Pharmacology, Biochemistry and Behavior 90 (2008) 534–539

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Behavioral effects of acute stimulation of κ-opioid receptors during lactation

Elizabeth Teodorov, Aline T. Tomita, Gabriela P.R. Banon, Iara G. Gil, Maria M. Bernardi, Luciano F. Felício *

Faculdade de Medicina Veterinária e Zootecnia, Departamento de Patologia, Universidade de São Paulo, Brazil

ARTICLE INFO

Article history:
Received 6 August 2007
Received in revised form 25 March 2008
Accepted 10 April 2008
Available online 16 April 2008

Keywords:
Maternal behavior
Motivation
Long-term effects
Adaptation

ABSTRACT

The behavioral effects of the κ-opioid receptor agonist U69593 were examined in lactating rats. On day 5 of lactation, animals were treated with 0.1 mg/kg of U69593 to determine whether it influences general activity and maternal latencies toward pups. Because little attention has been given to the possibility that pre-mating treatment with morphine may modulate the response to κ-opioid receptor stimulation, another group of animals was submitted to the same acute challenge after abrupt withdrawal from repeated treatment with morphine sulfate during the pre-mating period (5 mg/kg on alternate days for a total of five doses). Acute κ-opioid stimulation reduced total locomotion, rearing frequency, and time spent self-grooming and increased immobility duration. These κ agonist effects were not observed in animals pretreated with morphine. Similarly, latencies to retrieve pups were longer only in animals pretreated with saline and challenged acutely with U69593. None of these effects were observed in morphine sulfate-pretreated animals. The present results suggest that pre-mating repeated exposure to morphine produces a tolerance-like effect on behavioral responses to low-dose κ-opioid receptor stimulation in active reproductive females.

1. Introduction

Opioid receptors are involved in both exploratory and motivational behaviors. Recently, a role has been described for opioids in behavioral selection during lactation (Sukikara et al., 2006, 2007). When given a choice, a morphine-treated lactating dam hunts insects instead of caring for newborns. Opioid-induced inhibition of maternal behavior in lactating rats has been well established (Bridges and Grimm, 1982; Grimm and Bridges, 1983; Kinsley and Bridges, 1988; Mann et al., 1989, 1990, 1991). The expression of this phenomenon may be modulated by physiological state (Kinsley and Bridges, 1988) or other endogenous peptides (Felicio et al., 1991; Mann et al., 1995; Miranda-Paiva and Felicio, 1999; Miranda-Paiva et al., 2002, 2007). The role of κ-opioid receptors in these phenomena has not been established. κ-Opioid receptors have been reported to play a role in immunity, pain perception, neuroendocrine physiology, affective behavior, and cognition. Activation of these metabotropic receptors may modulate reproductive behavior in females (Simonin et al., 1995; Mansour et al., 1995).

Endogenous opioids interact with other neuropeptides to optimize maternal behavior (Brunton and Russell, 2008). Opioid receptors are upregulated by morphine treatment and pregnancy and down-regulated in different brain areas during lactation (Crain and Shen, 1998; Wise and Bozarth, 1982). Maternal behavior may be inhibited in dams treated with low doses of morphine that are otherwise ineffective for inducing such inhibition in morphine-naive lactating rats. It happens when opioid stimulation occurs during late pregnancy (Miranda-Paiva et al., 2001, 2003; Slamberova et al., 2001). Opioid systems are plastic and adaptive, and these characteristics may alter their responses to previous opioid treatments. Typically, chronic administration of morphine to animals produces a progressive and enduring increase in some behavioral parameters (Babbini and Davis, 1972). This phenomenon has been termed behavioral sensitization (Robinson and Becker, 1986; Felicio et al., 2001; Yim et al., 2006). Repeated treatment with morphine also can induce tolerance to its sedative effects. Thus, sensitization and tolerance may be mediated by different opioid receptors and by diverse biochemical pathways. Particularly, opioid receptors can change their expression and appear to play a role in these adaptive phenomena (Teodorov et al., 2006). The modulatory and adaptive roles of opioid receptors in motivation, however, are poorly understood (Nocjar and Panksepp, 2007). With the development of selective opioid agonists and antagonists, each type of opioid receptor has been found to have unique pharmacological properties and is differentially distributed and dimerized in the central nervous system (Mansour et al., 1995; Jordan and Devi, 1999). The κ_1-selective opioid agonist U69593 was chosen for use in the present study.

