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ORIGINAL INVESTIGATION

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Evaluation of the anxiolytic-like effect of NKP608, a NK1-receptor antagonist, in two rat strains that differ in anxiety-related behaviors

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Abstract *Rationale:* NKP608, a selective NK1 receptor antagonist, has been shown to produce anxiolytic-like effects in rodents tested in different anxiety models. However, most of these findings are based on social behaviors and, to our knowledge, there is no report concerning the effects of NKP608 in the elevated plus-maze (EPM) and the open field (OF), two classical models of anxiety/emotionality. Moreover, this compound has never been tested in rodent strains that display contrasting levels of anxiety-related behaviors. *Objectives:* To investigate the anxiolytic-like effects of NKP608 in Lewis and SHR inbred rats, proposed as a genetic model of anxiety for showing high and low indices of anxiety, respectively. *Methods:* Lewis and SHR rats of both sexes were tested in the EPM and OF tests following acute administration of NKP608 (0.003, 0.03 or 0.3 mg/kg) or chlordiazepoxide (CDZ, 5 mg/kg). Measures of approach/avoidance towards the open arms of the EPM and the central area of the OF were used as indices of anxiety. *Results:* All doses of NKP608 produced anxiolytic-like effects, similar to those of CDZ, in SHR males tested in the OF but not in the EPM. Conversely, this compound had a partial anxiolytic effect in LEW males (and, to a lower degree, in SHR females) in the EPM, but not in the OF. LEW females were unaffected following all pharmacological treatments. *Conclusions:* These findings indicate that the anxiety-related effects of NKP608 are strain-, sex- and test-dependent. Moreover, the present data confirm and extend the therapeutic potential of NK1 receptor antagonists for the treatment of anxiety.

Keywords NK-1 receptor antagonist · Spontaneously hypertensive rats · Lewis · Anxiety · Open field · Elevated plus-maze

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Introduction

Substance P (SP), a neuropeptide belonging to the tachykinin family, is widely distributed in the mammalian central nervous system, along with its preferring receptor NK1, including particularly those areas involved in the control of fear and anxiety such as the amygdala, hypothalamus and periaqueductal gray (Otsuka and Yoshioka 1993; Ribeiro-da-Silva and Hökfelt 2000). Studies on different animal species indicate that the injection of SP into these limbic areas, which are classical targets for anxiolytic and antidepressant drugs, provoke either anxiogenic- or anxiolytic-like effects, depending on the doses used and on the way of administration (Aguiar and Brandão 1996; Hasenöhrl et al. 1998; Gavioli et al. 1999).

Stimulated by the aforementioned evidence, several NK1 receptor antagonists have been synthesized and tested in a variety of animal models of anxiety and depression where they have been shown to display anxiolytic- and/or antidepressant-like effects (Kramer et al. 1998; Papp et al. 2000; Boyce et al. 2001; Cheeta et al. 2001; Varty et al. 2002). Moreover, as revealed by studies using knockout mice, the genetic deletion of either SP or its receptor NK1 caused a decrease in anxiety-related behaviors (Rupniak et al. 2000; Santarelli et al. 2001; Bilkei-Gorzo et al. 2002). In humans, the NK1 antagonist MK-869 has been reported to reduce indices of depression and anxiety in depressed patients (Kramer et al. 1998). Taken together, these findings suggest that the blockade of NK1 receptors is potentially useful in the treatment of anxiety- and depression-related disorders.

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NKP608 is a selective, potent, orally active and CNS-penetrant NK1 receptor antagonist both in vivo and in vitro, with a quite comparable high affinity at the rat, the gerbil and the human NK1 receptor (Vassout et al. 2000). Anxiolytic-like effects, similar to those of benzodiazepines, have been described for this compound in the social interaction and the social exploration tests in rats (Vassout et al. 2000; File 2000) and the social investigation test in gerbils (Gentsch et al. 2002). However, this compound showed only weak, partial anxiolytic-like effects in the stress-induced hyperthermia test in mice (Spooren et al. 2002), a paradigm that does not involve a social component. Taken together, these results suggest that the anxiolytic-like effects of NKP608 may depend on the context (social/non-social) of the test. Animal tests involving conflict (e.g. the elevated plus-maze) are often seen as models of generalized anxiety disorder, whereas social interaction tests have been suggested as models of social phobia and panic (Gyertyán 1992). Therefore, an evaluation of NKP608 in further classical models of anxiety/emotionality, such as the elevated plus-maze and the open field tests, might be helpful to provide a better evaluation of the therapeutic potential of this molecule.

