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Behavioral and immunological effects of substance P in female and male mice

Raquel M. Teixeira[a], Filipe S. Duarte[b], Thereza C.M. De Lima[b,*]

[a]Department of Clinical Analysis, CCS, Universidade Federal de Santa Catarina, 82, Florianópolis, SC 88015-420, Brazil
[b]Department of Pharmacology, CCB, Universidade Federal de Santa Catarina, Rua Ferreira Lima, 82, Florianópolis, SC 88015-420, Brazil

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Abstract

This study investigated whether the central injection of substance P (SP) promotes differential behavioral [elevated plus-maze (EPM), open-field and hole-board tests] or immunological effects [peripheral blood lymphocyte subsets and nitric oxide (NO) produced by macrophages] in male and female mice. The percentage of time spent on open arms was significantly reduced by SP treatment in female (87%) and male mice (68%). A similar effect was observed in the percentage of entries into open arms (55% and 30%, respectively), as well as in the head-dipping parameter (63% and 27%, respectively), suggesting that SP promoted an anxiogenic-like profile in both sexes, which are detectable only in the plus-maze test. Female mice showed a significant decrease (20%) in the absolute number of lymphocytes and leukocytes comparing with control group and male SP-treated animals (4% of reduction), although only SP-treated males presented an increase (100%) in NO production by macrophages. Thus, our data showed no apparent statistical difference on the anxiogenic-like profile of action induced by SP between female and male mice; notwithstanding, SP, depending on the immune parameter evaluated, differentially influenced both sexes. The implications of these findings, as well as the putative participation of proinflammatory cytokines in this phenomenon, are discussed.

Keywords: Anxiety; Sex; Lymphocyte distribution; Macrophages; Neurokinin; Nitric oxide; Plus maze

1. Introduction

The neurokinins are a family of peptides that includes substance P (SP), neurokinin A, neurokinin B and the neuropeptides γ and K. Neurokinins are widely distributed in the central and peripheral nervous system (Otsuka and Yoshioka, 1993) and are involved in various physiological mechanisms, including neurotransmission, vasodilatation, smooth muscle contraction (Iversen, 1989), as well as CNS disorders, such as Parkinson's (Cramer et al., 1989) and Huntington's diseases (Kanazawa et al., 1977), schizophrenia (Powell et al., 1973), anxiety (Aguiar and Brandão, 1996; Teixeira et al., 1996; File, 1997) and depression (Kramer et al., 1998; Hökfelt et al., 2000; Papp et al., 2000). Likewise, neurokinins, particularly SP, have been reported to modulate immunological responses, whilst specific cell surface receptors for SP have been demonstrated in lymphocytes and other immunological active leukocytes (Payan et al., 1986; McGillis et al., 1987; Eglezos et al., 1991; Maggi, 1997; Zhang et al., 2000). Actually, there is increasing evidence that SP plays a role in neuro-immunomodulation, i.e., in the control and regulation of the immune response by the central and peripheral nervous systems, which could be influenced by hormonal changes (Comsa et al., 1982), although the actual relevance and importance of the neurokinins in immunomodulation is uncertain (Severini et al., 2002).

The central nervous and the immune systems present a bidirectional influence (Ader et al., 2001), and neurokinins are known to be involved in the modulation of both systems (Maggi, 1997; Raffa, 1998). Thus, the general purpose of the present study was to analyze whether the central injection of SP promotes differential behavioral and immunological effects in male and female

* Corresponding author. Tel.: +55-48-222-2518; fax: +55-48-222-4164.
E-mail address: thereza@farmaco.ufsc.br (T.C.M. De Lima).

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mice and to investigate a putative correlation between these effects.

2. Materials and methods

2.1. Animals and procedure

Adult 3-month-old male and female Swiss mice, weighing 25–30 g, provided by the Animal House of the Universidade Federal de Santa Catarina (Florianópolis, Brazil), were housed in groups of 20 animals per cage and kept in a room with controlled temperature (22 ± 2 °C) and a 12-h light/dark cycle (lights on at 7:00 a.m.), with food and water ad libitum, except during the experiments. The animals were allowed to adapt to the laboratory conditions for at least 1 week before the beginning of the behavioral experiments. Each animal was used only once. All experiments were conducted in accordance with international standards of animal welfare recommended by Brazilian Society of Neuroscience and Behavior (1992), and the experimental protocols were approved by the Universidade Federal de Santa Catarina Animal Care and Use Committee (#23080.001156/2001-50/UFSC). This study used a minimum number of animals (7–13 for in vivo experiments and 3–4 for in/ex vivo experiments) and duration of observation required to obtain consistent data.

