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Neuropeptide S reduces mouse aggressiveness in the resident/intruder test through selective activation of the neuropeptide S receptor

Chiara Ruzza [a, *], Laila Asth [b], Remo Guerrini [c], Claudio Trapella [c], Elaine C. Gavioli [b]

[a] Department of Medical Sciences, Section of Pharmacology and National Institute of Neuroscience, University of Ferrara, Via Fossato di Mortara 19, 44121 Ferrara, Italy
[b] Department of Biophysics and Pharmacology, Federal University of Rio Grande do Norte, Natal, Brazil
[c] Department of Chemical and Pharmaceutical Sciences and LTTA, University of Ferrara, 44121 Ferrara, Italy

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ABSTRACT

Neuropeptide S (NPS) regulates various biological functions by selectively activating the NPS receptor (NPSR). In particular NPS evokes robust anxiolytic-like effects in rodents together with a stimulant and arousal promoting action. The aim of the study was to investigate the effects of NPS on the aggressiveness of mice subjected to the resident/intruder test. Moreover the putative role played by the endogenous NPS/NPSR system in regulating mice aggressiveness was investigating using mice lacking the NPSR receptor (NPSR(−/−)) and the NPSR selective antagonists [^{t}Bu-D-Gly^5]NPS and SHA 68. NPS (0.01−1 nmol, icv) reduced, in a dose dependent manner, both the time that resident mice spent attacking the intruder mice and their number of attacks, producing pharmacological effects similar to those elicited by the standard anti-aggressive drug valproate (300 mg/kg, ip). This NPS effect was evident in NPSR wild type (NPSR(+/+)) mice but completely disappeared in NPSR(−/−) mice. Moreover, NPSR(−/−) mice displayed a significantly higher time spent attacking than NPSR(+/+) mice. [^{t}Bu-D-Gly^5]NPS (10 nmol, icv) did not change the behavior of mice in the resident/intruder test but completely counteracted NPS effects. SHA 68 (50 mg/kg, ip) was inactive *per se* and against NPS. In conclusion, this study demonstrated that NPS produces anti-aggressive effects in mice through the selective activation of NPSR and that the endogenous NPS/NPSR system can exert a role in the control of aggressiveness levels under the present experimental conditions.

1. Introduction

Aggressiveness is a complex phenomenon, associated with environmental and psychosocial but also neurobiological and genetics factors. Despite most of psychiatric patients are not violent, some severe mental illness, particularly bipolar disorder, personality disorders and schizophrenia, are associated with an increased risk for aggressive behavior (Ballester et al., 2014; Volavka, 2013). Aggressiveness and social behaviors are known to be modulated by classical neurotransmitters, in particular dopamine and serotonin (Comai et al., 2012a), but also by neuropeptides, such as vasopressin and oxytocin (Albers, 2012; Bosch and Neumann, 2012; Calcagnoli et al., 2013; Neumann and Landgraf, 2012; Veenema and Neumann, 2008). Neuropeptide S (NPS, human sequence SFRNGVGTGMKKTSFQRAKS) is the endogenous ligand of a previously orphan G protein coupled receptor now named NPSR (Xu et al., 2004). The NPS/NPSR system regulates several biological functions, including wakefulness, stress and anxiety, food intake, memory processes, and drug abuse (Guerrini et al., 2010). Interestingly, NPSR is expressed in brain areas important for the control of aggressive behavior, such as the amygdala, the prefrontal cortex, the lateral and anterior hypothalamus (Clark et al., 2011; Xu et al., 2007). A recent study by Beiderbeck et al. (2014) reported that NPS reduces the aggressiveness of rats in the resident/intruder test. Anyway, the mechanism by which NPS exerts its anti-aggressive effect and the role of the endogenous NPS in modulating aggressiveness have not been addressed. Considering this, in this study the effects of NPS in

Abbreviations: NPS, neuropeptide S; NPSR, NPS receptor; NPSR(+/+), NPSR wild type; NPSR(−/−), NPSR knockout; VLP, valproate.

* Corresponding author.
E-mail address: chiara.ruzza@unife.it (C. Ruzza).

