers were blind to the treatment conditions during scoring of the videotapes. Behaviors observed included infant-directed activity (maintenance of proximity, looking toward infant, affiliative and aggressive behaviors) as well as other behaviors such as locomotion and stereotypies, vocalizations and exploration of the environment (see Table 1 for definitions). Videotapes were coded using The Observer 5.0 (Noldus Information Technology, b.v., Wageningen, NL) producing data on the frequency and duration of each behavior. Inter-observer reliability between the first two authors was established at greater than 85% for each behavior.

Results

Interest in the infant was expressed with contact-soliciting behavior (lipsmack and touch, figures 3A and 3B, respectively). In the first experimental series, compared to the first saline trial, the levels of attempts to touch the infant decreased at the 1 mg/kg dose and were further reduced at the 3 mg/kg dose, reflected in the frequency and duration of this behavior. A similar effect was found for proximity maintaining behavior (not shown). In the second experimental series, which included only saline trials, these behaviors increased after the first day of testing. In the first series, frequency of lipsmack decreased only with 3mg/kg dose of L368,899 (see figure 3A). In the second, saline-only series, lipsmack increased after the first trial. Locomotion decreased progressively from saline to 1 mg/kg and from 1 mg/kg to 3 mg/kg, whereas in the second experimental series, locomotion increased after the first day of testing (see figure 3C). A similar effect was found for pacing (data not shown). Other behaviors, including vocalizations, object exploration and stereotypies, did not show treatment effects in either series.

Study IV - L368,899 Decreased Interest in Sexual Behavior

This study tested the hypothesis that iv administration of L368,899 would reduce sexual behavior in female monkeys. The experimental subject was an adult parous, 12 yr old female who weighed 5.7 kg. She was paired with an adult male who weighed 13.8 kg and was sexually experienced, and served as a stimulus for female sexual behavior. These two monkeys had previously been in visual and auditory, but not physical, contact for several months.

The experiment took place in the same arena described above, and was videotaped for subsequent analyses of behavioral responses as described in Study III. The monkeys were individually habituated to the experimental room and testing cages prior to the study. To optimize the female's sexual interest, her menstrual cycle was monitored (perineal coloration and presence of menstrual blood) for two months prior to testing, and sex tests were conducted during her peri-ovulatory week. Treatments were administered iv 60 min before each behavior test. Saline was given on Day 1, 1mg/kg L368,899 on Day 3, 3 mg/kg L368,899 on Day 5, and saline again on Day 8.

During each behavior test, the male was released into the arena just prior to introduction of the female. Test sessions lasted 60 min and were videotaped as in Study III. Again video files were assigned random identifying labels to ensure that the observers were blind to the treatment conditions during scoring of the videotapes. Behaviors observed for both individuals included location (proximity), sexual, agonist and affiliative behaviors, locomotion and stereotypies as well as environment-directed exploratory behavior (see Table 2 for definitions). Videotapes were coded using The Observer 5.0 (Noldus Information Technology, b.v., Wageningen. NL), producing data on the duration, frequency and latency of each behavior. Reliability was established between the first two authors at greater than 90% for each behavior.

Results

Results of this study are presented in figure 4. Proceptive behavior (hip-grab) was completely suppressed after the 3 mg/kg dose (figure 4A).

Receptive behavior was assessed by examining the response of the female to the male's mounting attempts. Although mounts occurred under each experimental condition, the average duration of refuse mounts by the female and of incomplete mounts by the male were longer after the 3 mg/kg dose of L368,899 than after the 1 mg/kg dose or saline (figure 4B). When the female was treated with 3 mg/kg L368,899, the male's latency to first ejaculation (figure 4C) was approximately 6 times longer than when the female was given 1 mg/kg and 3 times

longer than when the female was injected with saline during the last test (there was no ejaculation in the first test following the initial saline injection). Other behaviors, including grooming, proximity maintaining behaviors, object exploration, locomotion and stereotypies did not show treatment effects.

