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Short communication

Anxiolytic-like effect of neuropeptide S in the rat defensive burying

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ABSTRACT

Neuropeptide S (NPS) has been recently identified as the endogenous ligand of a previously orphan G-protein-coupled receptor now named NPSR. Both NPS and its receptor are expressed in the brain, where they modulate different functions. In particular, it has been demonstrated that intracerebroventricular (i.c.v.) injection of NPS in rodents increases wakefulness and promotes anxiolytic-like effects. In the present study we used the defensive burying (DB) test in rats to further investigate the action of human NPS (0.1–10 nmol, i.c.v.) on anxiety-related behaviors. Diazepam (1.5 mg/kg, i.p.) and caffeine (20 mg/kg, i.p.) were used in parallel experiments as standard anxiolytic and anxiogenic drugs, respectively. None of the tested drugs produced statistical differences in the latency to contact the probe, burying behavior latency, number of shocks received or immobility/freezing duration. Caffeine increased cumulative burying behavior and the buried bedding height in a statistically significant manner thus promoting anxiogenic like effects. Opposite results were obtained with diazepam that significantly reduced these behavioral parameters. The anxiolytic-like action of diazepam was mimicked by NPS that reduced cumulative burying behavior in a dose dependent manner. Collectively, robust anxiolytic-like effects were recorded in response to NPS in the DB test. These results are of particular interest since the outcome of this assay is marginally influenced by drug effects on locomotor activity. In conclusion, we provide further evidence that NPS evokes genuine anxiolytic-like effects in the rat; therefore NPSR selective agonists are worthy of development as innovative drugs for the treatment of anxiety disorders.

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1. Introduction

Neuropeptide S (NPS) is a newly identified peptide transmitter that is involved in the regulation of anxiety and wakefulness [33]. Reverse pharmacology studies led to the identification of

NPS as the endogenous ligand of the NPS receptor (NPSR), a former orphan G-protein-coupled receptor [33]. NPS has strong sequence conservation across mammalian species [22] and is named after a highly conserved N-terminal serine at position 1. As an agonist of NPSR, NPS produces an increase in

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free intracellular calcium and cyclic adenosine mono-phosphate accumulation [13,33] indicating a coupling with both Gq and Gs proteins [21].

NPSR is widely expressed throughout the nervous system: highest levels of the receptor are found in cortex, thalamus, hypothalamus and amygdala and low levels in the brainstem while the NPS precursor mRNA is expressed strongly in a few brain areas including the locus coeruleus (LC) area, the principle sensory trigeminal nucleus and the lateral parabrachial nucleus of the brainstem [33,32].

This pattern of expression may be consistent with a role for NPS in arousal, regulation of food intake and anxiety [3,27,33]. NPS appears to induce behavioral activation and induce wakefulness, but at the same time, produces an anxiolytic-like effect, a paradox shared also by nicotine [16]. This suggests that arousal and stress are not always closely linked indicating that diverse sets of responses may be involved and different neurotransmitter systems implicated in these conditions [12]. NPS was repeatedly shown to produce anxiolytic-like effects in mice exposed to several different anxiety-predictive paradigms and increase locomotor activity [33,23,18].

Indeed, centrally administered NPS could produce an anxiolytic-like profile that was independent of the motor-activating effects of the peptide [33]. Since hyperlocomotion may be associated to emotional consequences, particularly in stressful conditions, the present study was designed to investigate the effect of NPS with the aim of minimizing its possible confounding effect on locomotion. For this purpose, we chose the rat defensive burying (DB) test. This assay measures fear-like responses of composite nature, generated by exposure to stressful environmental conditions and is established for its sensitivity to conventional anxiolytic tranquilizers and anxiogenic compounds [11].

The DB test had been previously validated [30], under the present experimental conditions, with prototypical anxiolytic (i.e. diazepam) and anxiogenic (i.e. caffeine) compounds on the basis of dose-response curves in anxiety tests [15,14] and controlled for effects on sensorimotor function (results not shown). In the current experiments, the timing scheduled in this behavioral assay in the rat was assessed using the highest dose of NPS (10 nmol/rat) in preliminary time-effect experiments on the basis of personal and other groups' [27] observations. The effects elicited by NPS were compared to those evoked, under the same experimental conditions, by the aforementioned reference stimulant and anxiolytic drugs, caffeine (20 mg/kg, i.p.) and diazepam (1.5 mg/kg, i.p.).

