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Morphine disruption of maternal behavior and modifications of underlying neural activity

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MORPHINE DISRUPTION OF MATERNAL BEHAVIOR AND MODIFICATIONS OF UNDERLYING NEURAL ACTIVITY

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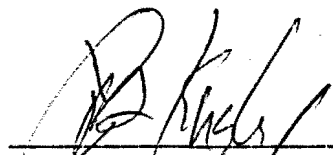
I certify that I have read this thesis and find that, in scope and quality, it satisfies the requirements for the degree of Master of Arts.



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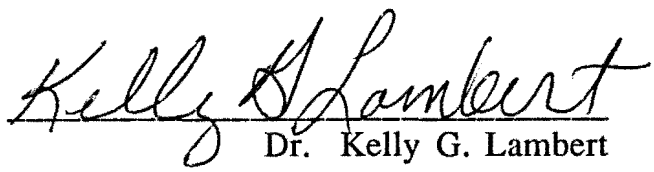

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Morphine Disruption of Maternal Behavior and Modifications underlying Neural Activity

Graciela Stafisso-Sandoz
Thesis Advisor- Dr. Craig Kinsley

Morphine significantly impairs maternal behavior; Naloxone, an opiate antagonist, restores it. Maternal behavior is associated with c-fos expression, an immediate early gene product, in medial preoptic area (mPOA) of females. In this series of experiments, the effects of morphine and Naloxone on the expression of c-fos were examined. On postpartum day 5 or 6, females were injected with morphine or saline (Exp. 1), or morphine+Naloxone or morphine+saline (Exp. 2) and placed back in the homecage, separated from their pups by a wire-mesh partition. Sixty minutes later processing for c-fos immunohistochemistry commenced. The c-fos positive cells in a proscribed portion of mPOA were counted. Morphine-treated females had fewer c-fos cells in mPOA compared to saline-treated females. Further, morphine+Naloxone-treated females expressed more c-fos cells compared to morphine+saline females. Morphine-treated females, therefore, may exhibit reductions in MB because of relative opiate-induced inactivation of areas of the brain devoted to the regulation of maternal behavior.

Morphine disruption of maternal behavior and modifications underlying neural activity

Maternal behavior is representative of the larger category of parental behavior, defined as any behavior of a member of a species toward a reproductively-immature conspecific which increases the likelihood that the latter will survive to maturity (Numan, 1994). In mammals, because it is the female that lactates, the primary infant caregiver is usually the mother and, therefore, we refer to such behavior as maternal (Numan, 1994). Maternal behavior involves two major classes of behavior, pup-directed patterns (e.g., retrieving, grouping, licking and nursing) and non-pup-directed patterns (e.g., nest building and maternal aggression), all of which are important for the survival of the young. For example, behaviors in which the mother carries the young in her mouth to a new site or back to the original nest, and collects them there (retrieval and grouping, respectively), function to keep all the pups close to the mother for protection, care, and feeding (Numan, 1994).

Most rodent young are dependent, essentially immobile and incapable of temperature regulation at birth, and the nest built by the mother prior to parturition serves to insulate the young in the absence of the mother. When the mother crouches over her young, she warms the young by transferring body heat. Additionally the crouching female exposes her mammary region to facilitate nursing (Numan,1994). The mother licks the young (who cannot void voluntarily) in the nest stimulating urination and defecation, thereby providing a significant source of water for the mother, to compensate for what is lost as a result of lactation (Baverstock & Green, 1975). In all, maternal behavior is a multi-faceted set of behaviors, heavily motivated, which ensures survival of the female's large genetic and metabolic investment.

The onset of maternal behavior is primarily hormonally based. Females of all species (though rats are the focus of the present study) will engage in maternal behavior upon proper endocrine and offspring stimulation. For example, during pregnancy, exposure to gonadal hormones such as progesterone and estradiol and the pituitary hormone prolactin stimulate a female to display maternal behavior (Kinsley,1994; Siegel & Rosenblatt 1975). The relationship

between hormones and maternal behavior involves both the initiation and maintenance of the behavior, and recruits different mechanisms through which the behavior is displayed. Several researchers have been able to stimulate maternal behavior in virgin rats through hormone regimens that mimic pregnancy (Mayer, Monroy & Rosenblatt, 1990; Bridges, 1984).