Little attention has been given to the possibility that pre-mating treatment with morphine may influence the behavioral effects of specific opioid agonists during lactation. Few studies have

* Corresponding author. Tel.: +55 11 3091 7934; fax: +55 11 3091 7829.
E-mail address: lfelicio@usp.br (L.F. Felício).

0091-3057/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2008.04.008

investigated the effects of morphine treatment during the pre-mating period and its consequences on maternal behavior. Yim et al. (2006) observed that morphine treatment in the pre-mating period produced sensitization-like effects in female animals with no effects on maternal behavior. These results are similar to findings obtained in adult male rats and mice (Felicio et al., 2001; Tao et al., 2006). κ-Opioid agonists can attenuate tolerance to and physical dependence on cocaine and morphine (Shippenberg et al., 1998; Tao et al., 2006). κ-Opioid receptor stimulation interferes with the effects of morphine. The present study, therefore, was designed (*i*) to test whether acute stimulation of κ receptors induces behavioral changes in lactating dams, and (*ii*) to evaluate whether a pre-mating treatment with morphine can interact with this post-partum acute challenge to modify exploratory behavior and mother–pup interactions. To investigate the effects of prior morphine treatment on the response to specific opioid receptor stimulation, a single injection of the κ-selective agonist U69593 was administered during lactation, and general activity and maternal latency to retrieve the dam's pups were tested in lactating rats that were submitted to morphine pretreatment during the pre-mating period. The results demonstrate that in lactating rats, κ-opioid receptor stimulation has behavioral effects that can be modulated by pre-mating morphine treatment.

2. Materials and methods

2.1. Animals

Female Wistar rats weighing 200–250 g at the beginning of the study were obtained from the Faculdade de Medicina Veterinária, Universidade de São Paulo, Brazil. Animals were housed in polypropylene cages (32×40×18 cm; three animals per cage) under controlled temperature (22±2 °C), with a 12 h/12 h light/dark schedule (lights on at 06:00 h). Animals had free access to food and water during the experimental procedure. The animals used in this study were maintained in accordance with the guidelines from the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA. To confirm the presence of κ-opioid receptors in the brain of lactating dams, a pilot test compared protein expression in the periaqueductal gray (PAG) of virgin and lactating rats. The PAG was selected because it has been found to play a role in opioid-mediated behavioral selection during lactation (Sukikara et al., 2006; Pavesi et al., 2007). The results confirmed the expression of the κ receptor in the PAG of lactating animals (Fig. 1).

After treatment with morphine or saline, two estrous female rats were placed overnight with one sexually experienced male for mating. The onset of pregnancy was confirmed by the presence of spermatozoa in vaginal smears on the following morning, which was considered gestational day 1 of pregnancy (GD1). Pregnant rats were housed two per cage until GD18 and then were individually housed until the end of the experiments. Parturition was considered postnatal day 0 (PND0), and all pups were examined externally, sexed, and weighed, with eight pups (four males and four females) left with each dam until weaning on PND21. An exception was the pilot study in which the dams were decapitated on day 5 of lactation. Behavioral studies occurred on PND5.

2.2. Confirmation of the expression of the κ receptor

Age-matched female virgins (n=7) and 5-day lactating females (n=7) were decapitated. Each PAG region weighing 40 mg was homogenized directly in sample buffer (1 M Tris–HCl buffer, pH 6.8; 10% sodium dodecyl sulfate; 10% glycerol). Later, Tris–HCl buffer (1 M, pH 6.8, containing 10% sodium dodecyl sulfate, 10% glycerol, 1 M dithiothreitol, 10 mM phenylmethylsulfonyl fluoride) was added, and the suspension was centrifuged at 11,500 ×g for 15 min at 4 °C. The supernatant was stored at −80 °C until analysis. Total protein concentration was quantified with Bio-Rad Protein Assay reagent (Hercules, CA, USA) before performing electrophoresis.