After the conclusion of experiments, vaginal smears were analyzed by light microscopy for identification of the estrous phase (Hoar and Hickman, 1975; Reddy and Kulkarni, 1999). Because of previous reports showing no significant differences in behavior between female at diestrus and males, and to avoid the influence of ovarian hormones fluctuations across the estrous cycle, only female mice in the diestrus (90% of total) were used in the experiments (Diaz-Véliz et al., 1989). This phase is characterized by (1) a low level of ovarian steroids that is associated with the lowest level of baseline exploration in the plus-maze and (2) a linear dose-response to diazepam, the reference anxiolytic drug (Bitran and Dowd, 1996), a pattern also observed in males. The other 10% of females were in different hormonal phases, mainly proestrus, and basically, the animals present the same profile of drug effect, but a greater variance, when compared with animals in diestrus (Rocha et al., 1998).

2.2. Drugs and solutions

Phosphate-buffered saline (PBS) from Sigma (St. Louis, MO, USA) was used as control solution. SP was purchased from Peninsula Laboratories (Belmont, USA) and diluted daily to the desired concentrations in PBS (pH 7.4). LPS from *Salmonella typhosa* (0901) was obtained from Difco (Detroit, MI, USA); murine recombinant interferon-γ was purchased from Genzyme (Cambridge, MA, USA). Fetal calf serum (FCS), and DMEM were obtained from GIBCO (São Paulo, SP, Brazil).

2.3. Drug treatments

Intracerebroventricular injections were given under light ether anesthesia (i.e., just sufficient for loss of the postural reflex), as described by Laursen and Belknap (1987) and used previously by our group (Teixeira et al., 1996; Ribeiro and De Lima, 1998, 2002). In brief, a 27-gauge needle attached to a 10-μl Hamilton syringe was inserted perpendicularly 3 mm deep through the skull, at a position 2 mm lateral from the midline on the line drawn through the anterior base of the ears. Each animal received only one intracerebroventricular injection. SP was injected at 10 pmol, an effective dose, as determined by our previous studies (Teixeira et al., 1996; Baretta et al., 2001; Ribeiro and De Lima, 2002), in a volume of 2 μl given over 30 s, the needle remaining for a further 30 s to allow the drug to spread. Control mice were similarly treated with vehicle (PBS). After the injection and recovery of the righting reflex (1 min later), the animals underwent behavioral evaluation (5 min), followed 1 h later (a period determined from preliminary studies, Teixeira and De Lima, 2000) by the collection of blood samples. Upon the termination of the experiment, each mouse was decapitated, and its brain examined a fresco. Results from mice presenting cannula misplacement or any signs of cerebral hemorrhage were discarded from the statistical analysis (less than 5% of the animals overall). In the plus-maze test and for the blood collection, naive animals were also used to verify the influence of intracerebroventricular injection procedure per se.

2.4. Anxiety evaluation

2.4.1. Elevated plus-maze test

The putative anxiogenic activity of the neuropeptide SP was assessed using the elevated plus-maze test (EPM), as adapted for the mouse by Lister (1987). This test is based on the natural aversion of rodents for open spaces. The EPM was made of clear Plexiglas and consisted of two opposed open arms (30 × 5 × 0.25 cm) and two opposed closed arms (30 × 5 × 15 cm), all facing a central platform (5 × 5 cm), elevated 45 cm from the floor. The apparatus was placed in a small closed room lit by a 15-W red light. Right after the recovery of righting reflex, each mouse was placed on the central platform, facing a closed arm, and observed for a 5-min period. The frequencies of entries into either open or enclosed arms, as well as the times spent in each arm type, were recorded (in s). An entry was scored as such only when the animal placed all four limbs into any given arm. The incidences of ethological parameters, such as grooming, unprotected head dipping (i.e., an exploratory forward head/shoulder movement over the side an open arm of the maze directed down towards the floor), protected stretch-attend postures (SAP; i.e., when animal stretches forward and retracts to original position without actually

walking forward, a behavior which occurs in or from the relatively security of the closed arms or central platform of the maze) and defecation (number of fecal boli), were also recorded (Cole and Rodgers, 1994). The use of ethological observation increases the sensitivity of EPM as an experimental model of anxiety, and anxiolytic drugs generally increase head dipping and decrease SAPs, besides affecting other behavioral parameters (Rodgers et al., 1997).