Unlike the onset of maternal behavior, the maintenance of the behavior during the postpartum period seems to be more directly related to pup exposure, and can be elicited in the absence of hormones. Hormones stimulate a high level of maternal responsiveness near the time of parturition, but, once maternal behavior is established, it is subsequently maintained at high levels by stimulation from pups (Rosenblatt & Siegel 1980). Consequently, many studies have focused on the role of sensory information coming from the young and its role in the maternal response (Sewell, 1970; Haskins, 1977; Allin & Banks, 1972; Smotherman, Bell, Hershberger, & Coover, 1974). Auditory and olfactory stimulation, (the latter, which is of some interest to the present research), are primary, and have been the center of many investigations (Allin & Banks, 1972; Smotherman et al, 1974).

Research has demonstrated that the medial preoptic area (mPOA) regulates maternal behavior in females (Numan 1974; Fahrbach & Pfaff, 1986; Numan, Rosenblatt & Komisaruk, 1977). Several studies have shown that lesions of the mPOA disrupt maternal behavior in the rat, which includes, as described above, retrieving, nest building, and nursing behaviors (Numan, 1974; Jacobson, Terkel, Gorski & Sawyer, 1980; Numan et al., 1977). The mPOA contains a high concentration of estrogen-and progesterone-binding neurons, and may be the site where steroids act to affect the onset of the behavior because, as Pfaff and Keiner (1973) have shown ovariectomized virgin female rats treated with estradiol implants directly in the mPOA , respond very rapidly to pups.

Hormones in combination with the "enriched" postnatal environment of motherhood (pup stimulation) may produce structural changes in the mPOA, facilitating maternal behavior. Data from our laboratory suggest a relationship between pregnancy (steroid exposure) and neuronal soma size in mPOA and hippocampal neurons (Keyser, Stafisso-Sandoz, & Kinsley, 1994;

Stafisso-Sandoz, Hearon, Keyser, & Kinsley, 1996).

When neurons and other cells are influenced by extracellular and intracellular signals, a class of genes, referred to as immediate early genes, may become activated (Sagar, Sharp, & Curran, 1988). The proto-oncogene c-fos is one of a class of immediate-early genes which is expressed in response to a variety of stimulus conditions and that co-acts with other immediate-early gene products to activate the expression of other genes (Sagar, et al, 1988). Fos is a nuclear protein that serves as a transcriptional factor that can alter the expression of target genes (Morgan & Curran, 1991). The immunohistochemical (double antibody procedure) detection of Fos can give a picture of neurons that are activated as a result of particular forms of neural stimulation (Morgan & Curran, 1991; Sharp, Gonzalez, Sharp & Sagar,1989). The proto-oncogene c-fos is expressed in neurons in response to direct stimulation by growth factors and neurotransmitters (Sagar et al, 1988).

Though Fos protein does not appear indiscriminately in neurons when they are active, it has been shown to be expressed in a variety of neurons responding to sensory, hormonal, neurochemical and behavioral events (Baum & Everitt,1992; Bullitt,1990; Cattaneo & Maggi,1990; Dragunow & Robertson, 1987; Sharp, Gonzales, Sharp & Sagar, 1989). Thus, c-fos -- it is believed-- is a marker for behaviorally important neural activation. For instance, the mPOA of virgin females exposed continuously to test pups contained many more Fos-labeled cells than did the mPOA of the non responding females (Numan & Numan,1992). Fos, therefore, may be a critical marker for the neurobiological activity underlying maternal responsiveness.

Maternal behavior can be disrupted by various treatments, among them opiates. For example, endogenous opiate systems may play an inhibitory role in the control of maternal behavior (Bridges and Grimm, 1982; Grimm and Bridges 1983). The physiological actions of opioid and opioid peptides appear to be mediated by opioid receptors and involve diverse functions including, for example, analgesia, stress, feeding behavior, neuroendocrine regulation and maternal behavior (Mann, Kinsley, Bridges,1991).

Bridges and Grimm (1982) found that systemic administration of morphine (5.0 mg/kg)

blocks maternal behavior and that when rats were treated with morphine and Naloxone (an opiate antagonist) the disruptive effect of morphine was blocked. One site of opiate action appears to be the mPOA. When morphine is administered to the mPOA, disruption of maternal behavior is observed in both steroid-primed, pup-induced virgins and lactating rats (Rubin & Bridges, 1984).

Therefore, given that mPOA and neural activation are necessary for the display of maternal behavior, that morphine administration can block the response, and that Naloxone can restore it, the goal of the current investigation was to explore whether, and to what extent, morphine and/or Naloxone administration could affect c-fos activation in the mPOA. If indeed reductions occur, the data will suggest that c-fos activation may, in part, regulate morphine disruption of maternal behavior.