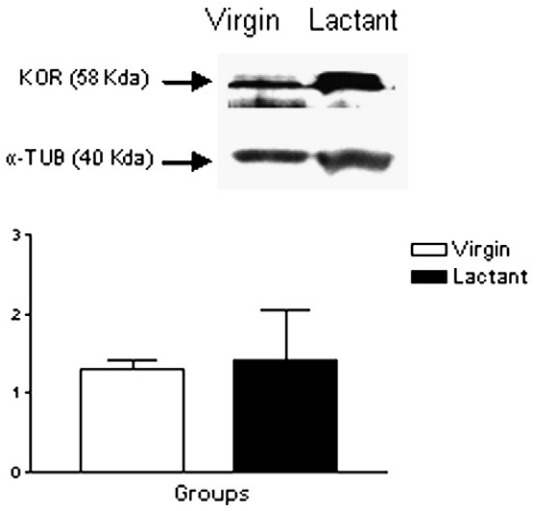

Fig. 1. Immunoreactivity for κ-opioid receptor (KOR) protein by immunoblot analysis (Western blot) in the PAG region from virgin and lactating female rats. Data are expressed as the ratio of the densitometric intensity of the immunoreactive band for the κ receptor divided by the immunoreactive band of α-tubulin. Data are expressed as mean±SEM (n=7/group). Student's t-test.

Western blot analysis of the κ-opioid receptor was performed with 10 μg of lysates obtained as described above, using rabbit polyclonal anti-κ-opioid receptor (1:1000) antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibody incubations were performed at room temperature for 1 h with anti-rabbit antibody 1:10,000 (Sigma, St. Louis, MO, USA). Protein expression was visualized using an enhanced chemiluminescence assay kit (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions. Signals were normalized to α-tubulin signals (1:30,000 Sigma, St. Louis, MO, USA) for 1 h at room temperature. The horseradish peroxidase-conjugated secondary anti-mouse antibody (Amersham Biosciences, Buckinghamshire, UK) was diluted 1:6000, incubated for 45 min at room temperature, and developed as previously described. κ-Opioid receptor expression was observed both in virgin and in lactating rats (Fig. 1).

2.3. Morphine sulfate and selective agonist for κ opioid receptor treatment

Adult female rats were randomly divided into four groups for each general activity and maternal behavior experiment. The four groups included: SS (saline/saline), MS (morphine/saline), Sκ (saline/κ agonist), and Mκ (morphine/κ agonist). The groups Sκ and Mκ were divided in five subgroups each according to the dose of the κ agonist, 0.05; 0.1; 0.25; 0.5 or 1.0 mg/kg. All animals were pretreated with morphine sulfate (5 mg/kg, s.c.; Cristalia, Sao Paulo, Brazil) or saline at 48 h intervals for a total of five doses before mating. Experimental groups were formed as described in Table 1. On the 5th day of lactation, 30 min before testing, dams in the Sκ and Mκ groups received one subcutaneous injection of the selective κ agonist U69593 (+)(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pirroldinil)-1-oxaspiro[4,5]dec-8-ilbenzeneacetamide) obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.4. General activity

General activity was observed in an open-field arena. The open-field was 97 cm in diameter and 28 cm in height. The arena was washed with a water–alcohol (5%) solution before behavioral testing to eliminate possible bias due to odors left by previous subjects. Pups

were removed between 08:00 and 11:00 h. Thirty minutes later, animals received U69593 (0.1 mg/kg, s.c.) or saline injections. Thirty minutes after the injection, rats were placed in the open-field arena and evaluated for locomotion frequency (number of floor units entered), rearing frequency (number of times the animal stood on its hind legs), immobility duration (total number of seconds without movement), and total grooming duration (measured in seconds for 5 min) (Felicio et al., 1987).