The influence of genetic factors on sub-clinical forms of anxiety as well as on anxiety-related psychopathologies has been repeatedly demonstrated (van de Wetering et al. 1999; Lesch 2001). Therefore, the use of genetic models (i.e. inbred strains of rodents that differ for anxiety-related traits) has proven to be a useful tool for the study of the biological and molecular mechanisms underlying anxiety (Gray et al. 1999). As pointed out by Spooren et al. (2002), the anxiolytic effects of NKP608 were so far seen only in "normal" animals, whose behavioral reactions could be considered as adequate from the adaptive point of view. Hence, it remains to be determined whether NKP608 and other NK1 receptor antagonists will be equally effective in animal strains with contrasting baseline levels of anxiety, which might represent a better model of psychopathological states in humans.

It has been recently proposed that the inbred rat strains Lewis (LEW) and spontaneously hypertensive rats (SHR) constitute a useful genetic model for the study of anxiety (Ramos et al. 1997, 1998, 2002). These two strains display contrasting behavioral responses when submitted to a variety of tests of anxiety/emotionality such as the elevated plus-maze, open field, black/white box and elevated T-maze. LEW rats of both sexes display higher levels of anxiety-related behaviors when compared with SHR rats, without differing in relation to several measures of general locomotion in either novel or familiar environments (Ramos et al. 1997, 1998, 2002; Ramos and Mormède 1998).

An F2 intercross between the LEW and SHR strains has been successfully used to identify and map, for the first time in rats, QTLs (quantitative trait loci) affecting emotionality-related behaviors (Ramos et al. 1999). This study revealed a locus on chromosome 4 which strongly affected the locomotion of rats in the central area of the open field test (a putative measure of anxiety). This genomic region was found to also harbor the gene *Tac1r*, which codes for the NK1 receptor (Ramos et al. 1999). This finding points to a possible implication of the NK1 receptor in the genetic differences in emotionality observed between LEW and SHR rats. If this hypothesis holds true, one could expect to find a different reactivity between these two strains to an antagonist of NK1 receptors.

The aim of the present study was thus to evaluate the effects of NKP608 in LEW and SHR rats of both sexes in two behavioral tests widely used to measure anxiety/emotionality, namely the elevated plus-maze and the open field tests, two paradigms which do not involve social stimuli.

Materials and methods

Animals

Colonies of the two inbred rat strains Lewis (LEW) and spontaneously hypertensive rats (SHR), were maintained in our laboratory under a system of brother-sister mating. In total, 200 rats (50 per sex and strain) were used in this study. The animals were weaned and separated by sex at 4 weeks of age and, thereafter, were kept in collective plastic cages (40×32×16.5 cm) with five rats/cage having food and water available ad libitum under a L12:D12 cycle (lights on at 0700 hours) at 23±2°C. At 9 weeks of age the average (±SEM) weight of the animals was 232±3 g and 160±1 g for male and female SHR and 234±4 g and 172±2 g for male and female LEW rats, respectively. These rats were submitted to the elevated plus-maze and, immediately after, to the open field test with five different pharmacological treatments being applied prior to test. Males and females were tested in alternate days. On each day, both LEW and SHR rats treated with any of the five treatments were tested in alternation, with the five treatments being randomly assigned. All tests were carried out between 1300 and 1800 hours. The present studies were in accordance with the local regulations for the ethical use of animals in research (CEUA/UFSC) and covered by the valid permission No. 23080.002412/2001-26.