Our hypothesis was to demonstrate a clear cut anxiolytic effect of NPS using the rat defensive burying test, an assay that is marginally affected by locomotor changes.

2. Materials and methods

2.1. Animals

Fifty-four adult male Wistar rats, weighing 180–200 g (Harlan Italy, S. Pietro al Natisone, UD, Italy), were housed in Plexiglas® cages in groups of three-four in controlled conditions: free access to food (also purchased from Harlan), and water; 12-h light/dark cycle (lights off at 18:00); temperature,

22 ± 1 °C; humidity, 60%. All testing sessions were performed between 9:00 and 14:00. Ethical guidelines for investigation of experimental pain in conscious animals were followed, and procedures were carried out according to EEC ethical regulations for animal research (EEC Council 86/609; D.Lgs. 27/01/1992, No. 116).

2.2. Drugs

Human NPS (SFRNGVGTGMKKTSFQRAKS) was synthesized according to published methods [25] using Fmoc/tBu chemistry with a SYRO XP multiple peptide synthesizer (Multi-Syntech, Witten Germany). Crude peptide was purified by preparative reversed-phase HPLC and the purity checked by analytical HPLC and mass spectrometry using a matrix-assisted laser desorption ionization time of flight (Bruker BioScience, Billerica, MA, USA) and a ESI Micromass ZMD-2000 mass spectrometer (Waters Corporation, MS, USA). All other substances (diazepam and caffeine) and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.3. Surgery

The rats were randomly divided into groups of eight animals each. For intracerebroventricular (i.c.v.) injections, stainless-steel guide cannulae (23 ga) (Plastic One, Roanoke, VA, USA) were stereotactically implanted in the right lateral ventricle, to a depth of 0.5 mm above the ventricle (in mm from the bregma: AP = −0.8; L = 1.4; V = 3.25), under ketamine plus xylazine anesthesia (115 + 2 mg/kg, i.p.; Farmaceutici Gellini, Aprilia, Italy and Bayer, Milan, Italy, respectively) and fixed in place with acrylic dental cement and one skull screw. A removable plug was kept in place except during the drug injections. All i.c.v. injections were in a volume of 5 µl. After the end of the experiment, rats were i.c.v. injected with 5 µl of Evans blue dye and sacrificed under anesthesia. The correct placement of the cannula was ascertained by inspection of dye diffusion in the right lateral ventricle.

2.4. Experimental procedure

Human NPS 0.1, 1 and 10 nmol/rat, or saline (CTRL) was i.c.v. injected in a single administration by means of an injector (1 mm longer than the guide cannula) at an infusion rate of 5 µl in 10–15 s; the animals were then subjected to the defensive burying test 15 min later. Other groups of rats were administered with diazepam (1.5 mg/kg, i.p.), caffeine (20 mg/kg, i.p.) both dissolved in sterile saline, or saline (2 ml/kg, i.p.), 30 min before the behavioral test. Since the comparisons between i.c.v. and i.p. saline-treated animals demonstrated no differences in any behavioral measures, these data have been pooled together and indicated as controls.

2.5. Defensive burying test

A rectangular Plexiglas burying apparatus consisting of an acrylic cage (27 cm wide × 38 cm long and 38 cm high), with the floor covered with fine sawdust, was used. A continuously electrified probe consisting of wires wrapped around a wooden dowel was positioned in the apparatus so that it protruded

Table 1 – Effect of increasing doses of NPS (nmol/rat, i.c.v.) on various behavioral parameters displayed by rats in the defensive burying test; comparison with diazepam (1.5 mg/kg, i.p.) and caffeine (20 mg/kg, i.p.)

Treatment	Latency to first shock (s)	Latency to bury following the shock (s)	Received electric shock counts (N)	Immobility/freezing (s)
Saline	25.4 ± 4.1	96.4 ± 15.7	4.1 ± 0.52	500.4 ± 98.3
Diazepam	35.7 ± 6.2	116.1 ± 20.8	5.4 ± 0.76	394.6 ± 93.1
Caffeine	18.3 ± 2.9	84.9 ± 14.4	3.8 ± 0.48	639.6 ± 100.9
NPS 0.1	21.8 ± 5.6	106.6 ± 13.8	4.7 ± 0.47	462.4 ± 94.8
NPS 1	19.2 ± 4.1	88.2 ± 13.9	5.2 ± 0.67	386.7 ± 91.4
NPS 10	26.6 ± 4.8	123.6 ± 14.8	5.6 ± 0.63	354.3 ± 88.2

Values are expressed as mean ± S.E.M. for eight rats for each group. NS = $P > 0.05$ vs. SALINE. One-way ANOVA never reached significance: $F(5, 42) = 1.82$; $P = 0.13$ for latencies to contact the probe; $F(5, 42) = 0.97$; $P = 0.45$ for burying behavior latencies; $F(5, 42) = 1.49$; $P = 0.21$ for the number of times rats received shocks from contacting the probe and $F(5, 42) = 1.22$; $P = 0.32$ for immobility behavior, respectively.