The ratios "time spent in the open arms/time spent in all (i.e., open and closed) arms" and "frequency of entries into open arms/total entries into all arms" were calculated and multiplied by 100 to yield the percentages of time spent in and of frequency of entries into open arms, respectively. Both parameters are considered to reflect fear-induced inhibition from entering the open arms and can be related to the anxiety level experienced by the animal. Drugs with anxiolytic-like activity usually increase the time spent in and/or frequency of entries into open arms, whereas the reverse holds true for anxiogenic-like drugs. Furthermore, the number of entries into closed arms was used as an index of general activity (Rodgers and Dalvi, 1997).

2.4.2. Open field

Mice were tested for their spontaneous locomotor activity after the intracerebroventricular treatment and the recovery of righting reflex. The test was carried out in a transparent Plexiglas arena ($30 \times 30 \times 15$ cm). The apparatus floor (black Plexiglas) was divided into nine equal squares, and the number of squares crossed, number of rearing and grooming behaviors, besides the defecation, in 5 min were recorded (Archer, 1975). Multifactorial analysis studies have demonstrated that exploration behaviors in the inner part of the open field is a reliable index of anxiolytic-like action, whereas the outer and the total locomotion reflects the locomotion in novel environments (Ramos et al., 1997).

2.4.3. Hole board

Spontaneous locomotor activity, as well as the exploratory activity, were recorded in a hole-board apparatus ($35 \times 35 \times 15$ cm) for 5 min. The walls were made of clear Plexiglas with a black floor divided in nine equal squares with a hole (2 cm diameter) in the middle of each square, and the apparatus was elevated 10 cm from the floor. After the intracerebroventricular treatment and the recovery of righting reflex, the animals were placed on the central square of the arena, and the following behavioral responses were recorded for 5 min: number of crossings (i.e., number of floor sections transversed), number of rearing, grooming behavior and number of nose poking (using as nose-poking criteria when animals insert their heads in one hole until the level of their ears). An increase in the number of nose poking is positively correlated to an enhanced locomotor activity or an anxiolytic-like profile of action, and the reverse holds true for a sedative effect (File and Wardill, 1975).

The experimental devices were thoroughly cleaned with 10% ethanol solution before placing the animals to obviate possible biasing effects due to odor clues left by previous mice. To minimize the influence of possible circadian changes on behavior, control and experimental animals were alternated for observation during the same period of day (7:00 a.m. and 1:00 p.m.; Scheme 1).

2.5. Immunological evaluation

2.5.1. White blood cell (WBC) counting

Blood samples were obtained through orbital bleeding as described by Harkness et al. (1995). Under ether anesthesia, mice were placed on their side on a flat surface. A small-bore Pasteur pipette was inserted into the medial corner of the eye and rotated into the infraorbital venous vessels. Blood from the ruptured sinus flowed through the pipette and was collected in tubes coated with 5% EDTA. When the blood ceases to flow, the tube was withdrawn. The differential WBC count was obtained using a Coulter counter (Coulter Electronics, Hialeah, FL, USA).

2.5.2. Nitric oxide (NO) production by macrophages

The method used to study macrophage activation was based on that described by Fernandes and Assreuy (1997).

EXPERIMENTAL GROUP	*IN VIVO* EXPERIMENTAL PROCEDURES	*EX VIVO / IN VITRO* EXPERIMENTAL PROCEDURES
Naive	-	-
Control	Open-field or Hole-board	Macrophages activation*
SP 10 pmol	Open-field or Hole-board	Macrophages activation*
Naive	Elevated plus-maze	Blood collection
Control	Elevated plus-maze	Blood collection
SP 10 pmol	Elevated plus-maze	Blood collection

Scheme 1. Experimental design and animal groups. * Macrophage activation was induced by adding IFN-γ (10%) and LPS (1 μg/ml) to the macrophages.