Drugs

NKP608 (quinoline-4-carboxilic acid [*trans*-(2R, 4S)-1-(3,5-bis-trifluoromethyl-benzoyl)-2-(4-chloro-benzyl)-piperidin-4-yl]-amide) was synthesized by Novartis Pharma AG (Basel, Switzerland). The drug was suspended in 0.5% methylcellulose (Methocel) and administered by oral gavage (2 ml/kg) at doses of 0.003, 0.03 or 0.3 mg/kg; note that the doses were selected based on the findings of Vassout et al. (2000). The control group received a similar volume of 0.5% Methocel. An additional group treated with 5 mg/kg chlordiazepoxide-HCl (Profarmaco S.R.L., Italy) served as positive standard. All treatments were given 90 min prior to the first test. Ten rats were used per treatment per strain and per sex.

Elevated plus-maze (EPM)

The apparatus was made of wood covered with a layer of black Formica and had four elevated arms (52 cm above the floor) 50 cm long and 10 cm wide. The arms were arranged in a cross-like disposition, with two opposite arms being enclosed (by 40 cm high walls) and two being open, having at their intersection a central platform (10×13.5 cm) that gave access to any of the four arms. The open arms were surrounded by a raised ledge (1 mm thick and 5 mm high) to avoid rats falling off the arms. The central platform was under 70 lux of illumination. Each rat was placed onto the

central platform facing an open arm and the following behaviors were registered for 5 min: the number of entries and the time spent (with all four paws) inside each type of arm and the percentage of open-arm entries in relation to the total number of arm entries. The behavior of each animal was recorded via a video camera positioned above the maze and monitored in another room via a closed circuit TV camera. The floor of the apparatus was cleaned with a wet sponge and then dried with a paper towel between rats.

Open field (OF)

The apparatus, made of wood covered with impermeable Formica, had a white floor of 100×100 cm (divided by black lines into 25 squares of 20×20 cm) and 40 cm high white walls. Illumination inside the open field was 330 lux. Each rat was placed in the center of the open field and the following variables were registered for 5 min: number of peripheral squares (adjacent to the walls) crossed (peripheral locomotion), number of central squares (away from the walls) crossed (central locomotion), time in the central area (central time) and total number of faecal boli (defecation). Also the floor of the apparatus was cleaned with a wet sponge and then dried with a paper towel between rats.

Statistics

Statistical analysis was performed separately for each sex using a two-way ANOVA for strain and treatment effects. Duncan's multiple range test was used for post-hoc comparisons of means whenever a significant treatment or treatment/strain effect was detected by the ANOVA. Specific comparisons between vehicle-treated LEW and SHR rats were performed for each sex by using a Student *t*-test. For one specific variable (time spent in the open arms of the EPM), for which the two-way ANOVA detected an almost significant effect of treatment, we also performed a one-way ANOVA for treatment effect in male LEW rats only.

Results

Elevated plus-maze

For males, the results grouped by strain and treatment are presented in Table 1 and Fig. 1. The two-way ANOVA revealed a significant effect of the treatments on the time spent in the open arms (F=3.73, P<0.01), the number of open-arm entries (F=12.43, P<0.01), the percentage of open-arm entries (F=6.09, P<0.01) and the time spent in the closed arms (F=3.46, P<0.05). Post-hoc comparisons indicated that LEW rats treated with CDZ (5 mg/kg) spent more time in the aversive open arms (P<0.05) and made a higher percentage of open-arm entries (P<0.01) than their vehicle controls. In addition, LEW rats treated with CDZ or NKP608 (0.3 mg/kg) made more open-arm entries (P<0.01 for CDZ and P<0.05 for NKP608) and spent less time in the closed arms (P<0.01 for CDZ and P<0.05 for NKP608) than their control group. A post-hoc comparison following a one-way ANOVA for treatment effects on LEW males only, revealed that both CDZ and NKP608 (0.3 mg/kg) had significantly increased the time spent in the open arms in this specific sub-group. SHR rats treated with CDZ made a higher percentage (P<0.05) and number (P<0.01) of entries into the open arms than their vehicle controls. The ANOVA also revealed overall strain effects for the time spent (F=25.75, P<0.01) and percentage of entries (F=6.47, P<0.05) in the open arms (SHR>LEW) and time spent (F=57.34, P<0.01) and number of entries (F=8.03, P<0.01) in the closed arms (LEW>SHR). The interstrain comparisons indicated that SHR rats treated with vehicle spent more time in the open arms (t=−3.04, P<0.01) and less time in the closed arms (t=4.21, P<0.01) when compared with their LEW counterparts.

