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DOTTORATO DI RICERCA IN
FARMACOLOGIA E ONCOLOGIA MOLECOLARE

CICLO XXIII

COORDINATORE Prof. Antonio Cuneo

PHARMACOLOGICAL AND NEUROBIOLOGICAL STUDIES ON NEUROPEPTIDE S AND ITS RECEPTOR

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List of abbreviations

5-HT	**serotonin**
aa	**amino acid**
Aib	**amino isobutyric acid**
ANOVA	**analysis of variance**
BSA	**bovine serum albumin**
cAMP	**cyclic adenosine monophosphate**
CHO	**chinese hamster ovary**
CNS	**central nervous system**
CRF	**corticotropin release factor**
DNA	**desoxyribonucleic acid**
dNTP	**deoxribonucleotide triphosphates**
EPM	**elevated plus maze**
FIU	**fluorescence intensity units**
FLIPR	**fluorometric imaging plate reader**
FST	**forced swimming test**
GABA	**γ-aminobutyric acid**
GPCR	**G-protein coupled receptor**
GPR154	**G-protein coupled receptor 154**
GPRA	**G-protein coupled receptor for asthma susceptibility**
GTP	**guanosin 5'-triphosphate**
HBSS	**Hanks' Balanced Salt Solution**
HEK	**human embryonic kidney**
HEPES	**2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid**
hNPSR	**human neuropeptide S receptor**
HTS	**high throughput screening**
i.c.v.	**intracerebroventricular(ly)**
i.p.	**intraperitoneal(ly)**
IL-1β	**interleukin-1 beta**
IL-6	**interleukin-6**

IP_3	**inositol 1,4,5-trisphosphate**
IUPHAR	**International Union of Pharmacology**
LA	**locomotor activity**
LC	**locus ceruleus**
mNPSR	**mouse neuropeptide S receptor**
mRNA	**messenger ribonucleic acid**
NA	**noradrenaline**
NOR	**novel object recognition**
NPS	**neuropeptide S**
NPSR receptor	**neuropeptide S receptor**
NPSR(+/+), NPSR(-/-)	**NPSR receptor wild type and knockout**
OF	**open field**
OX	**orexin**
PCR	**polymerase chain reaction**
PLC	**phospholipase C**
ppNPS	**neuropeptide S peptide precursor**
RR	**righting reflex**
RT-PCR	**reverse transcriptase polymerase chain reaction**
s.e.m.	**standard error of the mean**
SAR	**structure-activity relationship**
SHA 68	**(9R/S)-3-oxo- 1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide**
SIH	**stress induced hyperthermia**
SNP	**single nucleotide polymorphism**
TNF-α	**tumor necrosis factor α**
VRR1	**vasopressin receptor-related receptor**

Abstract

Neuropeptide S (NPS) is the last neuropeptide identified via reverse pharmacology techniques. NPS selectively binds and activates a previously orphan GPCR, now named NPSR, producing intracellular Ca^{2+} mobilization and stimulation of cAMP levels. Biological functions modulated by the NPS/NPSR system include anxiety, arousal, locomotion, food intake, learning and memory, pain and drug addiction. In our laboratories we provided further evidence that NPS injected supraspinally in mice acts as a stimulatory anxiolytic. In fact, in the mouse righting reflex (RR) test, NPS (0.01- 1 nmol, i.c.v.) was able to reduce in a dose dependent manner the percent of animals losing the RR in response to diazepam (15mg/kg, i.p.) and their sleep time. Furthermore, NPS in the same range of doses caused a significant increase in locomotor activity (LA) in mice. These effects were associated with a clear anxiolytic-like action elicited by NPS in the mouse elevated plus maze (EPM) test, open field (OF) test and stress-induced hyperthermia (SIH) assay. Thus NPS evokes an unique pattern of behavioural effects: stimulation associated with anxiolysis. To deeply investigate the biological roles played by the NPS/NPSR system the development of pharmacological (i.e. selective NPSR ligands, particularly antagonists) and genetic (i.e. receptor knockout animals) tools are needed. In collaboration with the medicinal chemistry group of the University of Ferrara, we performed a series of classical structure-activity (SAR) studies on NPS sequence. Specifically, NPS positions 2, 3, 4 and 5 were investigated in details, since they were demonstrated to be crucial for NPS bioactivity. Studies focussed on NPS position 5 led to the identification of the first generation of NPSR peptide antagonists. *In vitro*, in HEK293 cells stably expressing the mouse NPSR, [D-Cys(^{t}Bu)^5]NPS up to 100 μM did not stimulate Ca^{2+} mobilization but was able to counteract in a competitive manner the stimulatory action of NPS (pA_2: 6.44). *In vivo*, in the RR test, [D-Cys(^{t}Bu)^5]NPS at 10 nmol was inactive *per se* but dose dependently antagonized the arousal-promoting action of NPS 0.1 nmol. [D-Val^5]NPS acted *in vitro* as a pure NPSR antagonist, with a pK_B of 6.54 in inhibition experiments. *In vivo*, in LA test, [D-Val^5]NPS at 10 nmol completely blocked the stimulatory effect evoked by NPS. In a further medicinal chemistry study, the potent NPSR antagonist [^{t}Bu-D-Gly^5]NPS was identified. *In vitro*, [^{t}Bu-D-Gly^5]NPS did not stimulate calcium mobilization but blocked the stimulant action of NPS with a pK_B of 7.06. *In vivo*, in RR assay, [^{t}Bu-D-Gly^5]NPS (0.1-10 nmol, i.c.v.) was inactive per se but dose dependently antagonized the arousal-promoting action of NPS 0.1 nmol. Similarly in the LA assay [^{t}Bu-D-Gly^5]NPS (0.1-10 nmol, i.c.v.) was inactive per se but was able to counteract the stimulatory effect

evoked by 0.1 nmol NPS in a dose dependent manner. SHA 68 has been previously identified as the first non peptide NPSR antagonist. In our laboratories we further assessed the pharmacological profile of SHA 68 *in vitro* and *in vivo*. *In vitro* SHA 68 was inactive per se up to 10 μM while it antagonized NPS-stimulated Ca^{2+} mobilization in a competitive manner showing a pA_2 value of 8.06. *In vivo*, in the mouse RR assay, SHA68 50 mg/kg i.p. fully prevented the arousal promoting action of NPS 0.1 nmol. In LA experiments, SHA 68 50 mg/kg i.p. was able to partially counteract the stimulant effects elicited by NPS 0.1 nmol. Instead, the anxiolytic-like effects of NPS 0.1 nmol in mouse OF test were slightly reduced by SHA 68. Collectively these data demonstrated the exclusive involvement of NPSR in the arousal promoting and locomotor stimulant effects of NPS. Finally, we backcrossed on the CD-1 strain the NPSR knockout mice originally generated on the 129Sv/Ev genetic background. A first phenotype analysis revealed no locomotor differences between NPSR(+/+) and NPSR(-/-) mice, with the exception of rearing behaviour that was reduced in knockout animals. Furthermore, the behaviour of NPSR(+/+) and NPSR(-/-) mice in the EPM, OF and SIH tests is superimposable. Similarly no differences were detected in the novel object recognition, forced swimming, RR and formalin assays. However, the stimulant actions of 1 nmol NPS in RR and in LA test could be detected in NPSR(+/+) but not in NPSR(-/-) mice. Collectively these data demonstrated that endogenous NPS/NPSR system does not play a role in the control of locomotion, anxiety, depression and memory, at least under the present experimental conditions. These results demonstrated that the NPS stimulant effects are selectively due to NPSR activation, corroborating the findings obtained with NPSR antagonists. In conclusion, the research activity performed during the PhD program led to the identification of the first generation of NPSR peptide antagonists. The use of these research tools in parallel with knockout studies generated converging evidence demonstrating that the biological effects of NPS are selectively due to the selective activation of NPSR.

1. INTRODUCTION

1.1 Orphan G-protein coupled receptors and the reverse pharmacology approach

G-protein coupled receptors (GPCRs) are one of the largest family of proteins acting as modulators of intercellular interactions and regulating biological functions in the human body, in particularly in the central nervous system (CNS). There are numerous GPCRs in living organisms, but the function of many of them is still unknown. The human genome encompasses ~ 800 GPCRs, of which more than half are olfactory and/or taste GPCRs. GPCRs are targets of most of the primary messengers including neurotransmitters, all neuropeptides, glycoprotein hormones, lipid mediators and other small molecules; thus they have considerable pharmaceutical interest. Drugs acting at GPCRs are used to treat numerous disorders. More than 30% of the approximately 500 clinically used drugs, are modulators of GPCRs function, representing around 9% of global pharmaceutical sales; this makes GPCRs the most successful of any target class in terms of drug discovery [1].