Murine peritoneal macrophages were harvested from Swiss mice of both sexes, which had been injected 1 h before with SP 10 pmol icv. Cells were plated onto 24-well plates at 10^6 cells/well, incubated for 2 h in 5% CO_2 at 37° C and washed with fresh medium to remove the nonadherent cells. Assays were carried out in DMEM supplement with 10% FCS, 20 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Macrophage activation was induced by adding IFN-γ (10%) plus LPS (1 μg/ml) to the macrophages, and NO production was assessed by nitrite concentrations in the medium using the Griess reaction (Schmidt et al., 1989). Briefly, 100 μl Griess reagent (equal parts of 1% w/v sulfanilamide in 5% H_3PO_4 and 0.1% w/v naphthylethylene diamine dihydrochloride in water) was added to 100 μl of each supernatant in a 96-well plate. The plates were read using a plate reader at 540 nm against a standard curve of sodium nitrite in culture medium. Three identical replications were done in accordance to GLP protocols and qualify assurance methods.

2.6. Statistical analysis

All data presented are expressed as the means ± S.E.M., and each value reflects the mean of 10 animals per group. The means were compared by a two-way analysis of variance (ANOVA), followed by Newman-Keuls' multiple comparisons test, using GraphPad Prism Version 3.0. Differences were considered significant when $P < .05$.

3. Results

3.1. EPM test

As shown in Fig. 1A, intracerebroventricular administration of SP, at the anxiogenic dose of 10 pmol, caused a significant decrease in the time spent into the open arms in both male and female mice without showing a sex difference in control or treated mice [$F(5,44) = 12.661$, $P < .05$]. Concerning the percentage of entries on open arms, the control groups of both sexes were not different, although both sexes presented a reduction on this parameter in relation to their control values; this reduction did not reach statistical significance (Fig. 1B). Entries on enclosed arms were not affected by SP treatment in both sexes, as depicted in Fig. 1C. Naive animals showed no significant difference between males and females in all behavioral parameters registered, as well as when compared with control groups within the same gender. Nevertheless, the time spent into the open arms of the male control group was significantly enhanced in comparison with naive male animals (Fig 1A).

ANOVA revealed sex differences some ethological parameters displayed in mice centrally treated with SP in the plus-maze test, such as in head dipping, which was

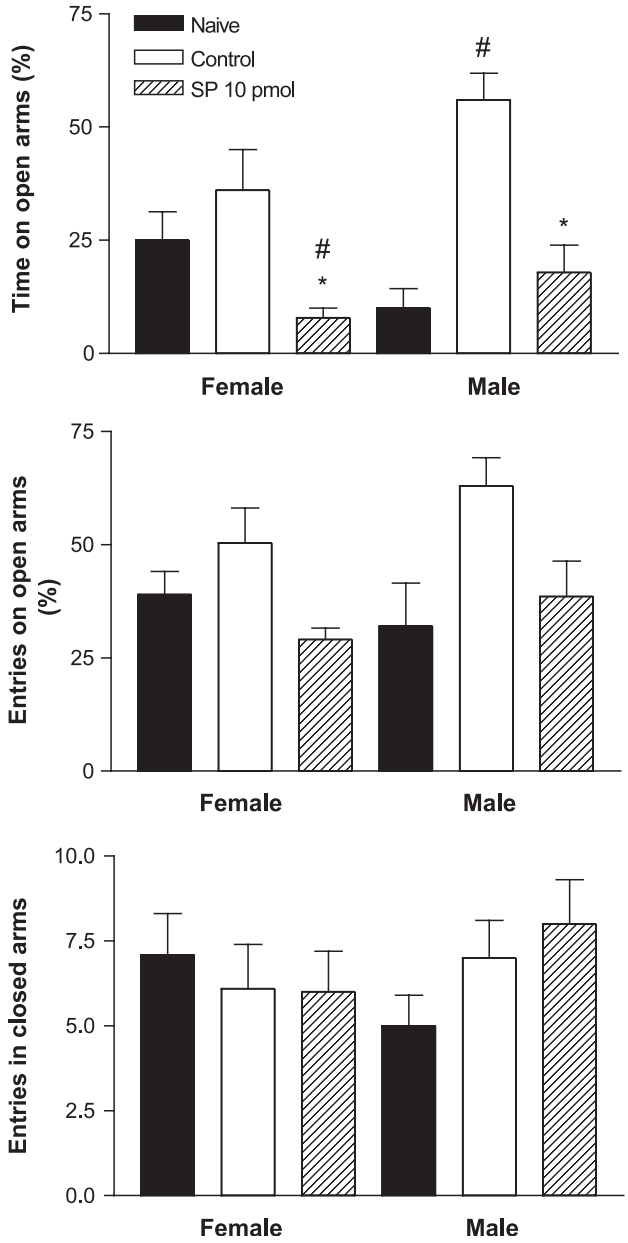

Fig. 1. Anxiogenic-like effect of SP (10 pmol) on the behavior of male and female mice evaluated in the EPM test. All behaviors were recorded for 5 min. Each value represents the mean ± S.E.M. of 7–10 animals. $^*P < .05$, as compared with the respective control group; $^{\#}P < .05$, as compared with the respective naive group (two-way ANOVA followed by Newman-Keuls' test).