Experiment 1

Method

Animals. Adult nulliparous and primiparous female Sprague-Dawley rats purchased from Charles River Laboratories, Inc. (Wilmington, MA) and bred in our laboratory served as subjects. All rats were mature adults, approximately 90 days of age, weighing 200-300 g. at the beginning of the study. They were randomly assigned to one of the two groups, saline (n=5) vs. morphine (n=6). Animals were housed in 20 x 45 x 25 cm. polypropylene cages filled with loosely packed wood shavings for bedding. The animals were maintained on a 14:10 light/dark schedule (lights on:0500), in rooms kept at approximately 20^0 C. Purina rat chow and water were available ad libitum. All animals used in this study were maintained according to the standards set forth by the University of Richmond Institutional Animal Care and Use Committee and the National Institutes of Health.

Procedure. On the afternoon of postpartum day 4 a wire-mesh partition was placed in the cage in order to habituate the mother to it. The partition was used in order to avoid confounding differences among the groups. That is, morphine-treated females are less likely to engage in maternal behavior; by placing the partition in the cage, we ensured that both groups had access to

the same pup-stimulation (i.e., the females were able to see, hear and smell but not have any tactile contact with the pups). On postpartum day 5 or 6, the female was injected subcutaneously with 5.0 mg/kg morphine sulfate or saline. The pups were removed from the cage for thirty minutes. At the end of this session, they were placed in the home cage separated from the mother by a wire-mesh partition for sixty minutes.

C-Fos Protocol. At the end of the sixty minutes of pup exposure, the females were given a lethal injection of sodium pentobarbital and perfused for 1 minute with heparinized PBS, followed by 20 minutes with cold 4% paraformaldehyde. The brains were removed and post-fixed in paraformaldehyde for 60 minutes, followed by immersion in 10% sweetened PBS. Within one week of the perfusion, the brains were blocked in the coronal plane (1 mm anterior to 2 mm posterior to optic chiasm). Blocks were attached to chucks and frozen sectioned (-17^0 C) at 60 um on a Zeiss Microm cryostat and washed 6 x 10 minutes in PBS. After this step the sections were incubated for 15 minutes in 1% H2O2. , followed by 6 x 10 min. washes in PBS. The sections were processed for c-fos immunohistochemistry in 24-well microliter plates where they were exposed to primary antibody (1:20,000 in PBS + 0.4% Triton-X) (Oncogene Science, Union Dale, NY) , for one hour at room temperature and then for 48 hours at 4 C.. This was followed by 10 x 6 min. rinse in PBS, then incubation in biotin-goat anti-Rabbit IgG (1:600 in PBS + 0.4% Triton-X) (Vector Labs, Burlingame, CA) for one hour. The sections were rinsed 5 x 10 min. in PBS, and incubated in A/B (ELITE) solution for one hour. After this they were rinsed 3 x 5 min. in PBS, followed by a 3 x 5 min. rinse in sodium acetate. The sections were then incubated in Ni sulfate-DAB chromogen solution 2-10 min. Next, the tissues were rinsed again in sodium acetate 3 x 5 min., to stop the reaction. To finalize the procedure, the embedded sections were rinsed 3 x 5 min. in PBS and then the sections were mounted onto chrome-alum-coated slides. They were left under a hood overnight, dehydrated in alcohol (70%, 90%, 100%), cleared in xylene, and cover slipped using Permount or Cytoseal. Alternate sections were saved for later verification using thionin. To control for specificity of binding, negative control section (the complete procedure described above with the exception of exposure to primary antibody) were saved, and

compared to the antibody exposed sections.

Image Analysis and Quantification. C-fos cells were counted in the mPOA by investigators blind to the treatment groups. The area selected for counting was the one with the highest concentration of well defined cells, in an area 300*u*m by 300*u*m from the ventral portion of the third ventricle. For accuracy the number of cells was counted in each section of a slide, by each investigator, and a mean was drawn from both numbers. The cells were counted, at X200, on a Zeiss Axioplan microscope fitted with an Optronics color camera. The camera was interfaced to a computer with a Bravado Frame Grabber board, and the cells were counted using the Neurolucida software package (Burlington, VT.).

Statistics. Statistical analysis were performed using a oneway ANOVA with treatment or drug exposure (saline vs. morphine) as the independent variables and the number of c-fos expressions as the dependent variable.