367 transmitter GPCRs have been identified within the human genome, the majority of these GPCRs have been identified on the basis of their sequence similarities, either by homology cloning or by bioinformatics analyses. Some of these receptors luck their endogenous ligand and they are defined 'orphans'. Currently the orphan GPCRs are ~ 140.

The first step in the characterization of an orphan GPCRs is the search of the activating ligand. As the genomes of most studied model organisms have now been sequenced, the process of discovery of GPCRs-ligand pairs has been reversed. Until recently, neuropeptides have been traditionally identified either on the basis of their chemical characteristics [2] or of their effects in particular assay systems [3]. Although highly successful, these approaches had reached a stand still by the mid 80's.

Through DNA recombination techniques, it is now possible to transfect the sequence of an orphan receptor of which the function is not yet known, into an appropriate cellular expression system. This leads to the use of orphan receptors as baits to isolate their natural ligands from fractions of tissue extracts in high-throughput screening (HTS). This approach has been named "reverse pharmacology". The expression system provides the necessary trafficking and G-protein-signalling machinery to enable the successful identification of the activating ligand. By exposing the transfected cell to a tissue extract containing the natural ligand of the orphan receptor, a change

in intracellular second messengers will be induced and will serve as a parameter to monitor orphan receptor ligand purification. Despite the logic of the theory, the process is not simple, since the chemical nature of the ligand and the type of the second messenger response that it will generate, are unknown. However, structural features in an orphan GPCR will determine its relationship to known receptors and will help in evaluating the nature of the receptor's ligand and its cellular action. Indeed, an orphan receptor which is related, even to a low degree, to a particular receptor family has a higher probability of sharing a ligand of the same physical nature and a coupling to similar G proteins. Notably this strategy has already led to several significant discoveries. The orphan receptor strategy was first proven to be successful with the discovery of the neuropeptide nociceptin/orphanin FQ as the endogenous ligand of the orphan GPCR opioid receptor-like 1 [4-5].

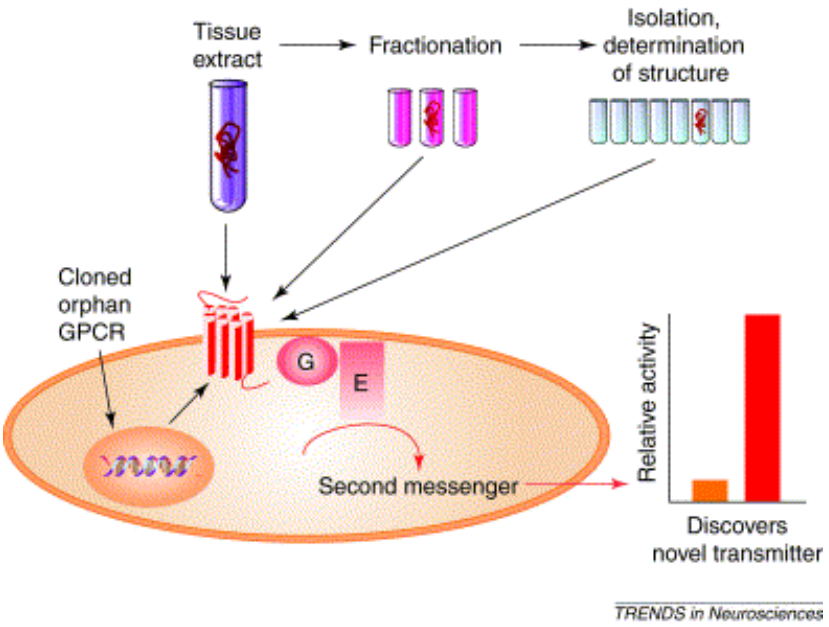

Figure 1 The orphan receptor strategy (Civelli *et al*.,TRENDS in Neurosciences Vol.24 No.4 April 2001). The orphan receptor strategy was developed to identify the natural ligands of orphan G-protein-coupled receptors (GPCRs) with the aim of discovering novel transmitters (defined in the main text). This strategy involves: (1) expression of the cloned orphan GPCR in an heterologous cell line; (2) exposure of this transfected cell line to a tissue extract that is expected to contain the natural ligand; (3) recording of the change in second messenger response elicited by activation of the orphan GPCR; (4) fractionation of the tissue extract and isolation of a surrogate, the active component; (5) determination of the chemical structure of the active component and (6) chemical synthesis of the active component and demonstration that it exhibits identical activity to that of the purified ligand.

This first successful example of orphan receptor strategy was followed by the identification of other novel bioactive peptides such as: hypocretins and orexins, prolactin-releasing peptide, apelin, ghrelin, melanin-concentrating hormone, urotensin II, neuromedin U, metastin, neuropeptide B, neuropeptide W. The last success of reverse pharmacology was in 2004 the identification of neuropeptide s, the subject of this thesis, as the endogenous ligand of the previously orphan GPCR GPR154, now named NPSR. Each of these discoveries was a landmark in its field [6]. The success in GPCRs deorphanization led to the approach being used by pharmaceutical industry [7], which had mastered the HTS of thousands of ligands. This led to thousands of potential transmitters and unexpected ligands (also of non-peptide nature) being tested on dozens of orphan GPCRs and a revival of the reverse pharmacology approach. The novel information emerging from reverse pharmacology discoveries substantially increased our knowledge on the diverse physiological functions modulated by peptidergic systems and most likely will open novel avenues for treating several diseases including food intake and sleep disorders, pain, anxiety and depression in the near future. Table 1 summarizes the transmitters of peptidic and non-peptidic nature identified as ligands of orphan GPCRs [6].

Table 1 Transmitters identified as ligands of orphan GPCRs after nociceptin/orphanin FQ [6].

Date	Transmitters found[b]	
	By synthetic ligand binding	In tissue extracts
1995	–	N/OFQ
1996	C3a	–
1997	LTB_4	–
	Latrotoxin	–
1998	S1P	Hypocretins and orexins
	LPA	PrRP
	–	Apelin
1999	LTD_4	Ghrelin
	MCH	MCH
	UII	UII
	Motilin	–
2000	NMU	NMU
	UDP-glucose	–
	SPC	–
	LTB_4	–
	Histamine	–
	Prostaglandin D_2	–
	LTC_4 and LTD_4	–
	NPFF and NPAF	–
	hRFRP-1 and hRFRP-3	–
2001	LPC	Metastin
	SPC	–
	ADP	–
	Psychosine	–
	Trace amines	–
2002	5-Oxo-ETE	NPB and NPW
	Bile acids	Adenine
	PK1 and PK2	PK1 and PK2
	BAM22	–
	Relaxin	–
2003	Bradykinin	Relaxin 3
	QRFP	–
	Cortistatin	–
	Medium and long fatty acids	–
	Nicotinic acid	–
	Proton	–
2004	β-Alanine	Succinate
	α-Ketoglutarate	NPS
	AMP and adenosine	–

1.2 Neuropeptide S

The identification of NPS as the endogenous ligand of the previous orphan GPCR GPR154 was first reported in the patent literature in 2002 [8]. The patent reported the sequence of GPR154 (GenBank accession numbers BD183774, BD183814, BD183773) and the isolation of NPS as its endogenous ligand, but nothing is provided about the biological functions and the pharmacological characteristics of this new system. Then, in 2004, an elegant study by Xu *et al*. [9] described for the first time some functional features of the NPS/GPR154 system. Following the description by Xu *et al*. [9], in the present work the GPR154 receptor will be indicated as NPSR. However this latter abbreviation has to be considered provisional since it is not in line with IUPHAR recommendations for nomenclature of receptors (the receptor name should not include the letter R as an abbreviation for receptor [10]).

The primary sequence of NPS in humans is SFRNGVGTGMKKTSFQRAKS. The amino acid at the N-terminus of the peptide is serine (S) in all animal species and this was the reason of naming the peptide NPS [9]. The NPS sequence is highly conserved among vertebrates with few variations located in the centre and C-terminus of the peptide [11] (figure 2). The N-terminal sequence Ser^{1}-Phe^{2}-Arg^{3}-Asn^{4}-Gly^{5}-Val^{6}-Gly^{7} is identical in all species, thus suggesting that this may represent the bioactive core of this peptide [11]. Curiously, the peptide is absent in fish genomes (e.g., zebrafish and fugu) and is also not found in amphibian or reptile DNA sequences, indicating that this transmitter arose relatively late during vertebrate evolution [11]. As other neuropeptides, NPS is cleaved from a larger precursor protein. NPS peptide precursor (ppNPS) is a typical neuropeptide precursor containing a hydrophobic signal peptide at the beginning of its sequence and a pair of basic residues (Lys-Arg) before the mature NPS sequence [12].