Discussion

In these studies, we demonstrated that the non-peptide OT antagonist, L368,899®, crosses the blood brain barrier after iv administration, indicated by accumulation in CSF and several brain areas, including hypothalamus, limbic brain areas and orbitofrontal cortex, but not in other brain areas. Most of the brain regions in which L368,899 accumulated are known to express OT receptors in mammals (Gimpl and Fahrenholz, 2001) and specifically non-human primates and humans (Boccia et al 2001; 2003). These results suggest that, in the primate brain, OT receptors may also be located in brain regions which have been implicated in the regulation of social behavior (Insel et al 1997; Witt, 1995). We further demonstrated that iv administration of this compound reduced one female monkey's interest in an infant and decreased another female monkey's sexual behavior. This is the first evidence that blocking central OT receptors in primates disrupts parental-like behavior and female sexual behavior. Before discussing the functional implications of these results, it is important to raise several potential factors that could account for the findings presented here.

The first factor relates to the small sample size in both behavioral studies. Although we had intended to test maternal interest and sexual behavior in both female monkeys, the parous female exhibited no interest in the infant, and the nulliparous female was attacked by the male during their first encounter. Thus, only one female monkey was used in each behavioral test situation. The behavioral data, therefore are promising but preliminary.

The second factor relates to the significant decline in locomotion produced by L368,899 which raises questions about the specificity of the diminished interest in the infant and in sexual behavior observed. This decline may reflect either a primary role of OT in facilitating locomotion as suggested by some rat studies or a secondary effect resulting from diminished arousal produced by exposure to the stimulus (Petersson, et al., 2005). It should be noted that this decline only occurred during the infant interest test, making this explanation less likely. Another possible cause of decreased locomotion is that this compound may be anxiogenic. Several studies indicate that central OT decreases anxiety in female rats (Windle et al., 1997; Neumann et al., 2000; Bale et al., 2001). However, pacing, an indicator of arousal or anxiety in monkeys, did not increase after the higher dose of the antagonist. Furthermore, our data are not consistent with a locomotion-diminishing effect producing lower interest in the infant or sex for two reasons. First, although both doses of L368,899 significantly diminished locomotion (figure 3C), most effects on infant interest (figure 3A and B) and sexual behavior (figure 4A-C) occurred only after the 3 mg/kg dose. If decreased locomotion was the main factor responsible for the reduction of behavioral responses after L368,899 injection, its effects on interest in the infant and in sexual behavior should have occurred after both the 1 mg/kg and 3 mg/kg doses, but it did not. In addition, the male's behaviors, such as incomplete mount and latency to ejaculation, were only significantly altered when the female received the highest dose of antagonist. These data suggest that, at least at the 1 mg/kg dose, the locomotor effects were not altering these responses toward either the infant or the male.

Finally, there is one inconsistency in the female's interest in the infant monkey that deserves comment. As shown in figure 4, the magnitude of all measures of female interest in the infant at the beginning of the second series of tests with only saline was much lower than the initial saline test in the first series. One likely explanation is that between the first and second series of infant interest tests, the nulliparous female monkey was introduced to and, unfortunately,

attacked by the male. When replaced several weeks later in the enclosure in which she had been attacked, although she was re-exposed to the empty test arena several times between the two series of tests, fear of encountering the male again may have suppressed her interest in the infant. By the second exposure to the arena and the infant during the second test series, her fear may have declined sufficiently for her interest in the infant to recover. Finally, although the female may have habituated to the infant due to repeated exposures, during the second series of tests her interest did return to levels higher than during the 3mg/kg dose antagonist test. This suggests that the lack of infant interest after drug exposure could not simply be due to such habituation, although this possibility cannot be completely ruled out at the present time.

Despite the concerns discussed above, the magnitude of the effects of L368,899 on the nullipara's interest in the infant and the parous female's sexual behavior are striking and suggest that endogenous central OT may indeed contribute substantially to maternal and sexual motivation in primates. Clearly larger numbers of animals must be tested to confirm these exciting results. Our findings suggest that non-peptide OT antagonists like L368,899 which penetrate the CNS after peripheral administration may be important tools for examining the significance of central OT in a variety of social and other behaviors, such as stress responses, as well as in other physiological processes. Such studies may provide invaluable clues about potential therapeutic applications of CNS penetrating OT agonists. It would first be necessary to more definitively prove that peripherally-administered L368,899 does indeed block central OT receptors. Such studies would include demonstrating that central administration of L368,899 has effects similar to peripheral administration but at lower doses that are ineffective peripherally. Autoradiographic binding studies with radiolabeled L368,899 may further clarify where OT receptors are located in the brain and if it corresponds with brain areas where L368,899 accumulates after peripheral administration. Furthermore, it would be important to determine whether OT and other OT analogues competitively diminish L368,899-binding in a selective manner especially compared to V1a receptor ligands.