significantly reduced in female SP-treated mice compared with its control group [$F(1,29) = 7.462$, $P < .05$] and to male SP-treated mice [$F(1,29) = 7.263$, $P < .05$; interaction: $F(1,29) = 0.531$, $P > .05$]. The SAPs were also significantly increased in SP-treated females only when compared with its control group [$F(1,29) = 7.719$, $P < .05$]. SP-treated male mice presented a similar profile of action, but this effect did not reach statistical significance [$F(1,29) = 0.270$, $P > .05$; interaction: $F(1,29) = 0.809$, $P > .05$]. No statistical differences were observed in the other parameters such as

Table 1
Effects of injections of SP into the lateral ventricle in the behavioral parameters of the open-field (OF) and the hole-board (HB) tests, in a 5-min session in mice

Treatment	Sex	OF				HB			
		Locomotion	Rearing	Grooming	Defecation	Locomotion	Rearing	Grooming	Nose poking
Control	Male	40.25 ± 1.15	22.83 ± 1.21	5.33 ± 0.75	1.08 ± 0.34	27.83 ± 0.83	16.25 ± 0.87	2.08 ± 0.47	8.58 ± 0.77
	Female	43.00 ± 2.67	19.40 ± 1.76	3.75 ± 0.99	0.75 ± 0.56	30.9 ± 2.23	18.73 ± 0.53	1.21 ± 0.49	9.40 ± 1.03
SP	Male	29.67 * ± 1.36	17.83 ± 1.99	3.42 ± 0.92	1.75 ± 0.54	28.5 ± 1.74	17.58 ± 1.08	2.17 ± 0.39	8.00 ± 0.97
	Female	30.67 * ± 2.03	17.85 ± 1.18	3.01 ± 0.71	0.88 ± 0.83	21.56 ± 4.35	17.75 ± 3.48	1.25 ± 0.49	7.88 ± 0.97

Each value represents the mean ± S.E.M. of 8–10 animals.
* $P<.05$ as compared with the respective control group (two-way ANOVA followed by Newman-Keuls' test).

grooming [$F(1,29)=2.378$] and rearing [$F(1,29)=0.779$]. Moreover, naive animals showed no sex difference in the ethological parameters registered in the EPM test (data not shown).

3.2. Open-field test

Central treatment with SP 10 pmol were able to diminish locomotion in both sexes without altering rearing, grooming behavior or defecation, as shown in Table 1.

3.3. Hole-board test

SP (10 pmol) intracerebroventricularly injected was unable to significantly change the parameters recorded at the hole board, such as locomotion, rearing, grooming or the number of nose poking (Table 1).

3.4. WBC counting

Changes in total leukocytes were characterized by a significant enhancement only in naive male mice 1 h after the plus-maze test compared with naive female animals [$F(5,61)=6.572$, $P<.05$] but not between the control groups or after intracerebroventricular injection of 10 pmol of SP in male and female animals (Fig. 2A). The same profile of action was observed for the lymphocyte cells, with only naive male mice presenting an increase in this parameter in comparison to the naive female group [$F(5,61)= 7.061$, $P<.05$], but there was no observed difference between the female and male mice of the control or SP-treated groups, as shown in Fig. 2C. The number of neutrophils (Fig. 2B), on the other hand, was not affected by any factor, i.e., sex or SP treatment [$F(5,61)=0.8351$, $P>.05$].

3.5. NO production by macrophages

Activation of mouse peritoneal macrophages with LPS and IFN-γ induced NO synthase activity, which results in the production of NO as assessed by the increased nitrite levels in the supernatants, as depicted in Fig. 3. Ex vivo SP treatment promoted per se an enhancement of NO production in male [$F(3,20)=17.012$, $P<.05$] but not in female mice [$F(3,20)=0.990$; $P.05$] without modifying the levels of NO produced by macrophages activated by LPS plus IFN-γ.