Figure 2 Alignment of NPS peptide structures from various species. Sequences were deduced from GenBank finished and unfinished genome sequences. Amino acids different from human NPS are highlighted in red [13].

The regional distribution of NPS in rat has been described in two publications by Xu *et al.* [9,14], using quantitative real time polymerase chain reaction (RT-PCR) and in situ hybridization techniques. Quantitative RT-PCR showed that NPS mRNA are expressed in various tissues and the highest levels were found in brain, thyroid, salivary, and mammary glands [9]. Xu and colleague focused their attention to the distribution of NPS and its receptor in the CNS. In situ hybridization experiments demonstrated that in rat brain ppNPS mRNA is expressed discretely in a few areas, with strongest expression in the locus (LC) coeruleus area, principle sensory trigeminal nucleus, and lateral parabrachial nucleus. Moderate expression was also found in a few scattered cells of the dorsomedial hypothalamic nucleus and the amygdala [9]. In particular, in the LC area NPS-expressing neurons defines a previously unrecognized population of cells located between the noradrenergic LC and the Barrington's nucleus [9]. Double label in situ hybridization studies demonstrated that most of the NPS expressing cells in the LC area are glutamatergic neurons, few are cholinergic, while none produce GABA [14]. In the principle sensory trigeminal nucleus many of the NPS expressing neurons use glutamate as a neurotransmitter [14]. Finally, in the lateral parabrachial nucleus NPS positive cells co-express CRF [14]. NPS seems to be co-expressed with excitatory neurotransmitters and on this basis it has been proposed that NPS may provide additional excitatory input to the postsynaptic target of these excitatory neurons.

1.3 NPS receptor

NPSR is also know as vasopressin receptor-related receptor (VRR1) [15] or G protein coupled receptor for asthma susceptibility (GPRA) [16]. NPSR is a typical GPCR, but it shows moderate homology to other members of the GPCR family, the closest relatives being the vasopressin (i.e., V1a and V2) receptors. Human and mouse NPSR were studied in heterologous expression systems, showing that NPS both stimulates intracellular calcium levels and cAMP accumulation with EC_{50} values in the low nanomolar range [9,17]. This indicates that the NPSR can signal via both G_q and G_s protein to increase cellular excitability.

Multiple single-nucleotide polymorphisms (SNP) and several splice variants have been identified in the human NPSR (hNPSR) gene that is located on chromosome 7p14-15 (figure 3). On note Laitinen *et al.* [16] described different polymorphisms in the hNPSR gene linked to an increased susceptibility for asthma and potentially other forms of allergy that are characterized by high IgE serum levels in Finnish and Canadian asthma patients [16]. In particular the study described two different C-terminal splicing variant of hNPSR, named "GPRA isoform A" (GenBank accession number NP_997055) and "GPRA isoform B" (Gen-Bank accession number NP_997056) [16]. The "GPRA isoform A" corresponds to the NPSR receptor extensively studied by Xu [17]. The most interesting of SNPs described for hNPSR gene is an Asn-Ile exchange at position 107 of the mature hNPSR protein (SNP591694 A>T; refSNP ID: rs324981) [17]. This receptor polymorphism seems to have functional implications since the hNPSRIle^{107} receptor displayed similar binding affinity but higher NPS potency (by approx. 10-fold) than hNPSRAsn^{107} [17]. This result has been replicated in different laboratories, using different assays (intracellular calcium and cAMP accumulation) as well as different cell types (HEK293 and CHO) [17-18]. Instead, the C-terminal splice variant of NPSR did not appear to cause measurable differences in the pharmacological profile of the receptor [17-18]. It is worthy of mention that the rat and mouse NPSR contain Ile at position 107. Different epidemiological studies reported a gender-specific association of the hNPSRAsn^{107}Ile polymorphism with panic disorder, sleep behaviour, bowel disease and asthma susceptibility [16,19-21], however, the pathophysiological implications of NPSR polymorphisms are far from being fully understood.

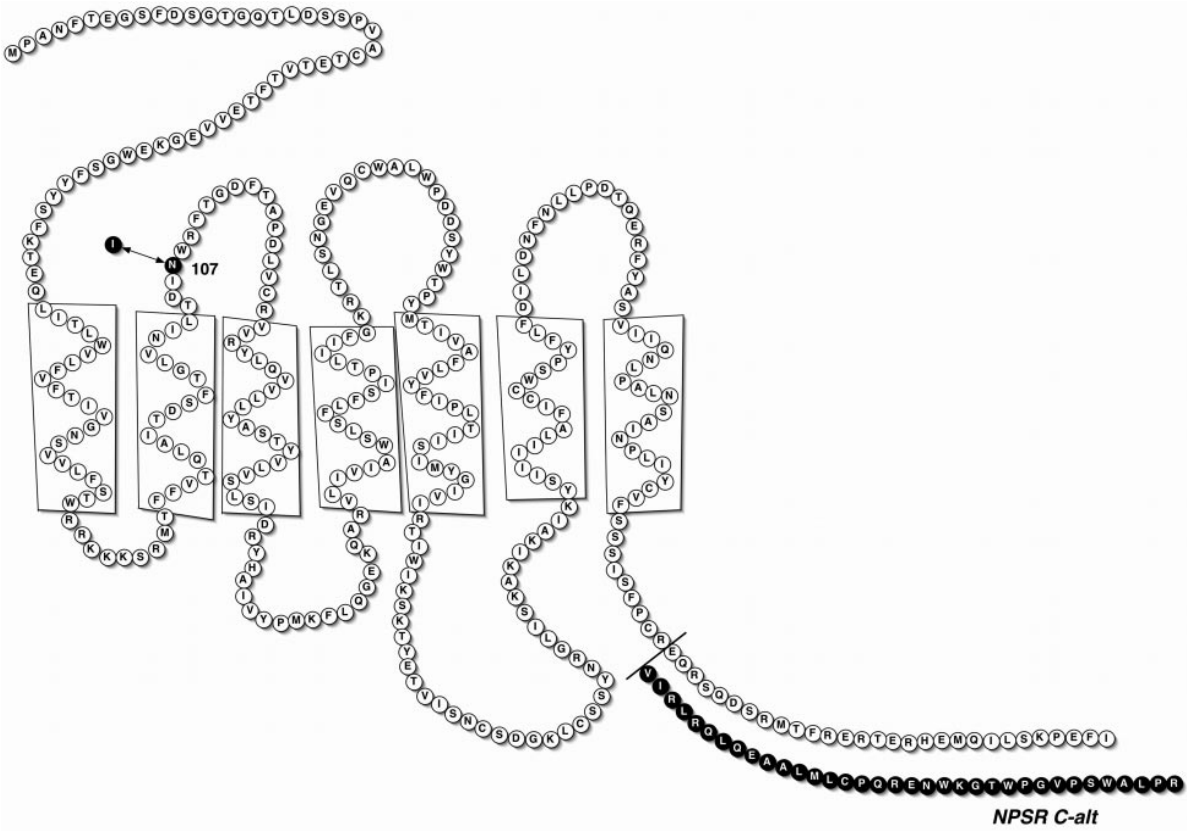

Figure 3 Schematic diagram of the hNPSR protein showing the presumed location of the $N^{107}I$ polymorphism and the sequence of the alternatively spliced carboxyl-terminal tail in NPSR C-alt [17].

Quantitative RT-PCR and in situ hybridization experiments reported that the NPSR mRNA is widely distributed in the brain [9,14]. High levels of expression are found in areas involved in olfactory processing, including the anterior olfactory nucleus, the endopiriform nucleus, and the piriform cortex. NPSR mRNA is also highly expressed in the amygdaloid complex, in the thalamus and in the preoptic region. Furthermore, multiple hypothalamic nuclei, including the dorsomedial and the ventromedial hypothalamic nucleus and the posterior arcuate nucleus, express high levels of NPSR mRNA. In addition, NPSR mRNA is strongly expressed in major output and input regions of hippocampus, including the parahippocampal regions, the lateral entorhinal cortex, and the retrosplenial agranular cortex. These data suggested that the NPS system may play a key role in modulating a variety of physiological functions, especially arousal, anxiety, learning and memory, and energy balance. NPSR transcripts were also found in peripheral tissues, including thyroid, salivary, and mammary glands, which might indicate additional endocrine functions [9]. Recently, Leonard and colleagues generated and validated a NPSR-specific antibody to determine the distribution of the NPSR protein in the rat brain [22]. The localization of the NPSR receptor identified by the use of the antibody was consistent with the mRNA distribution identified by Xu *et al*. using in situ hybridization techniques [14]. NPSR protein was in fact detected in the medial amygdala, substantia nigra pars compacta, subiculum, dorsal raphe, and several hypothalamic and thalamic

regions. Additionally, NPSR protein was localized in the pyramidal cell layer of the ventral hippocampus, the medial habenula, and was widely distributed in the cortex [22].