2.5. Maternal tasks

On day 5, pups were removed at 07:00 h and placed in another home-cage that was distant from their mother. Thirty minutes after removing the pups, the dams were acutely challenged with U69593 (0.05; 0.1 and 0.25 mg/kg, s.c.) or saline. The doses of U69593 were chosen based on previous sedation tests performed in our laboratory. Since the 0.5 and 1.0 mg/kg doses induced increasing sedative effects, leading to dose-dependent prostration in both the general activity task, they were not used for the maternal behavior test. Thirty minutes after the injections were given to the dams, all pups were placed back with their mothers, and maternal behavior testing began. Latencies in seconds for pup retrieval, grouping, crouching, and full maternal behavior were scored (Bridges and Grimm, 1982; Miranda-Paiva and Felicio, 1999). Animals were scored as fully maternal if they retrieved all eight pups to the nest and displayed nursing behavior with their back arched over the pups for 3 consecutive minutes. If animals were not fully maternal after 30 min of continuous observation, they were checked every 15 min until 60 min and then hourly until full maternal behavior was observed. Events observed after the first 30 min of continuous observation were recorded at the time of first observation (e.g., if full maternal behavior was first observed at 60 min, the full maternal behavior latency was scored as 60 min, or 3600 s). The same criterion was used for all other responses. For all behavioral tests, the observers were blind to the treatment of the test subjects.

2.6. Statistical analysis

Data of general activity and maternal tasks were analyzed by two-way analysis of variance (ANOVA) followed by Tukey test. In all cases, results were considered significant at $P<0.05$. Percentage data were compared using Fisher test.

3. Results

The 0.05 mg/kg dose showed no detectable effects. The 0.1 mg/kg dose induced no sedation. The 0.25, 0.5 and 1.0 mg/kg doses induced increasing sedative effects, leading to dose-dependent prostration in the general activity study (Table 1). Thus, only the 0.05, 0.1 and 0.25 doses were used in the maternal tasks experiment.

Previous treatment with morphine did not affect general activity by itself, indicated by the lack of significant differences between the SS and MS groups. Acute effects on general activity were observed for 0.1 mg/kg dose of the selective κ receptor agonist. Comparison among groups SS, MS, Sκ0.1 and Mκ0.1 showed that general activity in the Sκ0.1 group revealed decreased total locomotion ($F_{(3,40)}=2.99$, $P=0.036$), rearing ($F_{(3,40)}=7.50$, $P=0.0002$), and grooming ($F_{(3,40)}=10.21$, $P=0.0001$), as well as increased immobility duration ($F_{(3,40)}=10.21$, $P=0.0001$). Most of the time, the animals were immobile. No significant difference was observed for the Mκ0.1 group (Table 1).

In relation to maternal behavior, the 0.05 mg/kg dose showed no detectable effects. The 0.25 mg/kg dose induced sedative effects thus interfering with the expression of maternal behavior (Table 2).

Comparison among groups SS, MS, Sκ0.1 and Mκ0.1 showed that only Sκ0.1 group dams showed decreased percent of animals retrieving all pups, grouping, crouching and displaying full maternal behavior (Table 2). Moreover, animals from Sκ0.1 group presented increased latencies to retrieving the first pup ($F_{(3,40)}=3.79$, $P=0.0137$) and second pup ($F_{(3,40)}=4.68$, $P=0.0047$) compared with the other groups. Furthermore, mothers of the Sκ group showed longer latencies to retrieve the third pup ($F_{(3,40)}=40.53$, $P=0.0001$) and eighth pup ($F_{(3,40)}=4.23$, $P=0.008$) compared with the SS and MS groups (Fig. 2). Animals in the Sκ group continued digging in the wood chips and building nests instead of retrieving pups, resulting in longer latencies to retrieve the pups. Such disruptive effects were not observed in the Mκ group pretreated with morphine and challenged with U69593.

Table 1
Effects on general activity of morphine sulfate treatment before mating observed on 5 ° day of lactation, after selective agonist κ-opioid challenge (U69593)

Groups	CL	PL	TL	REA	GROO (s)	IMM (s)
SS (n=11)	2±0.33	106±7.16	121±7.44	23±1.65	11±3.25	41±5.87
MS (n=11)	2±0.31	107±5.84	124±6.72	29±1.82	12±1.97	45±5.71
Sκ0.05 mg (n=11)	2±0.11	101±6.64	120±7.12	21±2.15	15±2.32	53 ±6.31
Sκ0.1 mg (n=11)	1±0.50	76±6.28	85±6.05*	16±1.2*	2±0.94*	104±9.80*
Sκ0.25 mg (n=2)	1±0.50	0±0	1±0.2	1±0,5	0±0	250±0.31
Sκ0.5 mg (n=2)	0±0	0±0	0±0	0±0	0±0	300±0.22
Sκ1.0 mg (n=2)	0±0	0±0	0±0	0±0	0±0	300±0.22
Mκ0.05 mg (n=11)	2±0.31	117±5.84	122±5.82	26±2.21	14±2.55	36±7.21
Mκ0.1 mg (n=11)	2±0.52	103±8.58	115±10.46	19±2.24	13±4.93	72±7.58
Mκ0.25 mg (n=5)	2±0.33	0±0	2±0.13	0±0	0±0	278±0.31
Mκ0.5 mg (n=2)	0±0	0±0	0±0	0±0	0±0	300±0.22
Mκ1.0 mg (n=2)	0±0	0±0	0±0	0±0	0±0	300±0.22