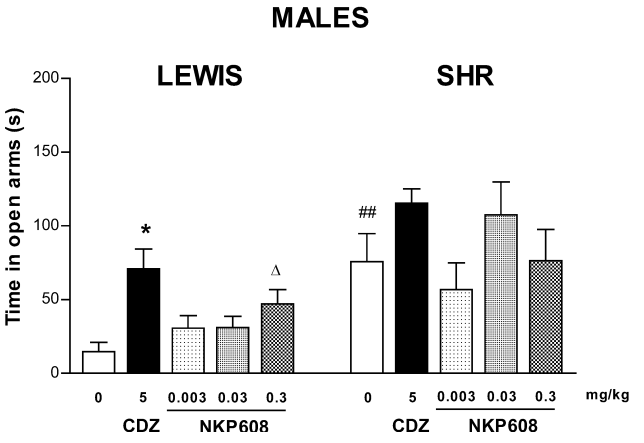

Fig. 1 Time spent in the open arms in the elevated plus-maze of LEW and SHR male rats treated with CDZ, methocel (0.5%) or NKP608 at three different doses. *Bars* and *vertical lines* represent the means and SEM of animals grouped by strain and treatment (n=10). For each strain, significant treatment differences in relation to the vehicle control group are represented by * (P<0.05). Significant interstrain differences for vehicle-treated rats are represented by ## (P<0.01, Student t-test). ΔSignificant treatment difference in relation to the vehicle control group when one-way ANOVA was applied for LEW rats only (P<0.05)

Table 1 Elevated plus-maze measures (mean±SEM) of 9-week-old LEW and SHR rats of both sexes

Treatment	Dose (mg/kg, GO)	Males				Females			
		Open-arm entries		Open-arm entries (%)		Open-arm entries		Open-arm entries (%)	
		LEW	SHR	LEW	SHR	LEW	SHR	LEW	SHR
Vehicle	0	2.0±0.7	3.5±0.7	20.5±6.0	34.4±6.3	6.4±1.0	6.6±0.9	40.2±4.2	43.2±3.0
CDZ	5	8.1±1.3**	8.8±0.7**	49.0±4.2**	55.6±1.2*	8.7±1.0	14.7±0.6**	47.7±3.0	68.4±2.6**
NKP608	0.003	3.6±1.1	3.1±0.6	25.6±6.9	32.5±5.0	6.0±1.2	7.5±0.9	40.8±5.7	50.0±4.0
	0.03	3.3±0.7	4.7±0.9	31.9±6.4	49.3±7.3	4.9±0.9	8.3±0.9	33.9±5.4	52.2±3.5
	0.3	5.1±1.0*	3.7±0.8	33.4±5.0	35.2±6.7	7.1±0.7	6.7±0.7	44.1±2.4	44.3±2.6

For each strain, significant treatment differences in relation to the vehicle control group are represented by * and ** (P<0.05 and P<0.01)

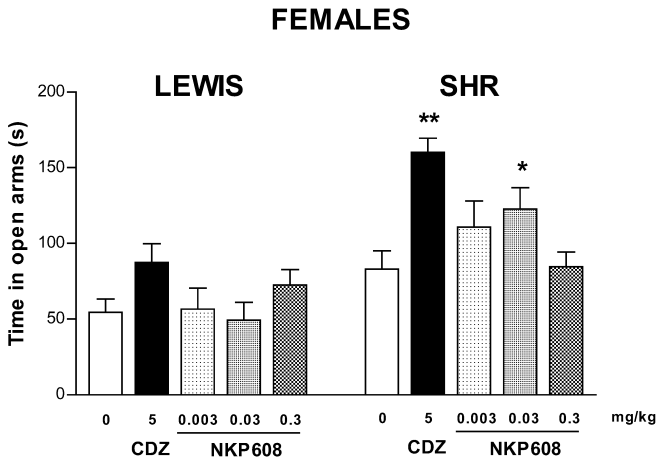

Fig. 2 Time spent in the open arms in the elevated plus-maze of LEW and SHR female rats treated with CDZ, methocel (0.5%) or NKP608 at three different doses. *Bars* and *vertical lines* represent the means and SEM of animals grouped by strain and treatment (n=10). For each strain, significant treatment differences in relation to the vehicle control group are represented by * and ** (P<0.05 and P<0.01)