1.4 Biological actions of NPS

1.4.1 *In vitro* studies

Most of the *in vitro* studies were performed with cells expressing the recombinant NPSR receptor. In these studies NPS was able to increase both intracellular calcium and cAMP levels, displaying similar high potency (EC_{50} in the low nanomolar range) at the rat, mouse and human NPSR. As mentioned above, NPS displayed 10 fold higher potency at the hNPSRIle^{107} isoform than hNPSRAsn^{107}.

Only limited information is available on the *in vitro* effects of NPS in cells/tissues expressing the native NPSR. In our laboratories we assessed the effects of NPS in a series of cell lines measuring intracellular calcium mobilization and in several animal tissues taken from the gastrointestinal, genitourinary, and respiratory system measuring myotropic effects; however, we were not able to find any NPS sensitive preparation. The only peripheral cell types reported to be sensitive to NPS are macrophages and lymphocytes [23-24]. Specifically, macrophages responds to NPS with reduced adhesion and increased phagocytosis, chemotaxis and production of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α [23-24]; moreover NPS stimulates lymphocytes proliferation [24]. These findings together with those coming from genetic studies, which provide evidence for association of NPSR gene polymorphism with chronic inflammatory diseases of the respiratory [16] and gastrointestinal [19] systems, indicate that the NPS/NPSR system may have a role in modulating innate immunity and chronic inflammatory diseases of epithelial barrier organs.

As far as CNS preparations are concerned, NPS has been reported to modulate synaptic activity in mouse brain slices [25-26] and neurotransmitter release in mouse synaptosomes [27]. In particular, two complimentary studies performed in mouse coronal brain slices reported distinct actions of NPS in the amygdala. Meis *et al.* [26] reported in response to NPS an increase in glutamatergic synaptic transmission onto basolateral amygdala GABAergic interneurons; this effect was sensitive to tetrodotoxin suggesting dependence from action potential propagation. The endopiriform nucleus was identified as the site of action of NPS. This NPS sensitive circuit might be responsible for the inhibitory effect of the peptide on the expression of contextual fear [26]. Using the same preparation, Jungling *et al.* [25] demonstrated that NPS increases glutamatergic transmission to intercalated GABAergic neurons in the amygdala via presynaptic NPS receptors onto connected principal neurons. This electrophysiological effect of NPS may likely be important for both the anxiolytic-like action of the peptide and for its ability to facilitate extinction of aversive

memories[25]. The electrophysiological actions of NPS modulating the activity of the circuit endopiriform cortex, lateral, basolateral, and central amygdala has been nicely summarized by Pape *et al.* [28]. Recently, electrophysiological studies demonstrated that NPS depolarizes and thereby excites ventromedial hypothalamic neurons [29]. This depolarization is postsynaptic and involves R- and T-type calcium channels and non selective cation channels [29]. The excitatory effect on ventromedial hypothalamic neurons can represent the cellular process by which NPS participates in the regulation of food intake and energy homeostasis [29]. Furthermore, NPS has been reported to behave as an extremely potent (pM range of concentrations) inhibitor of the release of 5-HT and NA from mouse frontal cortex synaptosomes. In parallel experiments NPS did not modify the release of GABA and glutamate and weakly reduced, only at high concentrations, dopamine and acetylcholine release [27]. However, no evidence was provided for the involvement of the NPSR in these neurochemical actions of NPS. Based on the reported cellular actions of NPS (i.e. increase in intracellular calcium concentrations and cAMP), the ability of NPS to inhibit neurotransmitter release should be considered unexpected. However, the existence of excitatory G protein coupled receptor mediating inhibitory effects on neurotransmitter release has been repeatedly reported (for detailed discussion see Raiteri *et al.* [27]). There is convincing evidence in the literature that elevation of 5-HT and NA levels is associated with anxiety like behaviours; therefore, the inhibition of 5-HT and NA release elicited by NPS may represent at least one of the mechanisms by which NPS promotes its anxiolytic-like effects. The results obtained by Raiteri *et al.* with mouse frontal cortex synaptosomes [27] are in contrast with the recent study performed by Si and colleagues [30] using *in vivo* microdialysis in freely moving rats. Si *et al.* [30] shown in fact that NPS administration increases release of dopamine in the medial prefrontal cortex but does not change 5-HT levels in this brain area. This stimulatory effect of NPS on dopamine release in the medial prefrontal cortex might be functionally connected to the effects of NPS on fear extinction and anxiety [30]. Furthermore, NPS given in the ventral tegmental area was reported to increase dopamine levels in the nucleus accumbens shell in rats, activating the mesolimbic dopaminergic pathway [31]. NPS induced stimulation might likely be mediated by the activation of the dopaminergic release in this circuit [31].

1.4.2 *In vivo* studies

Locomotor activity

In the pivotal study by Xu *et al*. [9] it has been reported that the supraspinal administration of NPS in mice stimulates locomotor activity (LA). This effect is evoked by low doses (0.1 nmol) of peptide, lasts for about 1 hr, and is similar in naïve mice and animals habituated to the test chamber. These findings were later confirmed in several studies both in mice [32-34] and rats [35], suggesting that the hyperlocomotor action of NPS is a robust phenomenon among experimental conditions and animal species. The involvement of NPSR in the locomotor stimulatory effect of NPS has been demonstrated with the use of the NPSR antagonist SHA 68 [33] that was able to counteract the stimulatory effect of NPS being *per se* inactive. These results were corroborate by the use of the NPSR knockout (NPSR(-/-)) mice. NPS was in fact able to increase animals LA in NPSR wild type (NPSR(+/+)) but not in NPSR(-/-) mice and no differences were recorded between NPSR(+/+) and NPSR(-/-) mice [36-38]. All these findings demonstrate that this peptide action is due to NPSR activation and suggest that the endogenous NPS system does not exert a tonic control on animal locomotor behaviour. It was demonstrated that a brain area important for NPS hyperlocomotor action is the ventral tegmental area [31]. Specifically, NPS seems activate the mesolimbic dopaminergic system, that origins from this area and projects in the nucleus accumbens. In fact the NPS stimulant effects were completely block by the administration in the shell of the nucleus accumbens of sulpiride, a dopamine D_2 –like receptor antagonist [31]. This finding indicates a role of the dopaminergic pathway in the neurochemical mechanisms responsible for NPS induced hyperlocomotion. Furthermore, a study by Boeck *et al.* [39] demonstrated that the pharmacological blockade of adenosine A_{2A} receptors by caffeine and the selective A_{2A} antagonist ZM241385 significantly attenuate NPS stimulant effects. Thus the endogenous adenosine system seems play a role in mediating this NPS function. Also corticotrophin releasing factor signalling via CRF_1 receptors seems to be involved in the hyperlocomotor action of NPS. In fact this effect of NPS is blocked by the selective CRF_1 antagonist antalarmin and is not evident in CRF_1(-/-) mice [34]. The microinjection of NPS into the paraventricular nucleus [35] or amygdale [25] mimicked the effects of the peptide given i.c.v. on food intake and anxiety states, respectively, but did not stimulate locomotion. Thus it is likely that these brain areas are not important for the locomotor stimulant effect of NPS.