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male mice subjected to the resident/intruder test have been investigated. Moreover the mechanism of action of NPS and the role played by the endogenous NPS/NPSR system in the modulation of inter-male aggressiveness has been studied using mice lacking the NPSR receptor (NPSR(−/−), Ruzza et al., 2012a) and the selective NPSR antagonists SHA 68 (Okamura et al., 2008; Ruzza et al., 2010) and [^{t}Bu-D-Gly^5]NPS (Guerrini et al., 2009a; Ruzza et al., 2012b).

2. Materials & methods

In this research, valproate and NPS dose response curve studies were performed at the Federal University of Rio Grande do Norte (Brazil), using male Swiss mice (8–24 weeks old, 28–45 g) obtained from the Federal University of Rio Grande do Norte (Brazil) breeding colony. Antagonism studies were performed at the University of Ferrara (Italy), using male CD-1 mice (Harlan, Udine, Italy). Phenotype studies were performed at the University of Ferrara (Italy), using, NPSR(+/+) and NPSR(−/−) congenic to CD-1 strain (8–24 weeks old, breed in the animal facility of the Department of Medical Science, Section of Pharmacology of the University of Ferrara). Mice NPSR(+/+) and NPSR(−/−) were generated as described by Ruzza et al. (2012a). A total number of 134 mice was used. All the *in vivo* experimental procedures were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used. Experiments performed with Swiss mice complied with the Brazilian law n°11.794/2008 and were approved by the Federal University of Rio Grande do Norte Ethics Committee (Protocol n° 035/2013). Experiments performed in Italy complied with European Communities Council directives (2010/63/E) and national regulations (D.Lgs, 26/2014) and were approved by the Animal Welfare Body of the University of Ferrara and by the Italian Ministry of Health (Authorization n° 120/2014-PR). This research was reported following the ARRIVE guidelines (Kilkenny et al., 2010). Mice were housed in 267 × 207 × 140 mm cages (Tecniplast, Varese, Italy), 5 mice/cage, under standard conditions (22 °C, 55% humidity, 12 h light–dark cycle, lights on 7.00 am) with food and water ad libitum for at least 15 days before experiments began. Each cage was also provided with a mouse red house (Tecniplast, Varese, Italy) and nesting material. Each animal was used only once. Neuropeptide S and [^{t}Bu-D-Gly^5]NPS were injected intracerebroventriculary (icv). For the experiments performed at the Federal University of Rio Grande do Norte icv injections were performed through a permanently implanted guide cannula. For the experiments performed at the University of Ferrara icv injections (2 μl per mouse) were given under light isofluorane anesthesia, into the left ventricle according to the procedure described by Laursen and Belknap (1986) and routinely adopted in our laboratory (Rizzi et al., 2008).

2.1. Surgery

Mice were anesthetized using a mix of ketamine and xylazine (100 and 10 mg/kg ip, respectively) and placed in a stereotaxic apparatus. A vertical incision was made in the skin to expose the skull. A stainless steel guide cannula was implanted into the lateral ventricle and fixed with dental cement. Coordinates toward the bregma were L + 1.1 mm, A − 0.6 mm, V − 1 mm. To prevent occlusion, a dummy cannula was inserted into the guide cannula. The dummy cannula did not protrude the guide cannula. After surgery, the animals were allowed to recover for at least 5 days. For the icv injection mice were gently restrained by hand and the drug solution (2 μl/mouse) was injectedat a rate of 1 μl/min. After completion of testing, mice were euthanized with sodium thiopental (>100 mg/kg, ip) and icv injected with methyleneblue dye (2 μl). Mice were perfused with saline solution and their brains were removed to verify the placement of the guide cannula. Only the data from those animals with dispersion of the dye throughout the ventricles (>95% of the animals) were used.

2.2. Drugs and reagents

Neuropeptide S and [^{t}Bu-D-Gly^5]NPS were synthesized according to published methods (Guerrini et al., 2009a, b). The compound SHA 68 was synthesized using the procedures described by Okamura et al. (2008). The anticonvulsant drug sodium valproate was used in this study as standard anti-aggressive drug. The mechanism by which valproate exerts anti-aggressive effects is still not clear, anyway it is reported to increase GABAergic transmission by inhibiting GABA metabolism, to block glutamatergic signaling and block voltage-gated Na^+ channels (Comai et al., 2012b). Valproate evokes anti-aggressive effects both in mice (Flaisher-Grinberg and Einat, 2010) and in humans (Comai et al., 2012b). Sodium valproate was purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved in saline. NPS and [^{t}Bu-D-Gly^5]NPS were dissolved in saline, while SHA 68 was dissolved in water containing 10% Cremophor (Sigma–Aldrich, St. Louis, MO, USA).