Previous studies have investigated the location of OT receptors in primates. Loup et al (1989; 1991) used autoradiography to locate OT receptors in human brain. They employed ^{3}H-OT and ^{125}I-d(CH2)$_5$ [Tyr(Me)$_2$,Thr$_4$,Tyr-NH$_{29}$] ornithine vasotocin (OTA) and found essentially the same binding distribution pattern with both ligands. ^{125}I-OTA selectively binds to OT receptors in rat brain (Elands et al, 1987). Loup and his colleagues found binding in the human hypothalamus, septal nuclei and numerous brain stem nuclei among other areas but not in several limbic structures, such as the amygdala and hippocampus, where ^{125}I-OTA autoradiography had identified OT receptors in rats (Gimpl and Fahrenholz, 2001). Subsequently, however, these results were brought into question by Toloczko et al. (1997) who found that almost all ^{125}I-OTA binding was competed off by a selective V1a receptor agonist but not an OT agonist indicating that ^{125}I-OTA principally binds to V1a receptors in primate brain. Furthermore, in entorhinal and cingulate cortex, where ^{125}I-OTA binding was found, they were unable to demonstrate the presence of OT receptor mRNA.

The L368,899 in the present study accumulated in hypothalamus, orbitofrontal cortex, amygdala, hippocampus, and septal nuclei but not other areas, including some for which there is evidence of OTRs in rodents. Gimpl & Fahrenholt (2001) have compared distribution of OTRs in rats and humans based on published reports to that time. They report evidence of OTRs in humans as well as rats in some of the sites of L368,899 accumulation, but not amygdala or orbitofrontal cortex. They also report OTRs absent in humans but present in rats in some of the sites where this compound did not accumulate, including caudate. Other sites studied here were not reported by Gimpl & Fahrenholt (2001).

The existence of OT receptors in primate brain, however, has more recently been confirmed by our immunohistochemistry studies employing 2F8, a monoclonal antibody directed against

the human uterine OT receptor (Kimura et al., 1994; Takemura et al 1994). We found immunostaining for OT receptors in cell bodies and fibers in the anterior hypothalamus and septum of a rhesus monkey (Boccia et al., 2001) and in the hypothalamus, preoptic area, amygdala, olfactory nucleus, cingulate gyrus and nucleus accumbens but not in the hippocampus of human brains (Boccia et al., 2003). Our current discovery that L368,899, after peripheral administration, accumulates in the hypothalamus, amygdala, hippocampus, septum and orbitofrontal cortex further suggests that OT receptors are expressed in monkey brain in areas that are in partial agreement with our previous immunohistochemical results. In conclusion, these findings suggest that L368,899 might be a very useful tool to investigate the involvement of endogenous oxytocin in primate social behavior.