Arousal and sleep

Electroencephalografic studies in rats demonstrated that the i.c.v. injection of NPS reduced all stages of sleep promoting wakefulness [9]. Similar to the locomotor stimulant action in mice, this effect of NPS lasted for about 1 h and can be evoked using low doses of peptide (0.1-1 nmol). The arousal promoting action of NPS has been also demonstrated using the righting reflex assay. In fact NPS dose dependently (0.1 – 10 nmol) reduced the sleep time of rat treated with ketamine (100 mg/kg) and thiopental (45 mg/kg) [40]. The NPSR antagonist [D-Cys(^{t}Bu)^5]NPS at the dose of 20 nmol was able to completely prevent the arousal promoting action of NPS 1 nmol giving evidence that the arousal promoting action of NPS is due to selective NPSR activation [40]. Moreover, [D-Cys(^{t}Bu)^5]NPS *per se* prolonged both ketamine and thiopental anesthesia duration, suggesting that the NPS/NPSR system tonically controls the anesthesia state [40]. These findings parallel the peptidergic arousal promoting system of the orexins. In fact, orexin receptor antagonists prolonged barbiturate sleeping time in rats [41] and emergence from general anesthesia [42]. Recent studies performed with NPSR(-/-) mice suggested a role played by NPSergic pathways in the modulation of circadian rhythm. In fact NPSR(-/-) mice displayed reduced late peak wheel running (an index of activity of the internal clock) [36] and reduced LA during the dark phase [37] compared to NPSR(+/+) mice. Clearly further studies performed both with NPSR ligands and NPSR(-/-) mice are needed to investigate the role of the NPS/NPSR system in the regulation of wakefulness and sleep physiology and pathology. As far as the brain areas possibly relevant for the arousal promoting effect of NPS are concerned, NPSR mRNA has been reported to be expressed in several structures known to play a major role in the regulation of arousal including the thalamus, hypothalamus, ventral tuberomammilary nucleus, substantia nigra and ventral tegmental area, and the pontine reticular nucleus [14]. In particular the thalamic midline nuclei which integrate the arousal circuit reticular formation-thalamus-cortex express high levels of NPSR mRNA. Microinjection and electrophysiological studies are now needed to establish the role of the above mentioned brain structures in the arousal promoting action of NPS. Interestingly enough, a recent genetic epidemiology study performed on 749 subjects found a clear association between the NPSRAsn^{107}Ile polymorphism and mean bedtime delay [20]. While these findings require replication in other samples, they provide evidence for a role of the NPS/NPSR system in regulating sleep physiology in humans.

Anxiety and mood

The studies by Xu *et al.* [9,14] demonstrated a strong expression of NPSR mRNA in different brain areas related to stress, including the amygdala, bed nucleus of the stria terminalis, hypothalamus, raphe nucleus and ventral tegmental area. This NPSR distribution suggests that the NPS system might influence emotional behaviours such as stress and anxiety responses. The pivotal study by Xu *et al.* [9] reported that the supraspinal administration of NPS in the 0.01 – 1 nmol range of doses evokes a clear anxiolytic-like effects in mice subjected to a battery of validated assays including the elevated plus maze (EPM), the light-dark box, and the open field (OF) [9]. These initial observations were later confirmed and extended to other assays such as the four-plate test and elevated zero maze [34,43]. These tests are based on the natural aversion of rodents for open or unfamiliar spaces and anxiolytic drugs increase exploration of these exposed areas. It should be considered that these assays are sensitive to the confounding effects of drugs, like NPS, that stimulate locomotor activity since in these tests anxiety levels are measured as inhibited behaviours. However NPS was reported to be able to reduce in a dose dependent manner the number of marbles buried in the marble burying test [9,34], a model which is not biased by locomotion since anxiety levels are measured as an active behaviour. Furthermore Leonard and colleagues [43] demonstrated that anxiolytic-like effects can be measured in response to NPS in mice in the stress induced hyperthermia (SIH) test. These results provide evidence that the anxiolytic-like action of NPS is a genuine effect. Subsequently the anxiolytic-like effects of NPS were confirmed and extended to a different species, the rat, and a different assay, the defensive burying test [44]. It was demonstrated that NPS (0.1 – 10 nmol) reduces cumulative burying behaviour in a dose dependent manner without modifying other parameters including latency to contact the probe, burying behaviour latency, number of shocks received or immobility/freezing duration [44]. Again, since the main parameter predictive of anxiolysis in this assay is the inhibition of an active defensive behaviour, motor activity can be considered a minor bias in the outcome of the defensive burying test [45].

The NPS effects were also investigated in the Pavlovian fear conditioning test [25], an established model for fear and emotional memory. In this paradigm, an aversive stimulus (unconditioned stimulus, i.e. short electric foot shock) is associated with a neutral stimulus (conditioned stimulus). After the training (pairing of unconditioned stimulus and conditioned stimulus), associative fear memory is tested by presentation of conditioned stimulus alone and fearful responses can be recorded reliably by measuring ‘‘freezing’’ behaviour. Repeated presentation of the conditioned stimulus alone without the aversive stimulus leads to a gradual decline in fear responses, which indicates extinction of fear memory. It was demonstrated that NPS

given into the amygdala produces a significant decrease of fear behaviour and facilitates the extinction of conditioned fear responses [25]. Next, Fendt *et al.* [46] reported that the intra amygdala administration of NPS completely block the expression of the fear-potentiated startle [46]. Thus the NPS/NPSR system seems to have a role in controlling the mechanisms of conditioned fear, in particular eliciting fear reducing effects.

The amygdala can be the brain area crucial for NPS anxiolytic action. In fact, the intra amygdala injection of NPS promoted clear anxiolytic effects in the mouse EPM and OF assays [25], mimicking the effects of the peptide given i.c.v [9]. Furthermore the NPS effects on fear expression were detected giving it into the amygdala [25,46]. Another brain region important for the NPS effects on anxiety and fear seems to be the medial prefrontal cortex, where NPS induces an increase in dopamine levels, probably through the activation of the dopaminergic neurons originated in the ventral tegmental area [30].

The endogenous NPS/NPSR system might tonically control anxiety levels since the intra amygdala injection of the NPSR antagonist SHA 68 produces robust anxiogenic effects in mice subjected to the open field [25]. Furthermore a study by Duangdao and colleagues [36] reported that NPSR(-/-) mice display an anxious phenotype compared to their wild type littermates in the light-dark box, EPM and OF. On the contrary Zhu *et al.* [38] and Fendt *et al.* [37] observed no differences between the behaviour of NPSR(+/+) and NPSR(-/-) mice in the OF, EPM and elevated zero maze tests, suggesting that the endogenous NPSergic pathway does not tonically control anxiety levels. The hypothesis that the NPS/NPSR system does not play a role in modulating anxiety is corroborated by the absence of anxiogenic effects *per se* of the NPSR antagonist SHA 68 in the EPM and defensive baring assays in rat [47]. These indications need however to be confirmed in future studies investigating the effects of different chemically unrelated NPSR receptor selective antagonists as well as performing systematic investigations of the phenotype of NPSR(-/-) and possibly ppNPS(-/-) [48] mice in different assays predictive of anxiety states. Interestingly NPS failed to elicit anxiolytic-like actions in OF and elevated zero maze assays in mice lacking the NPSR protein [38], demonstrating that the NPS anxiolytic activity is selectively due to the NPSR activation. This is also demonstrated by the fact that SHA 68 was able to counteract the NPS effects in the EPM and defensive baring assays in rats [47].

On note different epidemiological studies reported an association between NPSR SNPs and susceptibility to panic disorders. In particular the NPSR isoform NPSRAsn^{107} was found underrepresented in patients with panic disorder and the NPSRIle^{107} isoform was found associated with an over interpretation of fear reaction and a tendency to catastrophizing [21,49-51]. This association of the gain of function SNP hNPSRIle^{107} with panic disorder in humans is unexpected

and seems inconsistent with findings in rodent models, where NPS have been shown to exert a dose dependent anxiolytic effect. Thus the role plays by the NPS/NPSR system in the control of panic is still unclear and further studies are needed to address this issue.

Interestingly enough, the effect of NPS on emotional behaviour seems to be restricted to anxiety and fear since the peptide was found inactive in tests such as the tail suspension [43] or the forced swimming test (FST) (Rizzi *et al*., unpublished results) that are sensitive to the antidepressant- like effects of drugs. However preliminary results indicate that NPS may alter both anxiety- and depression-like behaviours in a rat genetic model of depression. In fact, in flinders sensitive rats NPS decreased depression-like behaviour in the forced swimming test and anxiety-related behaviour on the EPM in a dose-dependent manner (0.05 – 1 nmol, i.c.v.). In contrast, NPS did not alter the behaviour of flinders resistant animals [52]. A recent study performed with NPSR(-/-) mice reported that male mice lacking the NPSR receptor display increased depression-like behaviour in the FST. However Duangdao *et al.* reported no differences in the behavior of NPSR deficient mice compared to wild type mice in the FST [36]. Thus the role of the NPS/NPSR system in mood regulation is not well understood.