For females, the results grouped by strain and treatment are presented in Table 1 and Fig. 2. The two-way ANOVA revealed a significant overall treatment effect for the time spent in the closed arms (F=5.45, P<0.01), only. A significant treatment/strain interaction was found for the time spent in the open arms (F=2.49, P<0.05), the number of open-arm entries (F=4.27, P<0.01) and the percentage of open-arm entries (F=2.81, P<0.05). Post-hoc analyses showed that SHR females treated with CDZ made more entries into the open arms, displayed a higher percentage of open-arm entries and spent less time in the closed arms (P<0.01) than their vehicle control group. Yet, SHR females treated with both CDZ and NKP608 (0.03 mg/kg) spent more time in the open arms (P<0.01 for CDZ and P<0.05 for NKP608) than their controls. The ANOVA also revealed an overall strain effect (LEW>SHR) for the number of entries (F=4.90, P<0.05) and the time spent (F=67.23, P<0.01) in the closed arms. The interstrain comparison of vehicle-treated rats also indicated that SHR females spent less time in the closed arms (t=2.93, P<0.01) than their LEW counterparts.

Open field

For males, the results grouped by strain and treatment are presented in Fig. 3. The two-way ANOVA revealed a significant treatment/strain interaction for central locomotion (F=3.33, P<0.05) and central time (F=2.89, P<0.05). Post-hoc analyses showed that males from the SHR strain treated with CDZ or with any of the NKP608 doses crossed more squares and spent more time in the aversive central area of the open field than the vehicle-treated control rats. No treatment-effect was observed in