Results and Discussion

Morphine administration, in a dose (5.0 mg/kg) capable of significantly disrupting ongoing maternal behavior was found to depress c-fos activation in the mPOA (see Figure 1 and Table 1). The control group (saline-treated females), expressed significantly high levels of c-fos in the mPOA. ($F[1, 9]=7.052$, MSE=16811.67, $p< .026$). Figure 3 displays representative sections from a saline-treated (a) and a morphine-treated (b) female demonstrating the relative activation of c-fos in the mPOA following treatment. Note the marked under-activation of c-fos in the morphine-treated female (b).

These data follows from what one would expect based on the data from Bridges & Grimm, 1982, and Grimm & Bridges, 1983, that morphine disruption of maternal behavior. In the next experiment we administered Naloxone, an opioid antagonist, which has been shown to reverse morphine disruption of maternal behavior (Grimm & Bridges,1983; Bridges & Grimm,1982). We would expect, therefore, to see a diminution in the morphine effect observed in Experiment 1.

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Experiment 2

Method

Animals. Adult nulliparous and Sprague-Dawley rats purchased from Charles River Laboratories, Inc. (Wilmington, MA) and bred in our laboratory served as subjects. All rats were mature adults, approximately 90 days of age, weighing 200-300 g. at the beginning of the study. They were randomly assigned to one of the two groups, morphine plus saline (n=3) vs. morphine plus Naloxone (n=4). Animals were housed in 20 x 45 x 25 cm. polypropylene cages filled with loosely packed wood shavings for bedding. The animals were maintained on a 14:10 light/dark schedule (lights on:0500), in rooms kept at approximately 20^0 C. Purina rat chow and water were available ad libitum. All animals used in this study were maintained according to the standards set forth by the University of Richmond Institutional Animal Care and Use Committee and the National Institutes of Health.

Procedure. As in Experiment 1, a wire-mesh partition was placed in the cage. On postpartum day 5 or 6, the female was injected subcutaneously with morphine (5.0 mg/Kg) plus

saline or morphine plus Naloxone (0.5 mg/Kg). The pups were removed from the cage for thirty minutes. The pups were returned to the cage at the end of the thirty minutes, and remained there for sixty minutes, separated from the mother by the wire mesh partition.

C-Fos Protocol. The c-fos protocol and the image analysis and quantification were identical to those of Experiment 1.

Statistics. Statistical analysis were performed using a oneway ANOVA with treatment or drug exposure (morphine plus saline vs. morphine plus Naloxone) as the independent variables and the number of c-fos expressions as the dependent variable.

Results and Discussion

Simultaneous administration of morphine plus Naloxone (an opiate antagonist), in an attempt to block the effect of morphine on the behavior, revealed that the number of activated c-fos neurons was statistically higher than the control group, compared to morphine plus saline, ($F[1,5]=23.084$, $MSE= 735.103$, $p< .005$).

As predicted, the Naloxone reversed the effect of morphine and increased the number of c-fos cells. Hence the Naloxone antagonism of morphine disruption may reflect increased c-fos activation..

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General Discussion

The central role of the mPOA in the regulation of maternal behavior in females has been demonstrated through hormone implant and lesion studies. Further investigation in the area has shown, using fos immunocytochemistry, that mPOA neurons are activated during maternal behavior (Numan & Numan, 1992). Morphine administered to lactating females, as well as to pregnancy-terminated females (who normally would display rapid onset of maternal behavior) suppresses the behavior. When the opioid antagonist, Naloxone, is administrated following the morphine, the morphine's disruption is reversed, as would be expected (Bridges & Grimm, 1982).

The present investigation provides data that further corroborates the influence of morphine in the disruption of maternal behavior. In a more specific sphere, these data illustrate the relationship between morphine disruption of maternal behavior and the activity of c-fos activation in mPOA.

Experiment 1 showed how the number of active c-fos neurons decreased, when the female was injected with morphine, in comparison to saline treated animals. Therefore, the results of experiment 1 suggest that the morphine inhibition of c-fos activation may, in part, regulate morphine disruption of maternal behavior. One possible criticism of the current pup exposure procedure (a partition placed between mother and litter) has been that one cannot accurately determine that the subsequent c-fos activation is elicited through the hypothesized sensory input stimulation; it may be only an artifact of frustration (i.e., an inability to reach the pups). Numan's data in 1995, however, support the sensory input activation. The study showed that a higher degree of fos activation was observed in the mPOA in females who were allowed to interact fully with pups, compared to those who received only olfactory, visual, and auditory input from pups. These data explain the presumption that mPOA c-fos activation is directly related to the maternal behavior instead of any stress related factor. If indeed c-fos activation correlates with the display of maternal behavior, and morphine can decrease the activation, one would expect that a narcotic antagonist that restores the behavior would counter the decrease in underlying neuronal activity.