2.3. Resident/intruder test

The resident mouse (16 weeks old, ~40 g) was housed individually for 7 days before the experiment in a 267 × 207 × 140 mm plastic cage. Since territorially is strongly based on the presence of olfactory cues, the bedding was never cleaned during this period. Intruder mice (8 weeks old, ~25–30 g) were socially housed. Each resident mouse was tested twice: the first day of experiment it was tested with no treatment and its basal aggressiveness was recorded (control), after 3 days, the same mouse was re-tested with treatment. All the experiments were performed between 9:00 am and 1 pm. The test begins when the intruder is placed in the resident cage and lasts for 10 min. During this period the number of attacks and the time that the resident mouse spends attacking the intruder are recorded by an expert observer. The following behaviors are scored as attacks: bites, lateral threat, upright posture by the resident, clinch, keep down, tail rattles and chase. Mice that did not fight during the first test have been excluded from the study (~10%). The second day of test mice were randomly assigned to the different experimental groups. Saline, NPS (0.1–1 nmol) and [^{t}Bu-D-Gly^5]NPS (10 nmol) were given icv 30 min before starting the test, sodium valproate (300 mg/kg) and SHA 68 (50 mg/kg) were given ip 30 min before starting the test. When tested against NPS, [^{t}Bu-D-Gly^5]NPS (10 nmol) was co-injected icv with the natural peptide (0.1 nmol) 30 min before testing, while SHA 68 (50 mg/kg) was given ip 30 min before NPS.

2.4. Data analysis and terminology

Data are expressed as mean ± sem of n animals. Data were analyzed using paired Student's t test or two-way ANOVA followed by the Bonferroni's post hoc test, as specify in figure legends. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effects of the standard drug sodium valproate

A first series of experiments was performed to set up and validate the resident/intruder experimental conditions. The first day of the test (control) untreated cannulated resident Swiss mice spent 67 ± 13 s attacking the intruder mouse and their number of attacks was 23 ± 4. When, after 3 days, the same resident mice received an icv injection of saline, no statistically significant changes in their behavior were recorded (time spent attacking: 65 ± 14 s; number of attacks 24 ± 5). Similar results were obtained with non-cannulated CD-1 mice. In fact, the first day of test, they spent 92 ± 16 s attacking the intruder mouse and their number of attacks was 25 ± 2. The second day of the test, after the free hand icv injection of saline, CD-1 mice spent 87 ± 18 s attacking and their number of attacks was 19 ± 2. Of note, no statistically significant differences were detected between the behavior of Swiss and CD-1 mice, both naïve and saline treated (data not shown). Valproate (300 mg/kg), injected ip 30 min before starting the test, was able to significantly reduce the aggressiveness of resident mice, both in terms of total time spent attacking ($t_{(11)} = 4.92$) and number of attacks ($t_{(11)} = 4.63$, Fig. 1).

3.2. Effects of NPS

Neuropeptide S, injected icv the second day of test (30 min of pre-treatment), reduced, in a dose dependent manner, the total time the resident mice spent attacking ($t_{(10)} = 9.77$ for NPS 0.1 nmol, $t_{(10)} = 5.24$ for NPS 1 nmol) and their number of attacks ($t_{(10)} = 4.55$ for NPS 0.1 nmol; $t_{(10)} = 3.48$ for NPS 1 nmol, Fig. 2).

3.3. Phenotype and NPS sensitivity of NPSR(−/−) mice

In the resident/intruder test resident mice lacking the NPSR receptor were more aggressive than NPSR(+/+) mice. In fact they spent significantly more time attacking the intruder mice. No statistically significant differences were recorded in the number of attacks between NPSR(+/+) and NPSR(−/−) resident mice (Fig. 3). Importantly, NPS 1 nmol (icv, 30 min of pre-treatment) was able to reduce the time spent attacking in NPSR(+/+) but was totally inactive in NPSR(−/−) mice. Moreover NPS strongly decreased the number of attacks done by NPSR(+/+) but only weakly reduced the number of attacks done by NPSR(−/−) mice. Two way ANOVA (genotype × treatment) for repeated measurements revealed an effect of genotype ($F_{(1,24)} = 39.45$), of NPS ($F_{(1,24)} = 16.30$) and of the