4. Discussion

In the present study, we have systematically compared the effects induced by SP in female and male mice in behavioral and immunological parameters. In the plus-maze test, we have observed the same profile of action in both sexes, i.e., an anxiogenic-like profile of action for SP, whereas in the immunological evaluation, female and male mice presented differential effects which were affected by SP treatment depending on the parameter studied.

Anxiety is diagnosed far more often in women than in men (*DSM-IV*, 1994), and this finding has been underestimated in several preclinical and even clinical studies. Thus, there is a clear need for female representation in anxiety studies, as emphasized by Blanchard et al. (1995). The value of using both sexes in research is that general brain mechanisms underlying behavior are frequently highlighted by the differences between sexes. Sex differences are usually the result of complex interactions between the simple, direct, temporary influences of fluctuating gonadal hormones, known as activational effects, and the permanent organizational influences of steroid hormones (Kelly et al., 1999). Sexual differentiation of the mammalian brain is known to occur as a result of exposure to steroid hormones during critical periods of perinatal development. Functional differences, such as differences in neuronal concentration or expression of neurotransmitters, enzymes or receptors, may also underlie the sex-related differences manifested in adult rat brains (Searles et al., 2000). It is well documented that adult female rats show greater ambulatory and rearing activity and defecate less than do males in the open-field test (Blizard et al., 1975; Masur et al., 1980), although we have not observed any difference between the male and female mice evaluated in this test and in the hole board in the present study. In the EPM test, Johnston and File (1991) showed that female rats present reduced aversion for the open arms when compared with male rats and also tended to make more total arm entries, indicating a higher overall level of activity in this test. Moreover, estrogen has positive effects on activity levels and emotional reactivity

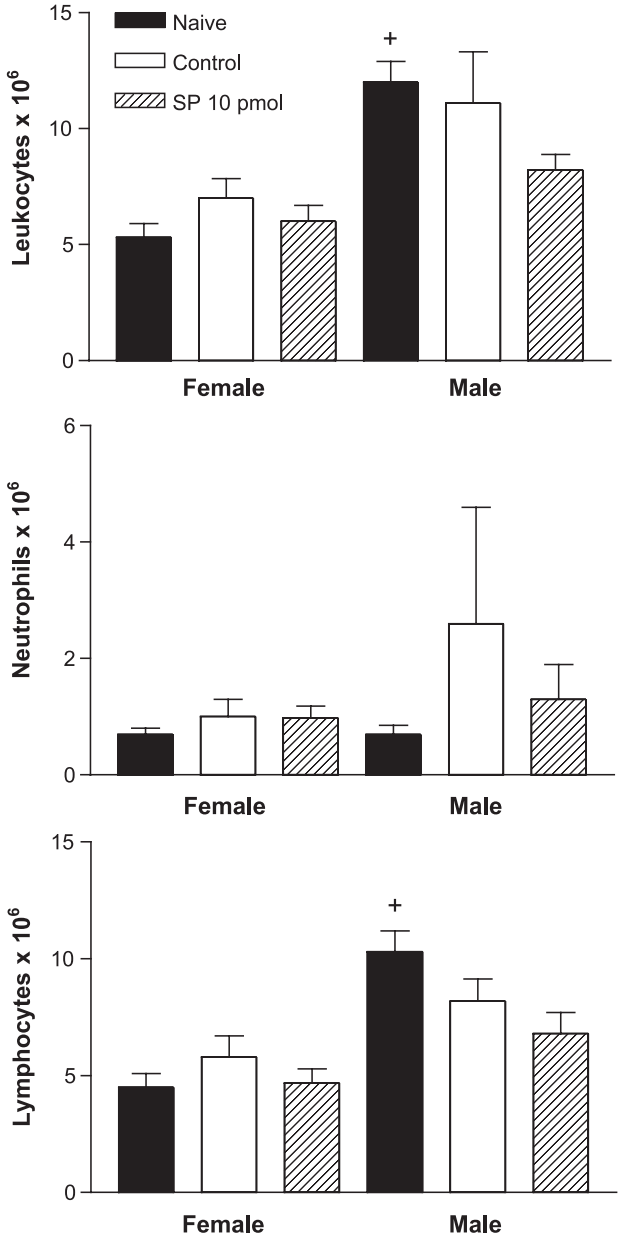

Fig. 2. Changes in peripheral blood leukocyte distribution induced by SP (10 pmol) centrally injected in female and male mice. Each value represents the mean ± S.E.M. of 9–13 animals. $^{+}P<.05$, to indicate sexes differences in the same group treatment (two-way ANOVA followed by Newman-Keuls' test).