In experiment 2, the group that received morphine plus Naloxone, showed an increase in

the number of activated c-fos cells. The morphine plus Naloxone group's mPOA neurons exhibited c-fos activation relative to, though smaller than, the number obtained for the saline group in experiment 1. This finding is to be expected due to the fact that the female rats in experiment 2 were also treated with morphine. Since saline has no effect on the opioid system, it was expected that the group treated with morphine plus saline would show a decreased number of activated cells, as indeed it did. It is also important to note that the combination of 5.0mg/kg of morphine plus 0.5mg/kg of Naloxone was sufficient to restore the behavior, but the dosage difference (0.5 vs. 5.0) may not be enough to activate the same number of c-fos cells because more morphine occupied receptors than could be displaced by Naloxone. Why were there fewer c-fos cells in Experiment 2 in morphine plus Naloxone than in Experiment 1? It may be that the number encountered in the second experiment, in the morphine plus Naloxone group, is sufficient for the display of maternal behavior.

The disruption of maternal behavior by morphine cannot be explained only on the basis of severe disruption of motor activity. The actions of morphine, at least in part, have been shown to have a direct relationship to the alteration of the perception of pup stimuli, in this case, odors. Previous data have suggested that the perception of olfactory signals, upon which the female is dependent to properly care for her young, are themselves dependent on the internal opiatergic milieu (Kinsley & Bridges,1990). Studies conducted by Kinsley and Bridges indicate that the actions of morphine are brought about, in part, through alterations in the perception of the stimuli. Maternally-behaving females, after having been treated with morphine, will display an aversion to the pup odor. Novel chemical stimuli from pups activates the central connections of both the main and accessory olfactory bulb of the lactating female (Numan, 1994). Further, treatment with the morphine antagonist, Naloxone, will reverse the effect of olfactory aversion (Kinsley, Morse, Zoumas, Corl & Billack, 1994), though, Naloxone does not, by itself, stimulate maternal behavior (Bridges & Grimm, 1982).

Fleming and Rosenblatt suggested in 1974 that virgin females typically find the odor of novel test pups aversive; therefore in order to show maternal behavior, virgin females must

overcome odor aversion. By removing olfaction, the aversive qualities of the pups are removed, and the female can now respond to those positive remaining characteristics of the pups that inspire maternal responsiveness. Because a primiparous female rat shows immediate maternal responsiveness to her own or to foster young, the hormonal (and other physiological) events of late pregnancy must operate to counteract the inhibitory effects of novel pup odors on maternal responsiveness (Numan, 1994).

Modifications in endogenous opioid activity may underlie the changes that occur in olfactory attraction from virgin and pregnant state through parturition and lactation. For instance, opiate receptors and ligand are relatively low in intact virgins. During early-to mid-pregnancy, beta-endorphin concentration rises in the hypothalamus, preoptic area (POA), and other brain regions (Kinsley & Bridges, 1990). Opioid activity is at its lowest during lactation (Bridges,1990), when maternal motivation is greatest.

The mPOA receives input from a variety of sources that may be relevant to maternal behavior control. Of these sources of neural input, probably the most important is the olfactory/vomeronasal input, which is capable of reaching the mPOA via the medial amygdala and bed nucleus of the stria terminalis. Therefore, if morphine is acting at the level of the olfactory bulb, the afferents to the mPOA may be decreased. Where morphine acts in order to disrupt maternal behavior is not clear. It seems possible, however, that by altering the perception or preference for the young, the input for the activation of the immediate early gene c-fos could be lower, therefore disrupting the display of maternal behavior.

In summary, the present investigation has demonstrated that in morphine treated females, the number of c-fos cells expressed in mPOA is reduced in comparision with saline treated females. Further, in morphine plus Naloxone treated females the number of c-fos neurons was greater than the number of c-fos neurons in females treated with morphine plus saline. Because of the effects of morphine on the perception of the pups, it is hypothesized that the blocking of maternal behavior could be a consequence of the c-fos activation. Stimuli coming from other areas of the brain involved in the regulation of maternal behavior may be decreasing the activation

of the c-fos neurons, therefore reducing the display of the behavior.