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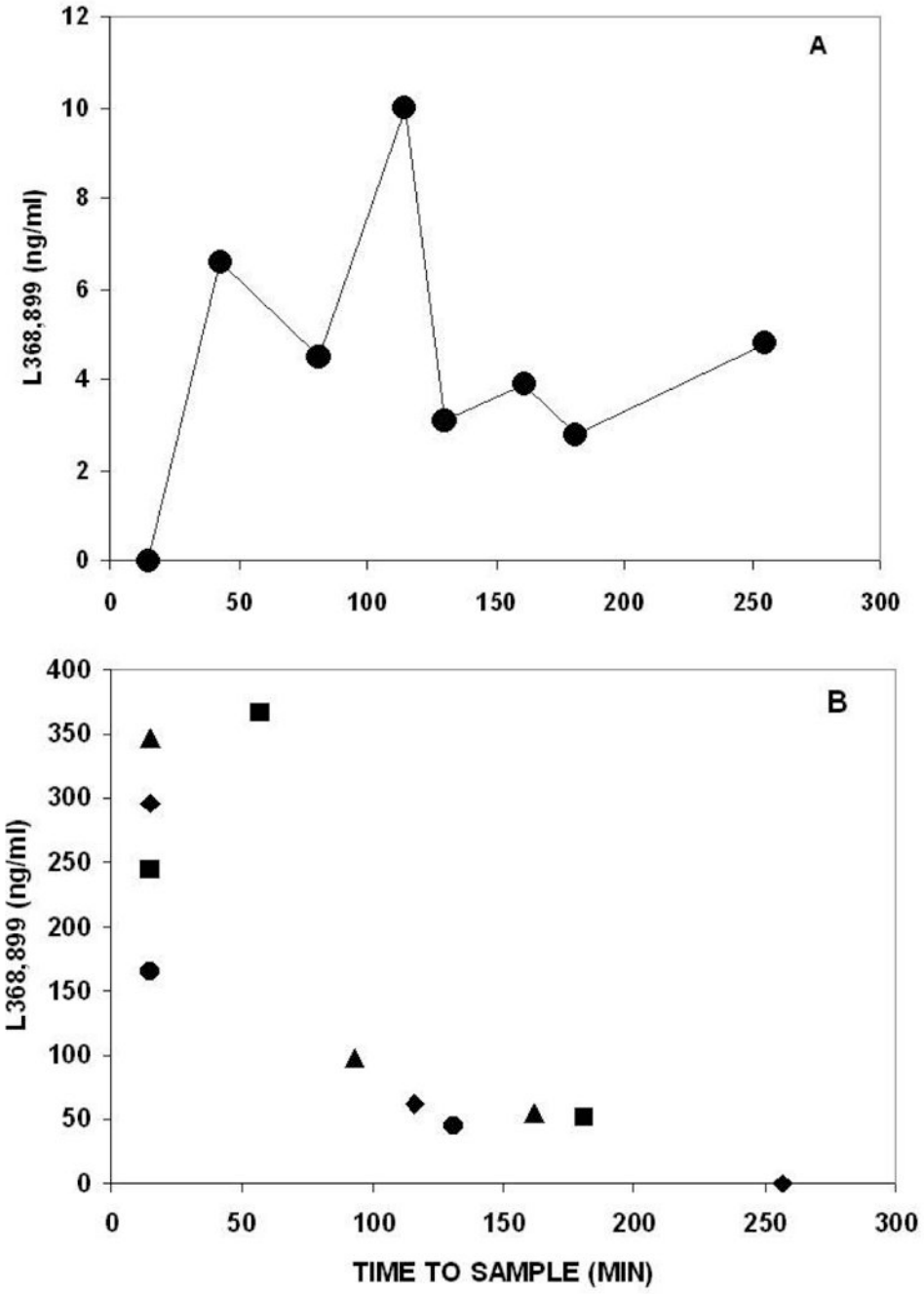

Figure 1.

(A) The concentration of L368,899 in CSF at time points following iv injection of 1mg/kg of the compound, in ng/mL. (B) The concentration of L368,899 in plasma at time points following injection of 1mg/kg of the compound, in ng/mL. In figure 1B, different symbols represent individual subjects, with corresponding points in figure 1A in points following 15 min sample.