10.3 cm into the chamber and was 7.5 cm from the bottom of the chamber. Contact with the probe produced a shock of 1 mA. Bedding in the chamber was approximatively 5.0 cm in depth. Animals were habituated (3–4 cagemates together) to the apparatus, in the absence of the shock probe, for 15 min each day for 4 consecutive days.

On fifth day, rats were tested individually for 30 min with the electrified probe in place. The height of bedding behind the probe was measured before testing and at the end of each 30-min test period and was expressed as the height of bedding above the 5.0 cm of bedding already placed in the chamber. Fresh bedding was placed in the apparatus before testing each animal. Behavior during the 30-min test period was recorded and later scored by two independent observers unaware of the treatments. Behaviors measured were: latency to first contact with the probe and to be shocked, latency to initiate burying (defined as pushing bedding material with the snout or forelimbs forward in the direction of the prod) from first contact, total number of shocks received, height of buried bedding and duration of burying behavior over the entire test period. In particular, latency to contact the probe is an indicator of potential changes in locomotion or exploratory activity [9]. Moreover, the latency intended as the time elapsing from the first shock to the display of the burying behavior inversely reflects the animal's reactivity [24] whereas the cumulative time of burying behavior, as well as the height of buried material, has been suggested to reflect the rat's anxiety levels in a specific manner [20]. Finally, the crouch/freeze duration (immobile stay at a counter side against the shock prod) was also counted as behavioral index since it

indicates a passive form of shock-prod avoidance behavior associated with fear and anxiety [9].

2.6. Statistical analysis

All values are expressed as mean ± S.E.M. of eight animals. One-way ANOVA followed by Bonferroni post hoc test was used to analyze the effects of a treatment with increasing doses of NPS or reference compounds in the defensive burying test. The level of significance was set at 0.05.

3. Results

In the DB, control rats spent about 103 s in covering the probe with bedding material (burying behavior) after the shock (corresponding to 5.7% of the total time) with an average height of buried bedding of 5.5 cm. The mean number of total electric shocks received during the 30-min experimental trial was about 4, the latency to be shocked for the first time being 25.4 s; after the first contact with the electrified probe, an average period of time of 96 s was registered before the beginning of the burying behavior (see Table 1).

Caffeine administration (20 mg/kg, i.p., 30 min before the test) produced a significant increase of both the height of buried bedding (+55% vs. controls) and the duration of burying behavior (+52% vs. controls) (Fig. 1). No statistical differences were detected in latency to contact the probe, burying behavior latency and number of received electric shocks, indicating that, under our experimental conditions, this drug

Fig. 1 – Effect of diazepam (1.5 mg/kg, i.p.), caffeine (20 mg/kg, i.p.) or increasing doses of NPS (nmol/rat, i.c.v.), on the duration of burying (a) and on the height of buried bedding (b) in the defensive burying test in the rat. Values are expressed as mean ± S.E.M. for eight rats for each group. * $P < 0.05$ vs. saline (CTRL) (one-way ANOVA followed by Bonferroni test).

failed to modify locomotion or exploratory behavior. Finally it displayed a non-significant tendency to increase the time spent in immobility/freezing postures (Table 1).

Diazepam (1.5 mg/kg, i.p., 30 min before the test) produced opposite results with respect to caffeine, reducing both the height of the buried material (–51% vs. controls) and the cumulative time of burying behavior (–70% vs. controls) (Fig. 1). Also diazepam was ineffective in producing any changes in the other parameters investigated, thus not interfering with the animal reactivity or exploration, at our dose used (Table 1).