Though further studies need to be conducted, the findings of this study should provide an opportunity to develop a clearer understanding of the underlying neuronal activity that controls behavior in general, and maternal behavior in particular. It is important to stress the potential for the fos technique to elucidate the diverse areas and many mechanisms of the brain devoted to the regulation of such an important behavior for the assurance of the survival of the individual and the species.

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Figure Caption

Figure 1. Number of c-fos neurons in mPOA following morphine or saline treatment.

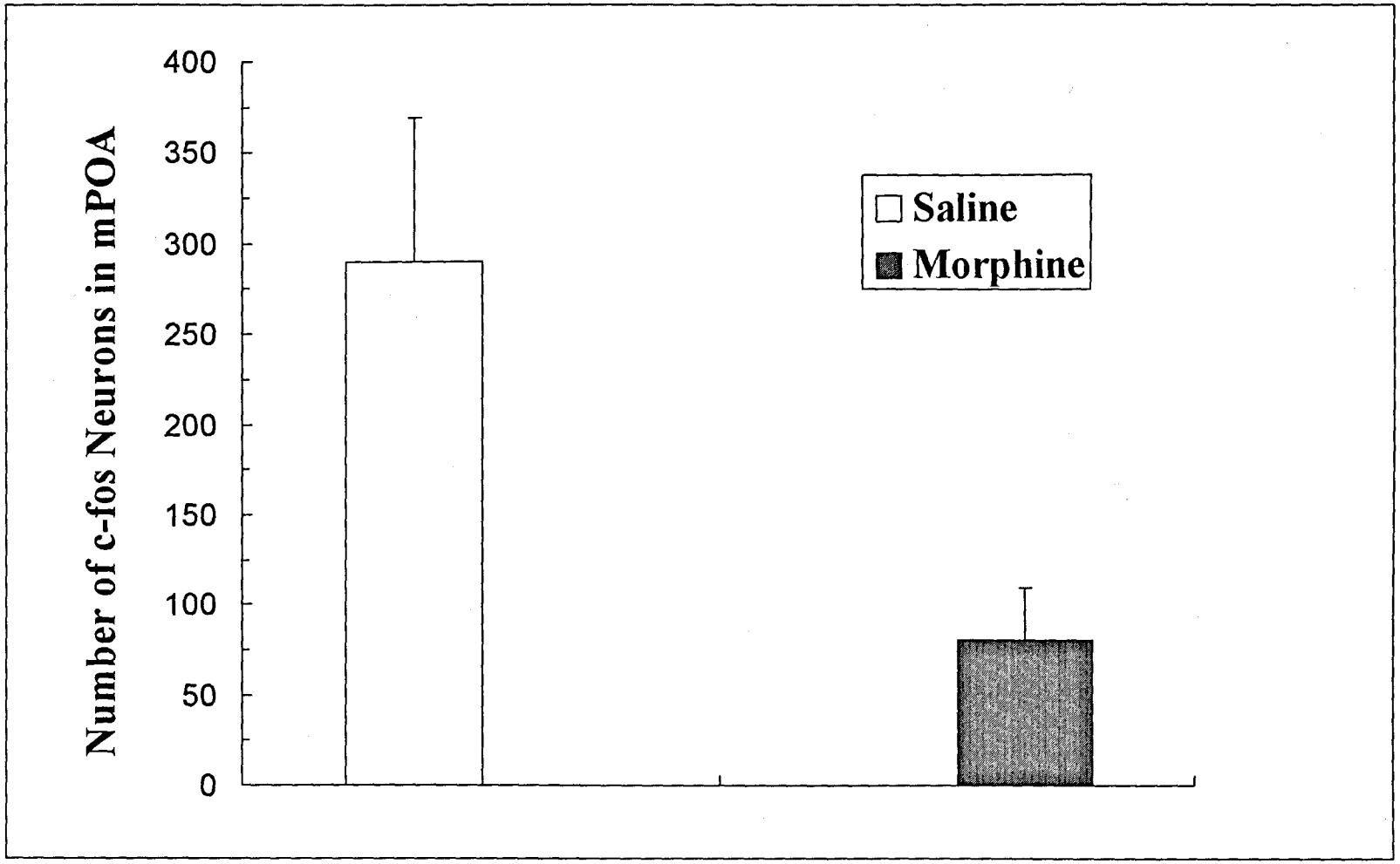
Number of c-fos Neurons in mPOA
400
350
300
250
200
150
100
50
0
Saline
Morphine

Table 1.