In conclusion, the initial findings by Xu *et al.* [9] were replicated and extended in different laboratories confirming that NPS promotes in the same range of doses stimulation of locomotor activity and arousal associated to a genuine anxiolytic-like action. Thus, the proposal of NPS as an activating anxiolytic [9], is confirmed after six years of research activities. This rather unique behavioral profile challenges the common idea that anxiolytics are also sedative (i.e. benzodiazepines) or that stimulants are also anxiogenic (i.e. caffeine, cocaine, and amphetamines). The only substance which shares this behavioral profile with NPS is nicotine which increases arousal and wakefulness and produces, at least in smokers, anxiolysis and antistress effects [53]. Interestingly enough, chronic nicotine treatment in rats increases both NPS and NPSR expression in the brainstem and NPSR in the hypothalamus [54]. Thus nicotine might produce some of its effects via regulation of the endogenous NPS/NPSR system.

Drug addiction

The unique pattern of behavioral effects elicited by NPS (arousal promoting action associated with anxiolysis) together with the expression of NPSR in brain areas involved in the rewording effects of drugs has prompted the investigation of NPS effects on drug addiction.

In conditioned place preference studies in mice, NPS neither induced place preference nor aversion. However, NPS blocked the acquisition of conditioned place preference to morphine. Moreover, the expression of conditioned place preference induced by morphine was also inhibited by NPS. These results revealed the involvement of NPS in rewarding activities of morphine [55].

The effects of NPS were also evaluated on ethanol drinking in alcohol-preferring and non preferring rats [56]. NPS given i.c.v. reduced ethanol intake in alcohol-preferring, but not in non preferring rats. The peptide neither altered anxiety-like behavior in the EPM test nor modified general motor activity. However, there was an increase in the amount of time spent in the center of the activity cage following infusions of 0.6 nmol of NPS in preferring, but not in non preferring rats, indicating anxiolytic actions of the peptide. Thus, this study suggests a role for NPS in the modulation of ethanol drinking and possibly anxiety-like behavior in rats selectively bred for high alcohol drinking [56].

Recently, two complimentary studies investigated the role of NPS in drug seeking behavior [34,57]. Cocaine-seeking behavior was evaluated in mice by Paneda *et al.* [34]. It was demonstrated that i.c.v. NPS reinstates extinguished cocaine-seeking behavior in a dose-dependent manner. At the highest dose tested i.e. 0.45 nmol, NPS increased active lever pressing in the absence of cocaine to levels that were equivalent to those observed during self-administration. This action of NPS involved corticotropin-releasing factor receptor signaling via CRF_1 receptor since CRF_1(-/-) mice did not respond to the cocaine reinstatement effects of NPS and the CRF_1 antagonist antalarmin blocked the increase in active lever responding in response to NPS [34]. Ethanol seeking behavior was investigated in rats by Cannella *et al.* [57]. In self-administration experiments, the stable response rates observed for ethanol reinforcement were not modified by i.c.v NPS (1.0 and 2.0 nmol). In reinstatement experiments, ethanol-associated cues induced robust rates of ethanol seeking. NPS i.c.v. injection resulted in a significant increase of ethanol seeking elicited by ethanol-associated cues. Site-specific NPS injection (0.1 and 0.5 nmol) into the lateral hypothalamus also reinstated extinguished responding to ethanol. This effect was selectively blocked by pre-treatment with the OX_1 receptor antagonist SB-334867 which did not modify alcohol reinstatement *per se*. This study provided the first demonstration that activation of NPSR in the lateral hypothalamus, via activation of orexin releasing neurons, intensifies relapse to

ethanol-seeking elicited by environmental conditioning factors [57]. Furthermore NPSR mRNA expression was found enhanced in rat with a history of ethanol addiction in different brain areas, suggesting an involvement of the NPS/NPSR system in controlling mechanisms of ethanol abuse [58]. Finally, a very recent study performed by Kallupi *et al.* [59] confirmed that NPS increases conditioned reinstatement of cocaine-seeking in rats through the activation of orexin immunoreactive neurons in the lateral hypothalamus. Interestingly the administration of the two chemically distinct NPS receptor antagonists SHA 68 and [D-Cys(^{t}Bu)^5]NPS potently and selectively prevented cue-induced cocaine seeking, suggesting that the endogenous NPS may have a role in the pathophysiology of drug relapse [59]. Based on these findings, the NPSR receptor represents an important and unique target for the treatment of drug craving and the prevention of relapses in addicted patients.

Food intake

Several studies demonstrated that NPS is able to reduce food intake acting as an anorexigenic signal in the brain. The first evidence for this NPS action was provided by Beck *et al.* [60] who demonstrated that the i.c.v. injection of NPS in the 0.4 – 4 nmol range strongly inhibited chow intake in overnight fasted rats with effects of longer duration with the highest dose. In the same study similar inhibitory effects were observed for the spontaneous intake of a palatable diet [60]. Smith and colleagues [35] later demonstrated that the injection of low NPS doses in the paraventricular nucleus of the hypothalamus produces robust anorexigenic effects. The observations in rats were confirmed in chicks where NPS inhibits food intake both injected i.c.v. and in the paraventricular nucleus or lateral hypothalamus [61]. In a follow up study [62] the same authors demonstrated that chickens lines selected for low or high body weight are differently sensitive to NPS. These data indicate that NPS may differentially affect appetite-related processes in hypo- and hyperphagic individuals. Subsequently, Fedeli *et al.* [63] further demonstrated that NPS elicits a marked inhibition of palatable food consumption in rats and that the paraventricular nucleus of the hypothalamus represents an important site of action for this NPS effect [63]. Moreover the NPSR antagonists [D-Cys(^{t}Bu)^5]NPS was able to completely counteract the NPS action on palatable food intake being *per se* inactive [63]. These results demonstrate that the NPSR protein is involved in the anorectic action of NPS and suggest that the NPS/NPSR system does not exert a tonic control on this biological function. This hypothesis were then confirmed in two parallel studies by Peng *et al.* [64] using the NPSR antagonists [D-Val^5]NPS and Cifani *et al.* [65] using the NPSR antagonists [D-Cys(^{t}Bu)^5]NPS and [^{t}Bu-D-Gly^5]NPS and the NPSR partial agonists

[Aib^5]NPS and [Ala^3]NPS. [D-Val^5]NPS was in fact able to block the NPS anorectic action being *per se* inactive in fasted mice [64], moreover [D-Cys(^{t}Bu)^5]NPS and [^{t}Bu-D-Gly^5]NPS counteracted the palatable food intake inhibition induced by NPS and they did not affect palatable food intake alone [65]. [Aib^5]NPS, like NPS, reduced food intake, instead [Ala^3]NPS blocked the NPS action being *per se* inactive [65]. All these results corroborate the evidence that NPS elicits inhibition of food consumption through selective NPSR activation and that the NPSergic pathway does not have a role in the tonic regulation of feeding behaviour. Obviously studies performed with NPSR(-/-) mice are needed to further confirm these findings.

Interestingly, the NPS effects on food consumption were not modified by the CRF receptor antagonists CRF 9–41 [63] and NBI-27914 [64], indicating that palatable food intake inhibition by NPS is independent from the activation of the CRF system.

While the above mentioned studies converge indicating that NPS reduce food intake, the report by Niimi *et al.* [66] provided opposite findings showing that the i.c.v. injection of NPS (1 nmol) in rats slightly but significantly stimulated feeding. In the same study it has been shown that centrally administered NPS increased Fos-like immunoreactivity in orexin-expressing neurons of the lateral hypothalamic area. Thus the orexin system may take part to the actions of NPS on food intake.