in both humans and rats, as a recently reviewed by Morgan and Pfaff (2002), who also showed a similar profile of action in mice. In the last years, we have been studying the anxiogenic-like effects of SP and other neurokinins in male (Teixeira et al., 1996; Ribeiro and De Lima, 1998; Ribeiro et al., 1999) and female mice (Baretta et al., 2001; Ribeiro and De Lima, 2002), with the assumption that there is no sex-related difference in the behavioral response to these neuropeptides. However, in our foregoing studies on the anxiogenic-like effect of SP, mainly represented by a reduction in open-arms exploration, adult females seemed to present a reduced aversion to the open arms of the plus-maze in comparison with our studies with males, a behavioral pattern previously described for rats (Johnston and File, 1991). Hence, this fact could indeed facilitate the observation of anxiogenic effects of this neuropeptide and their potentiation by specific drug treatments. Nevertheless, we have never systematically compared female and male subjects. Thus, in the present study, comparing the effects induced by SP in female and male mice, we have observed the same profile of action in both sexes, as shown by a reduction on the open-arm parameters, as well as in the head-dipping measure, whilst an increase in SAPs, although these effects seemed to be more evident in females.

As previously observed, this anxiogenic-like effect appeared to be specific and not merely a consequence of locomotor changes, since the SP did not alter the entries on the enclosed arms, a measure loaded to motor activity (Rodgers and Dalvi, 1997). These findings are supported by some studies that have failed to detect any significant sex differences in plus-maze behavior in rodents (Nomikos and Spyraki, 1988; Frick et al., 2000). Furthermore, the distribution and functions of regulatory peptides in the central nervous system have been widely studied over the last few decades, with most studies being based on the assumption that there are no sex differences in this regard. However, Rugarn et al. (1999) found sex differences in the expression of several neuropeptides, including neuropeptide Y and galanin, particularly in the hippocampus, striatum, hypothalamus and pituitary, but not for CGRP, SP and NKA, a

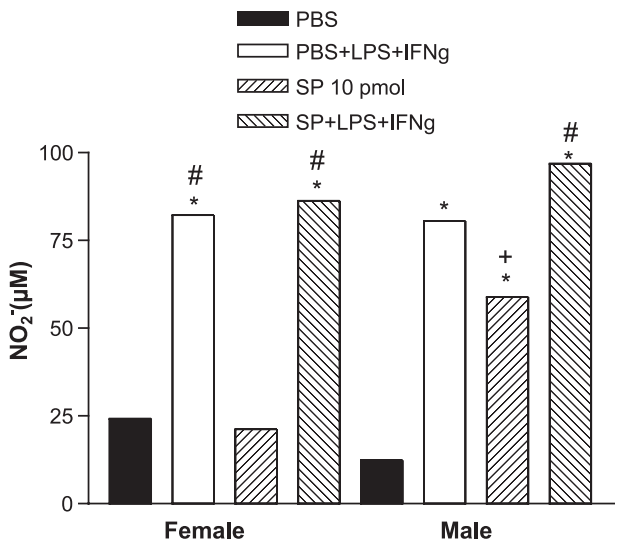

Fig. 3. Effect of macrophage activation induced by adding LPS (1 mg/ml) plus IFN-γ (10%) to macrophages 1 h after central SP treatment (10 pmol icv) in female and male mice as evaluated by nitrite production. Each value represents the mean ± S.E.M. of 3–4 animals in triplicate. $^{*}P<.05$, as compared with the respective control group; $^{\#}P<.05$ as compared with SP-treated groups; $^{+}P<.05$, to indicate sexes differences in the same group treatment (two-way ANOVA followed by Newman-Keuls' test).

finding which could explain our present results in the EPM, where no sex differences were observed. However, levels of SP, its precursors and receptors seem to vary during the estrous cycle in several brain regions (Duval et al., 1996; Gatreau et al., 1997), and perhaps, it is worthy to further investigate this aspect using ovariectomized animals treated with exogenous steroids, although the behavior among the different estral phases seemed to be similar, as we have observed in a previous study (Rocha et al., 1998).

Naive animals showed no sex difference, as well as control and SP-treated groups, although their levels of exploration of the plus-maze arms were lower than that of control animals submitted to the intracerebroventricular procedure. On this regard, handling, sham injection and physiological solution injection produced a significant reduction of the open-arms exploration, indicating a behavioral profile of stressed and anxious animals (Lapin, 1995).