Mean (+ SD) C-fos Neurons in mPOA

Treatment	n	Mean	SEM
Morphine	6	81.0	29
Saline	5	289.5	80

Figure Caption

Figure 2. Photomicrograph of c-fos immunoreactive neurons in medial preoptic area of saline- and morphine-treated females. a: Saline-treated; b: Morphine-treated. F: fos-immunoreactive neuron; IIIV: third ventricle. Bar = Approximately 150 um.

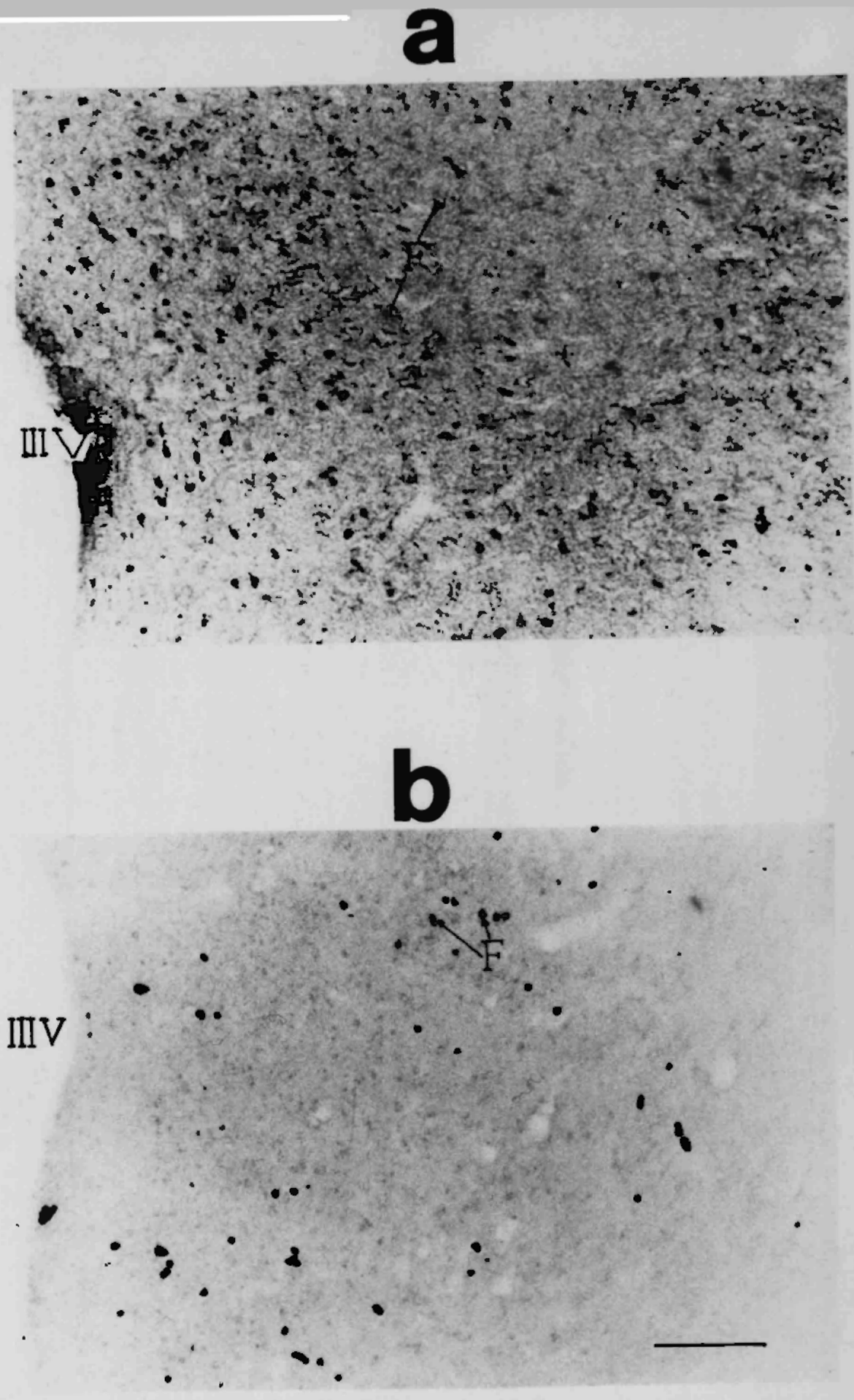
a
IIIV
b
F
IIIV

Figure Caption

Figure 3. Number of C-Fos neurons in mPOA following morphine + Naloxone or morphine + saline treatment.