Learning and memory

NPSR mRNA is expressed significantly in the major input and output regions of hippocampal formation, which are critical in the modulation of learning and memory. The first study aimed to investigate the effects of NPS on spatial learning and memory was performed by Han *et al.* [67] using the Morris water maze task. It was demonstrated that NPS (1 nmol) given i.c.v. before training trials facilitates spatial memory acquisition, in fact NPS treated mice spent significant more time in the target quandrant of the maze [67]. Furthermore NPS was able to mitigate the spatial memory impairment caused by the *N*-methyl-D-aspartate receptor antagonist MK801 [67] and by rapid eye movement sleep deprivation [68]. This first study by Han and colleagues [67] did not provide any indication regarding the role exerts by the endogenous NPS in regulating the mechanisms of spatial learning and memory. However a subsequent study performed with NPSR(-/-) mice reported no differences between the performance of NPSR(+/+) and NPSR(-/-) mice in the Morris water maze test, suggesting that endogenous NPS may not modulate spatial learning and memory under basal conditions [38]. A very recent study by Okamura *et al.* [69] demonstrated that NPS enhances long-term memory, possibly by acting during the consolidation phase. In the

inhibitory avoidance test NPS (1 nmol) was able to enhance emotionally aversive long-term memory until 96 hours after post training session [69]. Similarly, NPS increased the non-aversive memory in the novel object recognition (NOR) task until one week after the training trial [69]. Of note, NPS was effective when given after the training session, instead pre-training or pre-retrieval NPS administrations failed to enhance memory retention, suggesting that NPS may act during the consolidation phase [69]. Interestingly the NPSR antagonist SHA 68 completely counteracted the NPS effect in the inhibitory avoidance paradigm demonstrating that the mechanism by which NPS facilitate emotionally aversive memory is the selective activation of the NPSR protein [69]. Furthermore NPSR deficient mice shown impaired performance in the inhibitory aversive memory, novel object recognition, and novel place or context recognition, suggesting that activity of the endogenous NPS system is required for memory formation [69]. The central noradrenergic system seems to be involved in the NPS-induced memory enhancement, since the block of the noradrenergic receptors by propranolol attenuated this NPS effect [69]. In summary NPS system can have a significant role in the facilitation of various type of memory, such us spatial, emotional aversive and non aversive memory.

Pain transmission

NPSR mRNA is highly expressed in the periaqueductal gray [14], which is an important component of descending analgesic system [70]. Using the tail withdrawal and hot-plate test Li *et al.* [71] demonstrated that NPS (0.01–1 nmol, i.c.v.) caused a dose-dependent antinociceptive effect. The first findings by Li *et al.* were then confirmed by Peng and colleagues [72], using the formalin test. This is a very useful model of clinical pain in which the first phase seems to be caused by direct activation of peripheral nociceptors, while the second phase is considered to be dependent on an inflammatory reaction caused by peripheral and central sensitization. NPS injected i.c.v. significantly reduced both the first and the second phase of nociceptive behaviour [72]. Interestingly, the antinociceptive effects elicited by NPS in the tail withdrawal and hot-plate test were completely prevented by the NPSR peptide antagonist [D-Cys(^{t}Bu)^5]NPS [71], while the NPSR antagonist [D-Val^5]NPS was able to block the NPS actions in the formalin assay [72]. The NPSR antagonists given alone did not modify nociceptive transmission [71-72]. These results reveal that NPS may produce antinociceptive effects through NPSR activation and suggest that the endogenous NPS pathway does not control pain transmission under these experimental condition. Naloxone did not affect NPS antinociceptive activity in all the assays, indicating that opioid systems are not involved in this action [71-72]. The antinociception induced by NPS can be due to the

activation of neurons in periaqueductal gray, as suggested by the increased c-Fos expression in this brain area measured in response to NPS administration [72].

Colonic transit

It has also been reported that NPS inhibited, in a dose-dependent manner (0.001–1 nmol), distal colonic transit (measured as fecal pellet output and bead expulsion time) after i.c.v. but not i.p. administration [73]. The effect evoked by NPS 0.01 nmol was sensitive to the NPSR antagonist [D-Val^5]NPS (0.1 and 1 nmol, i.c.v.), demonstrating the involvement of NPSR in this biological action of NPS [73]. Of note, two different studies reported that NPS, in HEK293cells trasfected with the NPSR receptor, increases the expression of several peptide involved in the control of physiological motor and sensory functions in the gastrointestinal tract, such as somatostatin, cholecystokinin, vasoactive intestinal peptide and peptide YY [74-75]. Obviously further study are needed to verify this NPS action in native intestinal enteroendocrine cells.

In conclusion there is convincing evidence in the literature that the NPS/NPSR system regulates multiple biological functions including locomotor activity, arousal and wakefulness, fear expression and anxiety, food intake and gastrointestinal functions, drug addiction, memory processes, and pain transmission.

1.5 NPSR ligands

1.5.1 NPSR peptide ligands

Soon after the identification of the NPS/NPSR system, different research groups started medicinal chemistry programs directed to identify the NPS bioactive sequence and its crucial residues involved in NPSR recognition. To this aim classical Ala- and D-scans together with N- and C-terminal truncation studies were performed [17-18,76]. The systematic replacement of the NPS amino acid sequence with Ala residues can give information on the contribution of each amino acid side chain for NPS bioactivity. Nineteen Ala-substituted NPS analogues were synthesized and evaluated in a calcium mobilization assay using HEK293 cells expressing the hNPSR receptor by Roth *et al.* [76]. Results of these studies indicated that positions 2, 3, 4, and 7 are crucial for NPS bioactivity. In particular, the replacement of Phe^2 with Ala generated a completely inactive NPS analogue while [Ala^4]NPS and [Ala^7]NPS displayed a drastic loss of potency. Interestingly, [Ala^3]NPS bound to the NPSR receptor with about 10-fold reduced potency compared to NPS and was able to elicit maximal effects corresponding to 50% of those elicited by the natural peptide. Thus, [Ala^3]NPS behaves as a NPSR partial agonist. Ala substitution in the other NPS positions was fully tolerated and did not significantly modified either the potency or the efficacy of the [Ala^x]NPS analogs. Similar studies provided converging evidence demonstrating that the sequence Phe^2-Arg^3-Asn^4 is critical for NPS biological activity [18]. This study also confirmed the important role played by Gly^7 for NPSR recognition. D-scan investigation was applied to collect information on the contribution of the single amino acid chirality for NPS biological activity. Confirming Ala-scan results, these studies revealed that the NPS portion Phe^2-Arg^3-Asn^4 is of great importance for NPSR binding and activation [76]. In fact, replacement of these residues with their enantiomers consistently produced an important loss of peptide potency. Inversion of chirality of Val^6 generated a low potency NPS derivative. D-amino acid substitution of all the other chiral positions did not modify either the potency or the efficacy of the [D-Xaa^x]NPS analogues [76]. N- and C-terminal truncation studies [17-18,76] consistently identified in the N-terminal portion of NPS the crucial amino acid sequence needed for NPSR binding and activation. In particular, the deletion of Ser produced a moderate decrease in peptide potency while further deletion of Phe^2 generated an inactive analog [18,76]. In contrast, systematic deletion of up to ten residues from the C-terminal part of NPS did not produce major changes in peptide biological activity [17-18,76]. Further, C-terminal shortening of the NPS sequence produced different results with Roth *et al.* [76] reporting loss of activity while Bernier *et al.* [18]

reporting NPS(1–6) as a high potency NPSR agonist. The NPS(1–10) fragment was further investigated *in vivo* in locomotor activity experiments performed in mice. While NPS elicited a dose-dependent stimulatory effect in the range 0.01–1 nmol, NPS(1–10) was found inactive up to 10 nmol [76]. These results indicated that the NPS(11–20) sequence seems to be important for maintaining *in vivo* biological activity. Collectively, the data obtained with these first SAR studies demonstrated that: (i) the most important residues for NPSR recognition are Phe^2-Arg^3-Asn^4; (ii) the sequence Val^6-Gly^7 is also important for NPSR bioactivity; (iii) the C-terminal 11–20 sequence of NPS is not required for in vitro activity while it is necessary for its in vivo biological effects. Important suggestions for the design of novel peptide ligands can be obtained investigating the conformation of a given peptide in different environments. NPS conformational investigations performed by NMR experiments indicated that NPS presents a completely disordered conformation in water [77]. However, several NH-NH cross peaks were observed by Bernier *et al.* [18] in the NPS region 5–13; this lead these authors to hypothesize the presence of a nascent helix in this region, which, during the NPSR binding process, may favour the formation of a stable α helix structure. On the other hand, disordered NPS conformations were observed in different NMR solvent mixtures that may favour helicity [77]. This NPS behaviour has been ascribed, at least in part, to the presence in position 5, 7, and 9 of the flexible amino acid residue Gly. In order to force NPS to adopt a stable a helix structure, residues 7, 9, and 13 were replaced with Ala. NMR analyses confirmed that [$Ala^{7,9,13}$]NPS adopts a very stable helix spanning all the peptide sequence but this peptide was completely inactive at NPSR [77]. Single amino acid substitutions indicated that only the replacement of Gly^7 with Ala or Aib (2-amino-2-methylpropionic acid) is able to induce a significant helical structure, whereas [Ala^9]NPS and [Ala^{13}]NPS showed a limited degree of helicity [77]. Interestingly, [Ala^9]NPS and [Ala^{13}]NPS were almost as active as NPS at NPSR while [Ala^7]NPS and [Aib^7]NPS were found inactive. These results indicate that a helical conformation centred around position 7 is not compatible with NPS biological activity. Different results were obtained with substitution of Gly^5 with L- and D-Ala. In fact, both substitutions generated peptide derivatives that showed only slightly reduced potency compared to NPS [77]. This suggests that an a helix conformation (favoured by L-Ala but not by D-Ala) is, at least in this portion of the NPS sequence, not important for NPSR binding. Interestingly, [D-Ala^5]NPS behaves as a partial agonist at NPSR with efficacy corresponding to half of that of NPS. The replacement of Gly^5 with the achiral α helix promoting residue Aib did not produce a significant increase in peptide helicity and generated an NPS analogue with reduced potency and, more importantly, with a statistically significant reduction in efficacy [77]. These results may be interpreted assuming that the introduction of a methyl group (L- and D-Ala) or two methyl groups (Aib) on the Cα carbon of position 5 limits peptide flexibility and

this causes a reduction in potency. In addition, this reduction of conformational freedom may favour partial agonist ([D-Ala^5]NPS, [Aib^5]NPS), or full agonist ([L-Ala^5]NPS) pharmacological activities.