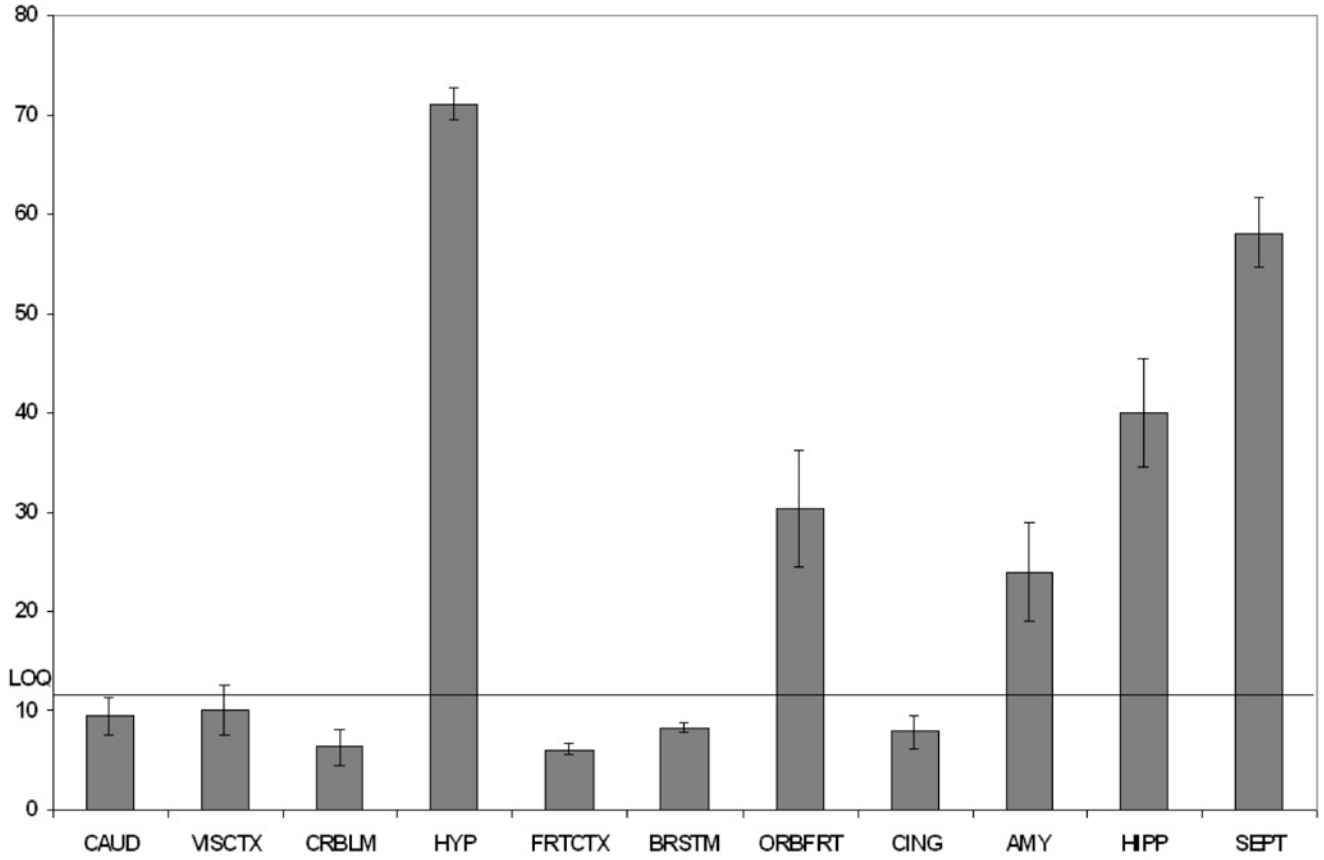

Figure 2.

Concentration of L368,899 (ng/gm tissue) in different brain regions of rhesus monkeys one hour following peripheral administration of 1 mg/kg of the compound, mean ± standard error of the mean. Some samples were lost in transit. The lowest level of detection (12ng/g) is indicated by a line, labeled LOQ. Numbers of samples are as follows: CAUD = caudate (n=4); VISCTX = visual cortex (n=4); CRBLM = cerebellum (n=3); HYP = preoptic and hypothalamic regions (n=2); FRTCTX = frontal cortex(n=2); BRSTM = brainstem (n=4); ORBFRT = orbitofrontal cortex (n=2); CING = cingulate (n=4); AMY = amygdala (n=4); HIPP = ventral hippocampus posterior to amygdala (n=4); SEPT = septum pelucidum with septal nuclei (N=2).