Data expressed as mean±S.E.M. Grooming and immobility were measured in seconds; *n* = animals per group.
CL: central locomotion; PL: peripherical locomotion; TL: total locomotion; REA: rearing; GROO: grooming; IMM: immobility.
* $P<0.05$ in relation to groups SS, MS and Mκ0.1 mg. ANOVA followed Tukey test.

Table 2
Effects on maternal behavior of morphine sulfate treatment before mating observed on 5 day of lactation, after selective agonist κ-opioid challenge (U69593)

Groups	Retrieve 1st pup	Retrieve all pups	Grouping	Crouching	Full maternal behavior
SS	100	100	100	100	100
MS	100	100	100	100	100
Sκ0.05 mg	100	100	100	100	100
Sκ0.1 mg	90	0*	0*	0*	0*
Sκ0.25 mg	0	0	0	0	0
Mκ0.05 mg	100	100	100	100	100
Mκ0.1 mg	100	70	60	40	40
Mκ0.25 mg	0	0	0	0	0

Data expressed as percentage of animals observed showing that behavior during the first 30 min. Fisher test.
* $P<0.05$ in relation to groups SS, MS, Mκ0.05 and Mκ0.1 mg Fisher test.

4. Discussion

Acute treatment with the κ-opioid receptor agonist U69593 decreased general activity and increased pup retrieval latencies in lactating dams, suggesting that acute stimulation of κ receptors might have important behavioral effects during lactation. These effects were not observed in animals pretreated with morphine, suggesting the existence of morphine-induced tolerance-like modulation of κ receptor-mediated responses. Throughout the observation period, these animals did not exhibit fear-related defensive responses and did not show any stereotyped behavior, hyperactivity, motor rigidity, or signs of sedation that would be expected with higher doses of morphine (Nasello et al., 1973). Because the body weight and litter size data suggest that morphine treatment did not affect pregnancy, the effects observed in the present study are interpreted as attributable to the pharmacological treatments. In addition, our results confirm the presence of κ-opioid receptors in the PAG of lactating animals, and the κ agonist-induced behavioral changes are interpreted to be a consequence of κ receptor stimulation.

Opioids have been suggested to play roles in both social behavior and behavioral selection during lactation (Byrnes et al., 2000; Sukikara et al., 2006, 2007; Nocjar and Panksepp, 2007). In the present study, the results obtained for exploratory activity in the open-field showed that acute treatment with U69593 decreased general activity. The same acute challenge induced a delay in pup retrieval. Curiously, the behavior displayed by dams in the Sκ group was very different in the two situations. While in the open-field, animals essentially remained still. When the dams were tested with pups, they continued digging in the wood chips and building a nest, which suggests that they may have been displaying indirect maternal behavior in the presence of the pups. Stimulation of κ receptors, therefore, may elicit different behaviors depending on the environment. In both situations, these effects were not observed in animals pretreated with morphine. Thus, pre-mating treatment with morphine may have induced tolerance in κ receptors.

κ-Opioid receptor stimulation caused a decrease both in exploration and in interactions with pups, reflected by longer latencies in retrieving pups. The two distinct behavioral patterns displayed in both situations might be part of a more general phenomenon—an opioid-induced change in motivational state. Stimulation of κ receptors may make the lactating animal less motivated to explore a new environment and to interact with pups. Nonetheless, this behavioral effect of κ receptor stimulation appears to be very adaptive and sensitive to plastic manipulations. A pre-mating morphine treatment (i.e., repeated stimulation of various opioid receptor subtypes) either attenuated or reversed all κ receptor agonist effects in a tolerance-like manner. Such a tolerance-like effect may be attributable to the pre-mating-specific stimulation, to the κ receptor, to stimulation of other opioid receptor subtypes, or to the simultaneous repeated stimulation of more then one opioid receptor subtype.