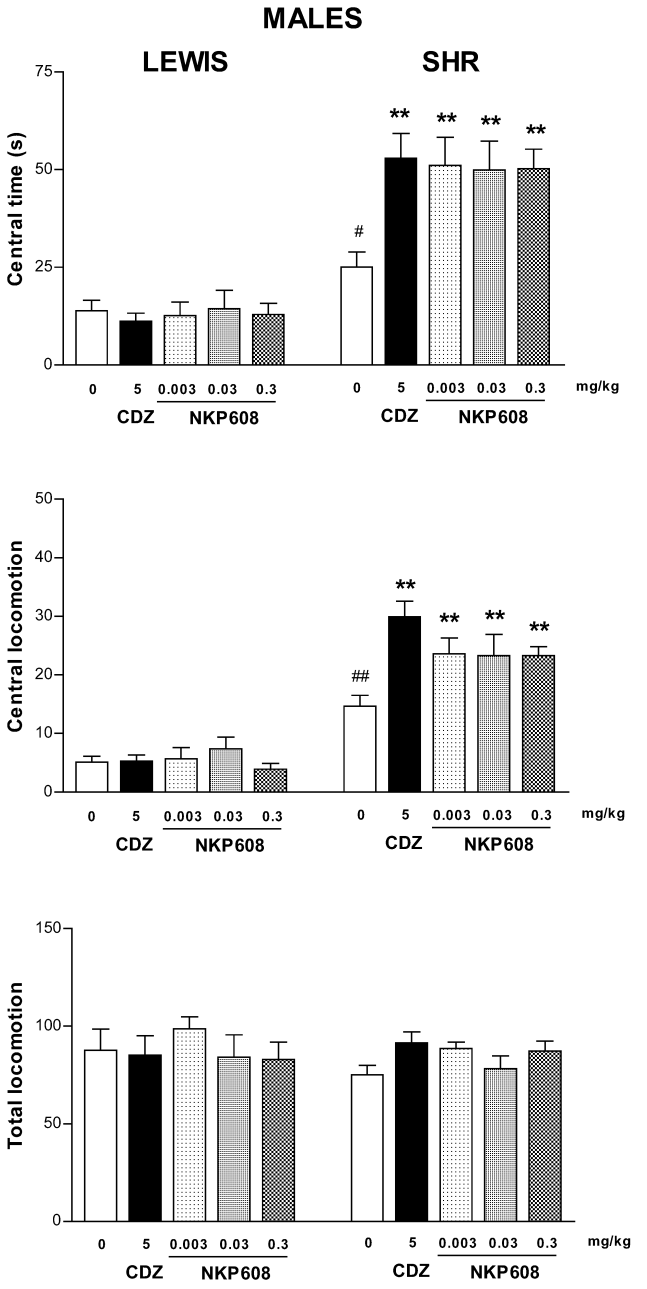

Fig. 3 Time spent in the central area and locomotion in the central and total areas of the open field of LEW and SHR male rats treated with either CDZ, methocel (0.5%) or NKP608 at three different doses. *Bars* and *vertical lines* represent the means and SEM of animals grouped by strain and treatment (n=9). For each strain, significant treatment differences in relation to the vehicle control group are represented by ** (P<0.01). Significant interstrain differences for vehicle-treated rats are represented by # and ## (P<0.05 and P<0.01, Student t-test)

LEW males. The ANOVA also revealed an overall strain effect for peripheral locomotion (LEW>SHR). The interstrain comparison of vehicle-treated rats revealed that SHRs crossed more squares (t=–4.79, P<0.01) and spent more time (t=-2.41, P<0.05) in the central area than LEWs.

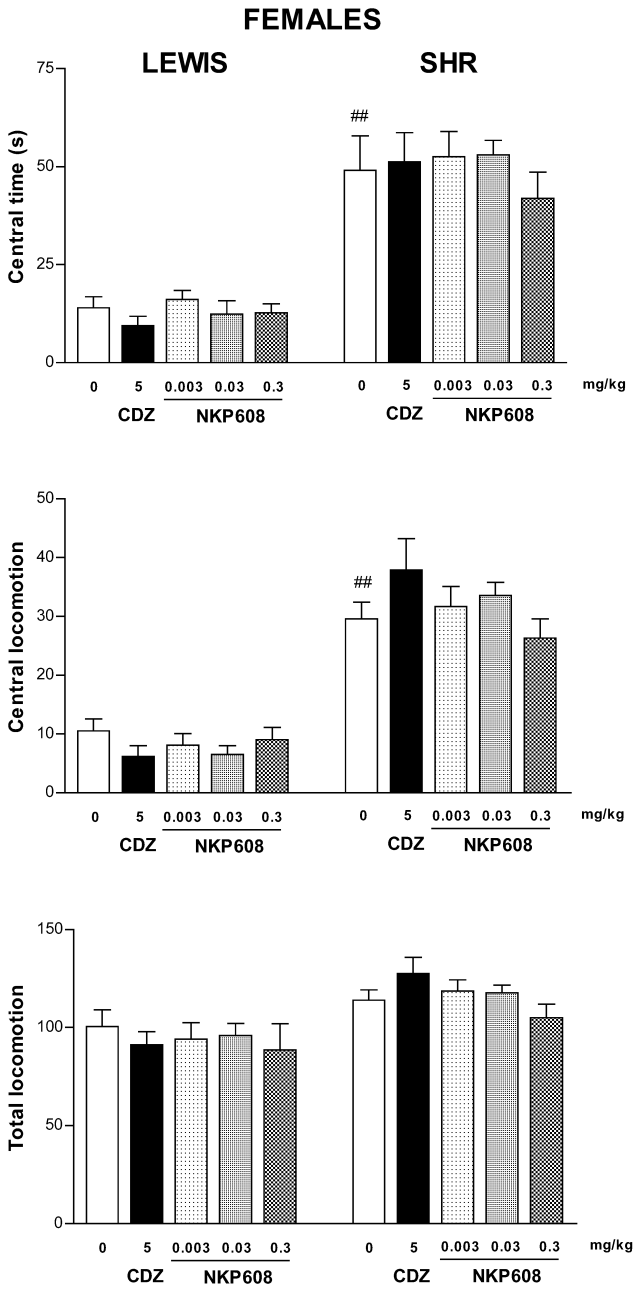

Fig. 4 Time spent in the central area and locomotion in the central and total areas of the open field of LEW and SHR female rats treated with either CDZ, methocel (0,5%) or NKP608 at three different doses. *Bars* and *vertical lines* represent the means and SEM of animals grouped by strain and treatment (n=10). Significant interstrain differences for vehicle-treated rats are represented by ## (P<0.01, Student t-test)