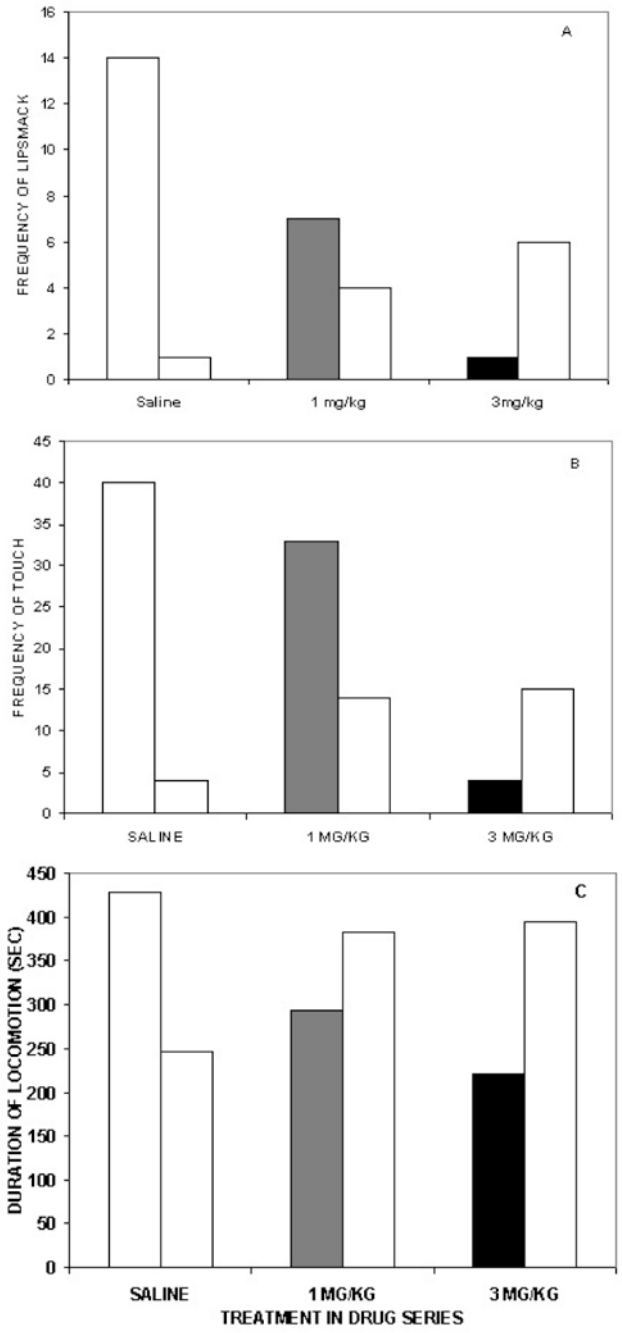

Figure 3.

Interest in infant. Behavior of female monkey in tests with infant: (A) Frequency of lipsmacks directed toward the infant. (B) Frequency of attempts to touch the infant. (C) Duration of locomotion. Open bars indicate saline treatment, grey bars indicates 1mg/kg of L368.899, and black bars indicates 3 mg/kg of L368,899. Drug series: for each treatment, the left bar represents the first series of trials (with saline, 1 mg/kg and 3 mg/kg separated by 48 hr) and the right bar represent the second series (one month after the first series, with saline administered three times separated by 48 hrs).