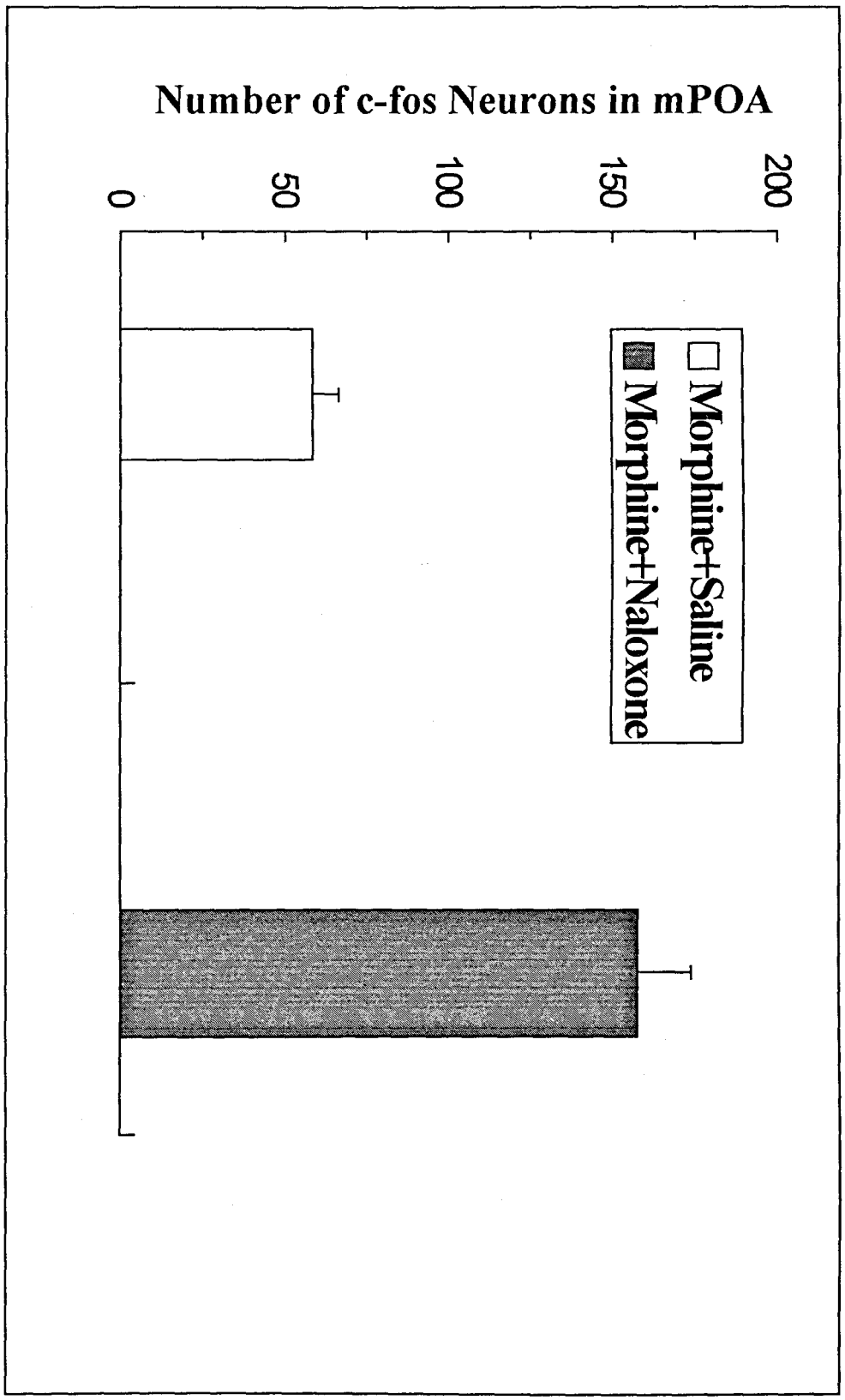
Number of c-fos Neurons in mPOA
0
50
100
150
200
Morphine+Saline
Morphine+Naloxone

Table 2.

Mean (+ SD) C-fos Neurons under Morphine + Naloxone or Morphine + Saline in mPOA.

Treatment	n	Mean	SEM
Morphine + Naloxone	4	157.5	16.5
Morphine + Saline	3	58.0	8.3

Biography

Graciela Stafisso-Sandoz was born on January 23, 1967, in General Roca, Rio Negro, Argentina. She received her Licenciatura in Psychology from the National University of Cordoba, Argentina in 1990. Graciela received a pre-doctoral fellowship from the Medical College of Virginia in January, 1995, under which she is currently working.