Previous studies have shown that maternal behavior is inhibited by opiates. Morphine disrupts maternal responsiveness in female rats, an effect reversed by naloxone treatment. β-endorphin, an endogenous opioid, infused into the ventricular system of lactating rats dose-dependently blocks normal maternal behavior, suggesting

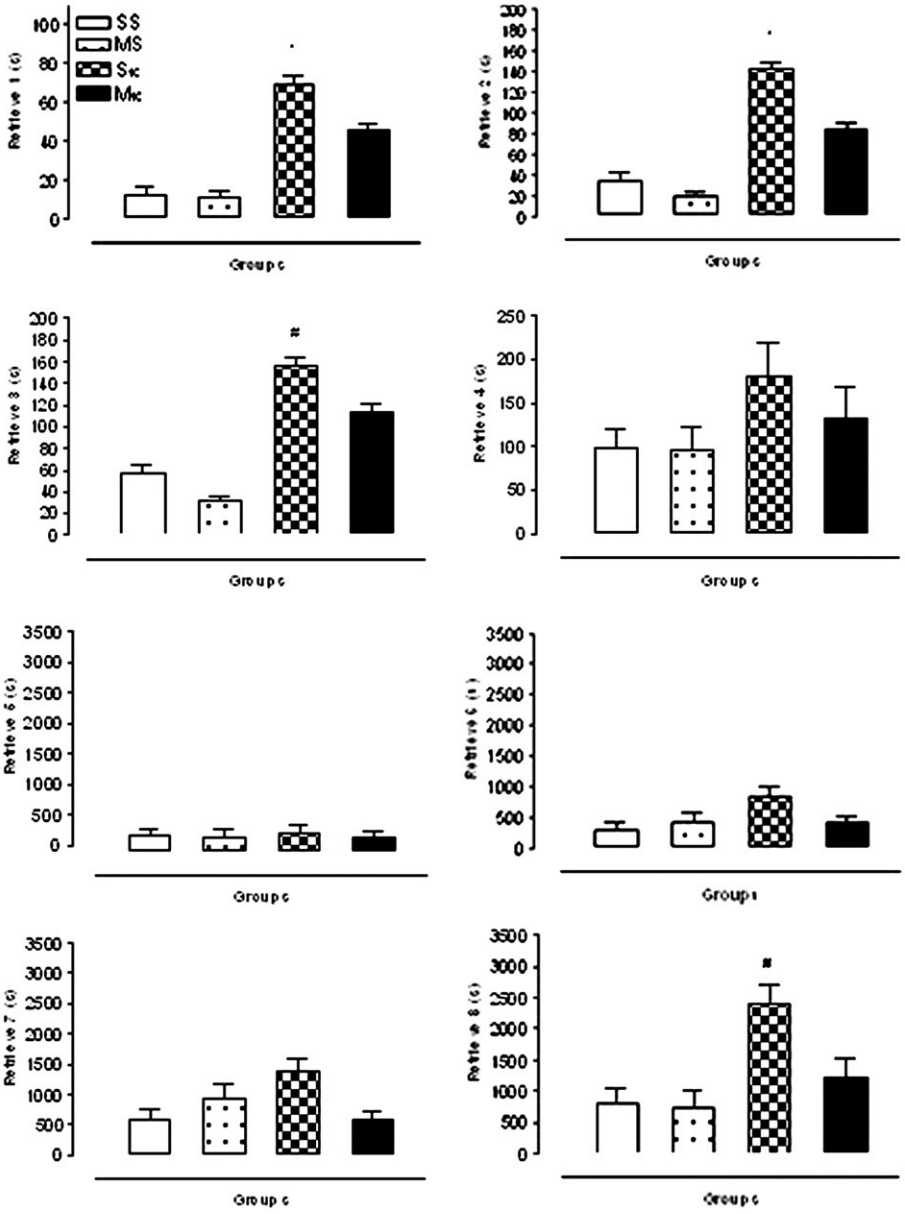

Fig. 2. Parameters of maternal behavior observed in female rats on day 5 of lactation treated in the pre-mating period with morphine and the selective κ-opioid receptor agonist U69593 on the test day. Latencies to retrieve pups were measured in seconds. Data are expressed as mean ± SEM. *$P<0.05$ compared with all other groups; #$P<0.01$ compared with the saline/saline (SS) and morphine/saline (MS) groups. ANOVA followed by Tukey test.