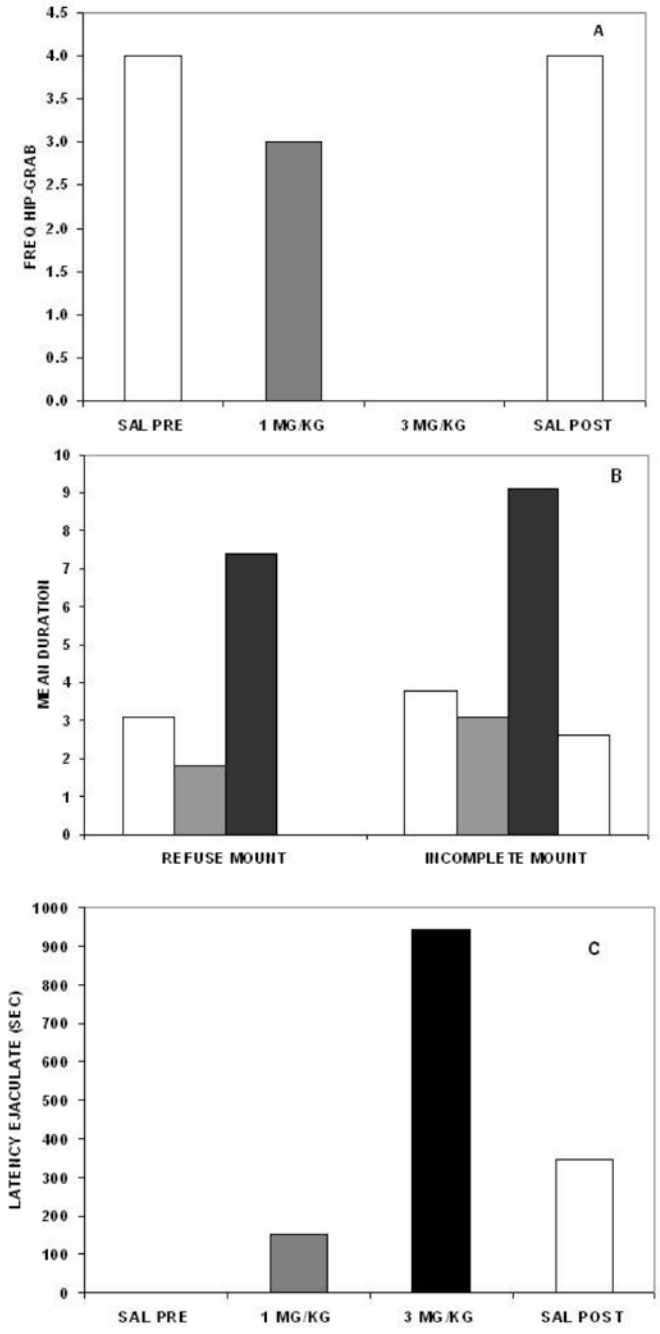

Figure 4.

Female and male sexual behavior. (A) Frequency of proceptive behavior (hip-grab) by the female. (B) Duration of mount refusals by the female and incomplete mounts by the male. (C) Latency from the start of the test to the first ejaculation. Open bar indicates saline treatment, grey bars indicates 1mg/kg of L368.899, and black bars indicates 3 mg/kg of L368.899.

Table 1

Behavioral Taxonomy used for Study III, Interest in an infant

Behavior	Definition
Infant-directed Behaviors:	
Touch or attempt to touch	manual contact or attempt to make manual contact with the infant through the wire mesh
Proximity	within a zone defined as 0.7 × 0.3 × 0.9 m adjacent to the enclosure wall near the infant
Look toward the infant	head oriented toward infant or its cage
Lipsmack	rapid opening and closing of mouth, often with tongue protrusion, and distinctive smacking sound, an affiliative facial expression
Threaten	open-mouth threat, lunge, stare or more subtle threats
Vocalizations:	
Coo call	smooth high pitch call
Alarm bark	lower pitched, short harsh call
Locomotion & Stereotypy:	
Locomotion	move all four feet, in any direction, including climbing, excluding pacing
Pacing	repetitive locomotion in stereotyped pattern
Other stereotypies	bizarre postures or movements (such as eye-poke, salute, self-mouthing, etc)
Exploration:	
Object exploration	manual, oral or close visual inspection or manipulation of objects or cage parts

Table 2
Behavioral Taxonomy used for Study IV, Sexual Behavior

Behavior	Definition
Female Sexual Behavior:	
Present	present rump to male with tail up and no locomotion
Refuse Mount	resist or ignore male attempts to mount
Hip grab	grasp waist of male with one or both hands (proceptive behavior)
Female Mount	approximation of male mount by female
Male Sexual Behavior:	
Mount	single- or double-foot-clasp mount, with or without intromission
Incomplete Mount	mount attempted but not completed to foot-clasp mount
Ejaculation	evidenced by rigid, tonic muscle contraction in characteristic mount posture, typically followed by extended interval without mount attempts
Other Social Behavior:	
Touch	in physical contact
Proximity	within arm's length of other animal
Approach (initiate/receive)	enter proximity
Withdraw (initiate/receive)	leave proximity
Lipsmack	rapid opening and closing of mouth, often with tongue protrusion, and distinctive smacking sound
Groom present	present area of body to other animal for grooming
Groom (receive/initiate)	oral or manual manipulation of hair or skin of other animal
Fear grimace	bared teeth expression
Threaten	open-mouth threat, lunge, stare or more subtle threats
Locomotion & Stereotypy:	
Locomotion	move all four feet, in any direction, including climbing, excluding pacing
Pacing	repetitive locomotion in stereotyped pattern
Other stereotypies	bizarre postures or movements (such as eye-poke, salute, self-mouthing, etc)
Exploration:	
Object exploration	manual, oral or close visual inspection or manipulation of objects or cage parts