NPS (0.1–10 nmol/rat, i.c.v., 15 min before the test) dose-dependently reduced the height of buried bedding and the cumulative burying behavior, the lowest effective dose being 1.0 nmol/rat for both parameters (–45% and –47% vs. controls, respectively), thus indicating a specific change in indicators directly reflecting an anxiolytic-like behavior (Fig. 1). On the other hand there was no significant difference in any other parameters evaluated (Table 1), confirming, also for NPS, a lack of alteration in locomotion/exploration or reactivity. Even at the highest dose used, NPS did not produce any significant change in immobility/freezing duration even if a slight dose-dependent decrease was detected. Thus, NPS paralleled the effects induced by diazepam and produced opposite results with respect to caffeine on the burying behavior (ANOVA: $F(5, 42) = 23.90$; $P < 0.001$; $P < 0.05$ vs. CTRL in post hoc Bonferroni test) and height of bedding (ANOVA: $F(5, 42) = 20.83$; $P < 0.001$; $P < 0.05$ vs. CTRL in post hoc Bonferroni test).

4. Discussion

In the present study, we used the DB test in rats for investigating the effects of NPS on anxiety, in comparison with diazepam and caffeine as standard anxiolytic and anxiogenic drugs, respectively. Injections of NPS (i.c.v.) dose-dependently attenuated burying behavior in the DB test mimicking the effects of diazepam while caffeine produced opposite results. Therefore we confirmed and extend previous findings [33,23,18] to a different species (the rat) and to a different assay (the DB), demonstrating that supraspinal administration of NPS produces robust anxiolytic-like effects that are very consistent among animal species and preclinical models of anxiety.

In the DB assay, drugs that are clinically effective in the treatment of anxiety disorders such as benzodiazepines or selective serotonin reuptake inhibitors dose-dependently shorten the burying behavior and height of the bedding material at low shock intensity. Moreover, the drug-class specificity of this test has been demonstrated [7]. Thus, the DB test can be regarded as a simple and reliable method to detect the activity of drugs able to modify anxiety states.

Up to now, no evidence is present in the literature regarding the effects of caffeine in the DB test in rats. However, the anxiogenic action of caffeine (10, 25 and 50 mg/kg, i.p.) has been investigated in rats using several assays [4]. Caffeine produced a dose-related anxiogenic profile in rodents comparable to that of yohimbine [4]. In our study caffeine increased the height of buried bedding as well as the duration of burying behavior thus demonstrating a clear

anxiogenic action. The present results regarding the anxiolytic effect of diazepam in the rat DB test are in line with those described by other groups [28,31] regarding the ability of diazepam to inhibit probe burying behavior of rats subjected to the DB task. At doses higher than those used in the present study, benzodiazepines produced an increase in burying behavior latency suggesting that this parameter reveals changes in animal reactivity [10]. On the contrary, under our experimental conditions, only parameters specifically related to anxiety were significantly modified by diazepam treatment. These differences may be explained by some features of the test design (intensity of the electric shock, duration of the behavioral recording, drug dose) and indicate that our experimental procedure induces less interference on the animal reactivity either using anxiolytic or anxiogenic substances. As regards locomotor activity, our data are in line with authors [15] who demonstrated that diazepam (from 0.5 to 2.0 mg/kg, i.p.) dose-dependently reduced the rat cumulative burying without modifying ambulatory behavior. Higher doses of diazepam (i.e. 4.0 mg/kg) produced stronger effects on cumulative burying but also depressed locomotion, indicating that this dose provoked sedative actions that may bias the interpretation of the results.

Under the same experimental conditions, the anxiolytic-like effects of diazepam were mimicked by the supraspinal administration of NPS. In fact, the peptide in the dose range of 0.1–10 nmol/rat produced a behaviorally selective inhibition of cumulative burying behavior and height of buried material, thus suggesting a reduction of the anxiety state of the rats. This result confirmed previous findings obtained in mice by different groups using a rather large panel of assays [33,23,18]. Interestingly enough, the dose range of activity of NPS in the present rat experiments (0.1–10 nmol) is very similar to that reported in mice studies (0.01–1 nmol) [33,23,18].