On the other hand, the open-field test was unable to detect any effect induced by SP, except for a reduction in locomotor activity, a measure loading to spontaneous exploratory behavior (Cruz et al., 1994; Ramos et al., 1997). The hole-board test, another putative validated model for evaluating experimental anxiety (File and Wardill, 1975), was also not adequate to detect any behavioral effect induced by SP. The reason for this lack of sensitivity of both tests to detect the behavioral effects produced by SP could be attributed to the fact that different tests may reflect or model different aspects of anxiety, as revealed by factor analysis studies (Belzung and La Pape, 1994; File, 1991). In this regard, Lister (1987) revealed that parameters recorded in several anxiety models (e.g., EPM, social interaction, hole board and Vogel conflict) produced distinct anxiety factors, thereby indicating that they reflect different emotional states. Moreover, it does not seem likely that "simple" exploratory tests, such as open field and hole board, could consistently satisfy the criteria of sensitivity and selectivity for assessing anxiolytic/anxiogenic drug actions (Treit, 1985). Locomotion in rodents is a motor behavior associated with spatial exploration (Kelley et al., 1993), being one of the most prominent in spontaneous behavioral repertoire of mice and influenced by other factors besides the fear promoted by the novelty of the equipment (Fonseca et al., 2002).

On the other hand, in humans, as well as in laboratory animals, psychological characteristics and behavioral traits play an important role in the pathogenesis of many diseases, including affective and immune disorders (Dantzer and Kelly, 1989). Our results also pointed to a clear difference in peripheral blood elements of female mice. Overall, females presented a significant reduction on leukocyte and lymphocyte numbers, which could suggest a natural decrease in the immune response, without being sensible to the anxiogenic challenge provided by SP. Female naive animals presented a similar profile, i.e., a leucopenia and a lymphopenia in parallel to neutrophilia, compared with male naive mice.

In sharp contrast, ex vivo treatment with SP promoted an enhancement of NO production by macrophages only in male mice. Macrophage activation is followed by several changes in cellular metabolism, including increased release of cytokines, reactive oxygen intermediates and NO, which can be affected by neuropeptides, among other neurotransmitters (Eglezos et al., 1991; Bernstein, 1991; Schedlowski and Benschop, 1999). Thus, it seems that SP centrally injected promotes a clear-cut effect on immunological function, which is sex different and dependent on the parameter evaluated.

The exact site and underlying mechanisms at which SP acts to achieve their immunological and behavioral effects is yet unknown, albeit we have observed the involvement of NO (Baretta et al., 2001) and GABA (Ribeiro and De Lima, 2002) in the anxiogenic-like effect induced by SP. Thus, we are presently investigating the role of cytokines in the behavioral and immunological effects of SP, since cytokines are multifunctional pleiotropic proteins that play crucial roles in cell-to-cell communication and cellular activation and are involved not only in immune mechanisms but also in a variety of physiological and pathological processes, including the mood modulation (Turrin and Plata-Salaman, 2000). Likewise, cytokines are released by activated macrophages and/or lymphocytes under stressful situations (Palermo-Neto et al., 2003), and neuropeptides, such as SP, are involved in stress modulation (Papp et al., 2000), as well as in the release of cytokines (Palma and Manzini, 1998). Cytokines, on its turn, interacting with their receptors can produce NO in activated cells (Nelson et al., 1997).

In summary, the present study did show no sexual difference in the behavioral effects of SP, as evaluated in the plus-maze test, although females seem to present more evident responses than males did. Females also presented lymphopenia and leucopenia compared with male mice, but this blood parameter was not altered by SP. Males, on the other hand, showed an increase on NO production by macrophages when challenged by SP central treatment. These sexual differences could be due to differences in the neuropeptide activity, which may have implications for immune but not mood regulation, a hypothesis that deserves to be further examined. Another possibility to explain these findings might be related to cytokines modulation, which can be changed by neurokinins (Maggi, 1997). Actually, it may be conceivable that neurokinin administration may lead to mood disturbances and emotional reactions, besides immunological responses, in subjects sensitive to physiological changes in sex steroid concentrations. In this respect, it is interesting to note that anxiety and autoimmune diseases are more frequent in females. The exact underlying mechanism of action for the effects here described remains to be elucidated, but the present findings are important because they point to a potential role for NK_1 antagonists in the therapy of these disorders, a possibility that is presently under investigation in several laboratories.

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