For females, the results grouped by strain and treatment are presented in Fig. 4. The two-way ANOVA revealed no effect of the pharmacological treatments but showed an overall strain effect (SHR>LEW) on central locomotion (F=172.21, P<0.01), central time (F=123.76, P<0.01) and total locomotion (F=21.19, P<0.01). For vehicle-treated animals, SHR females displayed higher locomotion (t=−5.46, P<0.01) and spent more time (t=−3.76, P<0.01) in the central area of the open field than LEW females.

Discussion

The potent and selective NK1 receptor antagonist NKP608 has been recently shown to produce anxiolytic-like effects both in rats and gerbils (File 2000; Vassout et al. 2000; Gentsch et al. 2002). These previous findings, as well as those of the present study, reinforce the emerging notion that NK1 receptor antagonists have an anxiolytic profile and they corroborate the hypothesis that tachykinins are involved in anxiety- and/or depression-related disorders (Kramer et al. 1998; File 2000; Papp et al. 2000; Stout et al. 2001; Gentsch et al. 2002; Spooren et al. 2002; Varty et al. 2002). The present results also suggest, however, that the psychopharmacological effects of NKP608 may depend on the genotype and gender of the animals tested and on the psychological context of the behavioral test.

In the EPM, the first test the rats were exposed to in the present study, the highest dose of NKP608 (0.3 mg/kg) showed an anxiolytic-like effect (though less pronounced than that caused by CDZ) in male LEW rats, by increasing their number of entries and time spent in the open arms and decreasing their time spent in the closed arms, without affecting their main measure of general locomotion (entries in the closed arms). For SHR females, the intermediate dose of NKP608 increased only one index of anxiety, the time spent in the open arms, again without altering general locomotion. These findings point to a weak and genotype-dependant anxiolytic effect of this NK1 antagonist in the EPM. In this same test, the classical benzodiazepine CDZ, at the dose of 5 mg/kg, showed a more robust anxiolytic effect in males of both strains and in SHR females, without causing any stimulant or sedative effect (as judged by the number of closed-arm entries) in any of the groups tested. No anxiolytic effect of CDZ was observed in LEW females, who may be less sensitive than the other groups to benzodiazepines.

In the OF test, all doses of NKP608 and CDZ (5 mg/kg) were similarly effective in SHR males, increasing their level of approach towards the aversive central area while causing no change in either total or peripheral locomotion, which is consistent with the anxiolytic profile of the two drugs. However, the same pharmacological treatments had no effect in any of the other groups of rats (LEW males; LEW and SHR females) in this test. As far as SHR females are concerned, it is possible that a ceiling effect had already been attained by the control group. For LEW rats of both sexes, on the other hand, the lack of anxiolytic-like effect suggests that the high-anxiety profile of these rats may be resistant to both CDZ and NKP608 in this specific test. It should be noticed, however, that when rats were submitted to this OF test they were no longer test naive, since they had been exposed immediately beforehand to an EPM apparatus. Taken together, the present results indicate that NK1

receptors may modulate fearfulness in the OF test and that such a modulation depends on the gender and genotype of the rats used. Both these findings would be in agreement with our aforementioned hypothesis that the gene for the NK1 receptor (which we have mapped, through the use of a LEW/SHR intercross, near a locus influencing central locomotion in the OF) may be involved in the control of emotionality in LEW and SHR rats (Ramos et al. 1999).