In the same range of doses NPS has been reported to consistently stimulate locomotor activity in mice [33,25,23,18]. This stimulatory effect of NPS which may interfere with the interpretation of the results obtained in the above-mentioned assays as well as in the present experiments, seems to be less robust in rats [27,2]. However, it is unlikely that the decrease in burying behavior in response to NPS could be somehow related to a general motor activity modification since no changes in locomotion (latency to contact the probe and burying behavior latency) were detected, indicating no general alteration in animal motor activity or reactivity under the present experimental conditions. As a matter of fact, motor activity may be considered a minor interfering parameter in the outcome of the DB assay since the main parameter that is predictive of anxiolysis is the inhibition of an active defensive behavior (burying) [9]. Indeed, the possible actions of NPS on locomotion, shown in other assays, may affect the DB test in the way of increasing the duration of the burying behavior rather than inhibiting it, as it actually happens in our case. Therefore we propose to interpret the behavioral changes induced by NPS in the rat DB test as a genuine anxiolytic-like effect. The results obtained in mice with other assays which are marginally influenced by locomotion such as the marble burying [33] and the stress-induced hyperthermia [18,23] corroborate this proposal.

Finally, recent findings obtained in female rats demonstrated only a slight anxiolytic-like behavior in alcohol-preferring animals, detected by an activity cage but non by the elevated plus maze test (EPM) [2]. The discrepancies between our and the above-mentioned results could be explained by the differences in strain and gender, by the parameters evaluated as well as by the differential sensitivities of the test.

Indeed, we did prefer to use the DB test instead of the more commonly used paradigm, the EPM, in the current experiments. In fact, the administration of anxiolytic drugs leads to the expression of opposite behaviors in these two situations: in the DB test, a decrease of anxiety is related to a behavioral inhibition (the rat stops burying the probe) whereas, in the EPM, a decrease of anxiety corresponds to a behavioral disinhibition (the rat exits more often in the open arms) [29].

Therefore, the value of the present study resides in the fact we confirm our hypothesis that NPS promotes a clear cut anxiolytic effect in rats when possible confounding changes in locomotion are minimized. Thus, NPS behaves as an important signal in the brain whose anxiolytic-like effects are similar to those elicited by diazepam and opposite to those of caffeine.

Indeed, for its high degree of face and construct validity, the defensive burying paradigm allows the measurement of several behavioral indices of fear/anxiety expression and can give valid information from which to draw reliable conclusions on the action of putative anxiolytic compounds such as NPS.

Surprisingly, no differences were found among the various groups of rats in the duration of immobility/freezing postures which indicates a different behavioral expression of anxiety (passive avoidance) and could have been a useful additional anxiety/fear parameter in this behavioral paradigm, this index being reciprocally related to the levels of burying [8,9]. On the other hand, previous studies demonstrated either no change or a paradoxical increase in immobility score using benzodiazepine-like compounds as well as some anxiogenic agents (i.e. corticotrophin-releasing factor), thus suggesting that passive and active (burying) coping responses are possibly affected by different neuronal networks and display distinct behavioral profiles [9]. Thus, in our case, the defensive burying active reaction fulfils the major pharmacological and sensitivity criteria to be considered a reliable response to be assessed for anxiety-related behaviors.

Little is known about the mechanisms by which NPS promotes its anxiolytic-like action. The expression of the NPS receptor in several brain regions that are known to be involved in anxiety [6,26], such as amygdala, thalamus and hypothalamic regions, is consistent with an important role of NPS in modulating the behavioral response to stress [32].

The receptor mechanism involved in the anxiolytic-like effects of NPS is also unknown. At this regard, the only information available up to now in the literature suggests that selective NPSR activation represents the mechanism by which NPS stimulates locomotor activity in mice. In fact, peripheral administration of the NPSR antagonist SHA 68 (50 mg/kg, i.p.) is able to antagonize mice NPS-induced horizontal and vertical activity [19].

The use of this compound as well as the identification of other NPSR selective and potent antagonists, together with the

study of the phenotype of mice knockout for the NPSR gene [1] will be of paramount importance, in the near future, for understanding the roles played by the endogenous NPS-NPSR system at the CNS level. In particular, the study of the regulation of various central functions including the response to stress, anxiety, locomotor activity, arousal and sleep, and food intake will be of interest for ultimately identifying therapeutic indications of novel drugs acting as NPSR selective ligands.

In conclusion, the present findings strongly support the hypothesis that the activation of NPS receptor acutely suppresses anxiety-like behavior and support the possibility that NPSR could provide an interesting target for the development of innovative drug for the treatment of anxiety.

Indeed, there is a clear need for more effective anxiolytic pharmacotherapy; in fact, some of the untoward side effects of classical anxiolytic drugs are associated with dependence, interaction with alcohol, or sedation [5]. Novel drugs targeting the NPSR could possibly represent an efficacious treatment for anxiety without the risk of sedation because of the unique role played by NPS in mediating both anxiolytic-like activity and arousal [18,17].

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