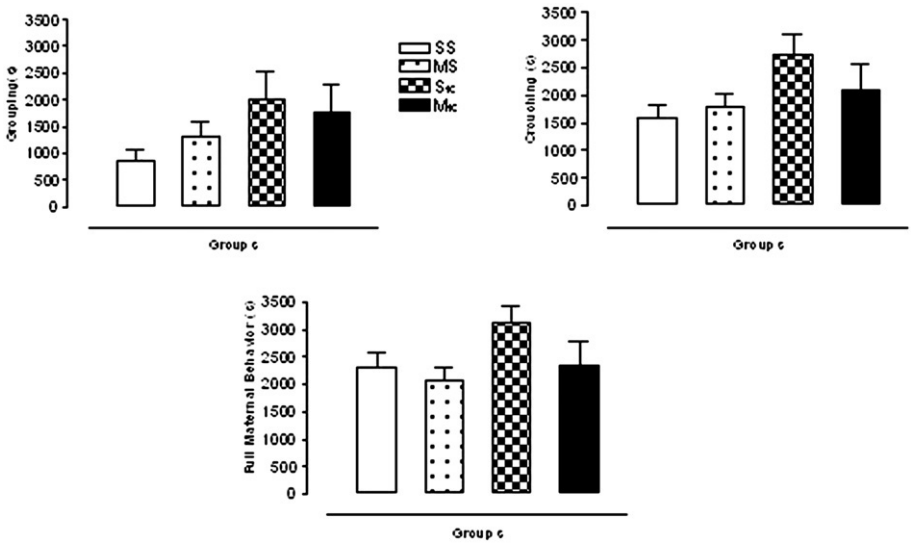

Fig. 2 (*continued*).

that the changes in endogenous opioid levels alter maternal responsiveness in rats (Felicio et al., 1991; Robinson and Becker, 1986). The inhibitory effects of opioids on maternal behavior are modulated both by other peptides and by previous opioidergic stimulation (Felicio et al., 1991; Mann et al., 1995; Miranda-Paiva et al., 2003). Previous studies from our laboratory show that repeated administration of morphine to female rats during late pregnancy inhibits ongoing maternal behavior, suggesting the existence of adaptive morphine-induced neuronal mechanisms that appear during late pregnancy (Wise and Bozarth, 1982; Miranda-Paiva et al., 2001, 2003, 2007; Yim et al., 2006). In contrast, the present results suggest the existence of an opioid-induced, tolerance-like, receptor-specific mechanism. Tardive effects of opioid stimulation in reproductively active dams may vary according to the physiological state. Alternatively, because the doses and time period of the treatments differed, these factors may have played a role in the present results. Another major difference between the present study and previous results is that the lactating animals were submitted to acute stimulation of one opioid receptor subtype. This unique approach allowed insight into the behavioral role of this receptor in the lactating female brain. Thus, specific κ receptor stimulation, among the various opioid receptors, would be necessary for the expression of tolerance-like phenomena.

The results presented here are consistent with previous work showing that pretreatment with morphine sulfate has effects on actively reproductive female animals that vary with the pretreatment period (Yim et al., 2006). In the present study, acute challenge with the selective κ agonist with no prior morphine treatment elicited behavioral effects during lactation. These effects were attenuated or abolished in dams submitted to morphine pretreatment.

Taken together, the present data support the hypothesis that κ-opioid receptor activation has important neurobehavioral effects. In addition, pretreatment with morphine may activate inhibitory mechanisms that result in a tolerance-like response to κ receptor activation in lactating dams. The functional role of this receptor in the behavioral adaptability during this critical period deserves further investigation.

Acknowledgments

Research was supported by funds from CAPES, CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico (119306/2004-2), and the Fundação de Amparo à Pesquisa do Estado de São Paulo (Grant number 2004/05557-4).

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