The EPM is a model of anxiety that is based on the natural tendency of rodents to avoid open alleys (Montgomery 1955; Pellow et al. 1985; Treit et al. 1993). The approach towards its open arms can be increased by benzodiazepines and decreased by anxiogenic substances (Handley and McBlane 1993), whereas serotonin-related compounds produce variable results (Pellow et al. 1985; Handley and McBlane 1993; Treit et al. 1993; Hogg 1996). The OF, on the other hand, was originally developed as a test of emotionality (Hall 1934, 1936). In this novel large arena, rodents tend to avoid the center and concentrate their ambulation in the peripheral area, where they can physically touch the walls (Treit et al. 1993; Ramos and Mormède 1998). The effects of anxiolytic drugs have not been universally verified in this test (Fisher and Hughes 1996; Angrini et al. 1998) but, as suggested by Prut and Belzung (2003), the OF seems to be a good model to test classical benzodiazepines and 5-HT_{1A} agonists.

Although the doses of NKP608 employed herein have not yet been demonstrated to cause blockade of NK1 receptors in the CNS of rats, the present findings are in general agreement with the demonstration that various NK1-receptor antagonists (including NKP608) produce anxiolytic-like effects in rodents (File 2000; Vassout et al. 2000; Gentsch et al. 2002). The NK1 receptor antagonists MK-869, L-742,694, L-733.060, CP-99,994 and CP-122,721 produced anxiolytic-like effects in the gerbil EPM (Varty et al. 2002). On the other hand, FK888 had anxiolytic effects in the EPM in mice (Teixeira et al. 1996) but not in rats (De Lima and Ribeiro 1996), whereas RP67580 produced anxiolytic effects in mice tested in the EPM and the ultrasonic vocalization test (Santarelli et al. 2002). Moreover, CGP49823 and L-760735 increased social interaction in rats (File 1997) and gerbils (Cheeta et al. 2001).

In agreement with our previous findings (Ramos et al. 1997; 1998, 2002), the present results suggested that SHR rats were less fearful than their LEW counterparts in both the EPM and OF tests. Nevertheless, there is increasing evidence for the fact that different tests may assess distinct types of emotionality in rodents (Ramos and Mormède 1998). Factorial studies have shown, for example, that the central locomotion in the OF may not correlate with measures of anxiety from the EPM (Ramos et al. 1997, 1998). Therefore, the effects of NKP608 that were shown to depend, in the present study, on the strain and behavioral test used, are likely to result from the different types of stress that the EPM and the OF tests may evoke in either LEW or SHR rats.

Previous studies had shown that NKP608 caused anxiolytic-like effects in rats and gerbils tested in a variety of models involving a social context (File 2000; Vassout et al. 2000; Gentsch et al. 2002). However, in the test of stress-induced hyperthermia, a non-social paradigm, Spooren et al. (2002) have reported only a partial anxiolytic effect of NKP608 in mice. To the best of our knowledge, this is the first study to investigate the effects of NKP608 in two widely recognized, non-social models of emotionality/anxiety, the EPM and the OF. In the latter, this NK1 antagonist has shown a strong anxiolytic-like effect in SHR males, whereas in the former, it produced a mild anxiolytic-like effect in males of the LEW strain. Since only a limited dose range of NKP608 has been tested, the present results should be interpreted with some caution. Moreover, similar studies with other NK1 antagonists will be necessary before one can infer that the effects (or lack of effects) reported herein are compound or class-of-compound specific.

In the present study, females were less sensitive to the pharmacological treatments than males, especially in the LEW strain, which may be related with the known influence that sex hormones have on mood and on the effects of psychoactive drugs (Fink et al. 1996; Godfroid 1999). Therefore, further studies aiming to investigate sex and sex/genotype differences in drug-induced emotional responses are needed and, ideally, they should also control for the stage of the females' estrous cycle.

In summary, the present study showed that an oral, acute administration of NKP608 produced clear-cut anxiolytic-like effects in SHR males when tested in the OF, but not in the EPM. Conversely, this compound had a partial anxiolytic effect in LEW males (and, to a lower degree, in SHR females) in the EPM, but not in the OF. LEW females were unaffected following all pharmacological treatments. The present results demonstrate that the complexity of emotional behaviors requires comprehensive studies and that ignoring sex, strain and test differences in pre-clinical research may lead to oversimplified explanations of drug action. The present data confirm and extend the therapeutic potential of NKP608 for the treatment of anxiety and provide further evidence for the involvement of tachykinins in the control of fear-related behaviors.

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