Collectively, this conformation-activity study together with SAR investigations demonstrated that: (i) the sequence Gly^5-Val^6-Gly^7 may represent a flexible peptide region important for inducing and/or stabilizing NPS bioactive conformations; (ii) an α helix conformation around position 7 is not compatible with NPS biological activity; (iii) modifications of Gly^5 may be critical for the design of NPSR ligand with reduced efficacy.

1.5.2 NPSR non peptide ligands

The first example of non peptide molecules able to interact with the NPSR was reported in the patent literature by Takeda researchers [78]. These compounds are characterized by a 3-oxo-tetrahydro-oxazolo[3,4-a]pyrazine scaffold mainly substituted in position 1 and 7. Among this series of compounds, SHA 68 (figure 3) i.e. the racemic mixture (9R/S)-3-oxo- 1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (reported as example 25 in Fukatzu *et al.* [78]) has been selected by Okamura *et al.* [33] for *in vitro* and *in vivo* pharmacological characterization. In radioligand ([^{125}I][Tyr^{10}]NPS) binding experiments SHA 68 displayed high affinity (pK_i 7.3) for human NPSR. In cells expressing the hNPSR SHA 68 was inactive *per se* while antagonizing the stimulatory effects of NPS on calcium mobilization in a competitive manner. Similar high pA_2 values were obtained at hNPSR Ile^{107} (7.6) and Asn^{107} (7.8) receptor isoforms. SHA 68 appears to be selective for NPSR since it did not affect signalling at 14 NPSR unrelated G-protein coupled receptors [33]. Pharmacokinetic analysis demonstrated that SHA 68 reaches pharmacologically relevant levels in plasma and brain after i.p. administration. Furthermore, peripheral administration of SHA 68 in mice (50 mg/kg i.p.) was able to antagonize NPS-induced stimulation of locomotor activity [33]. In a separate study SHA 68 given into the amygdala exerted functionally opposing responses compared to NPS [25]. Thus, available data demonstrated that SHA 68 behaves as a potent and selective NPSR pure antagonist. SAR studies performed on position 7 of SHA 68 indicate that a urea functionality is required for potent NPSR antagonist activity while alkylation of the urea nitrogen or replacement with carbon or oxygen generated less potent derivatives. In addition, compounds with a-methyl substitution or elongated alkyl chains had reduced potency, indicating a limited tolerance for position 7 substituents. The only chemical modification tolerated in this position was the elimination of the fluorine atom in the para position of the phenyl ring; this generates a molecule (SHA 66), which displays similar potency to the parent compound [33,79].

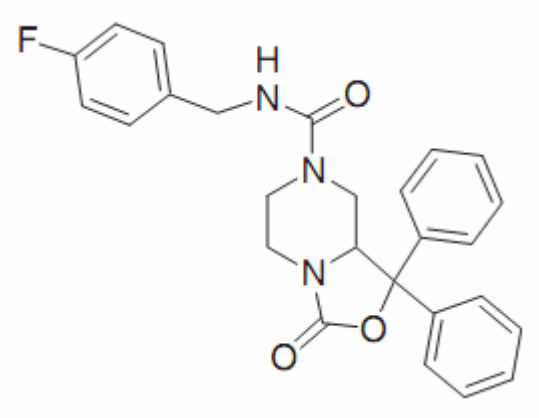

Figure 4 Chemical structure of the non peptide NPSR antagonist SHA 68

Subsequently, Merck researchers, using HTS techniques, identified and developed two new structural classes of potent NPSR antagonists, characterized by a quinoline [80] and a tricyclic imidazole [81] scaffold. From the first series of compounds the NPSR antagonist NPSR-QA1 has been identified. This compound showed potent binding to the rat NPSR receptor in *in vitro* binding displacement studies with an IC_{50} of 1.3 nM, furthermore it provided high occupancy of NPSR in the brain after i.p. dosing [80]. From the tricyclic imidazole series the compound NPSR-PI1 was identified, with an IC_{50} of 43 nM in FLIPR experiments. It exhibited significant plasma free fractions after i.p. administration, enabling *in vivo* central nervous system exposure [81]. Thus NPSR-QA1 and NPSR-PI1 are potent, highly selective and CNS penetrant NPSR antagonist and they represent a useful pharmacologic tool for further study of the effects of central NPSR antagonism *in vivo*. Up to now no data are reported on the effectiveness of these NPSR antagonists *in vivo*.

Recently GlaxoSmithKline researchers published a new series of NPSR antagonists, with 5-phenyl-2-[2-(1-piperidinylcarbonyl)phenyl]-2,3-dihydro-1H-pyrrolo[1,2-c]imidazol-1-one structure [82].

1.6 NPSR knockout mice

NPSR(-/-) mice were generated on 129S6/SvEv strain in Taconic laboratories as described in Allen *et al.* [83]. The inactivation of the NPSR protein was obtained by replacing the majority of exon 4 with the neomycin cassette. This deletion is predicted to remove the third transmembrane spanning domain and regions of the intracellular loop 2 [83]. Allen and colleagues investigated the functional significance of NPSR in the regulation of lung physiology and pathology. Despite the loss of functional NPSR, no differences were detected in the development of asthma and allergic lung pathology in NPSR(-/-) mice. Neither qualitative nor quantitative differences in the cellular infiltrate, nor the development of airway hyperresponsiveness in response to methacholine, distinguished the mutant mice from their littermate controls [83]. Thus, no evidence for abnormal respiratory or immunological functions were recorded in mice lacking NPSR. These first findings by Allen and colleagues were very recently confirmed in a study performed by Zhu *et al*. [84] using NPSR deficient mice in which the exon 2 was deleted. No differences in the lung inflammation and respiration in response to ovalbumine or A. fumigatus exposure were measured between NPSR(+/+) and NPSR(-/-) mice, suggesting that endogenous NPS is not directly involved in the development of experimental asthma [84].

In the last few years the behavioural phenotype of NPSR(-/-) mice was deeply investigated in different laboratories, using NPSR(-/-) mice on a 129S6/SvEv [36] or C57BL/6 genetic background [37-38,50]. The first of these studies was performed by Duangdao *et al.* [36]. They reported that NPSR(-/-) mice display lower late peak wheel running activity during the subjective evening at the end of the dark phase, suggesting that a functional NPS system is required for expression or maintenance of arousal. This hypothesis was corroborate by the subsequent results obtained by Fendt and colleagues [37], that recorded a decreased LA during the dark phase in mice lacking the NPSR protein.

Interestingly, NPSR(-/-) mice were found by Duangdao *et al.* [36] to display increased levels of anxiety-like behaviours in the OF, EPM and light dark box tests, providing evidence for a substantial role of the endogenous NPS system in regulating anxiety levels. On the contrary, no differences were observed in the OF and in the elevated zero maze assays by Zhu and colleagues [38] and in the OF and EPM tests by Fendt *et al.*[37]. Importantly, NPS failed to have anxiolytic effects in the elevated zero maze and OF tests in mice lacking the NPSR receptor [38]. Thus it was demonstrated that NPS elicits anxiolysis through the selective activation of the NPSR protein.

No differences were recorded between NPSR(+/+) and NPSR(-/-) mice by Duangdao *et al.* [36] in the FST, indicating that NPS/NPSR system is not involved in regulating depressive-like behaviour, instead Zhu *et al.* [38] found an increased depressive phenotype in male NPSR(-/-) mice in the FST but not in the tail suspension test. Furthermore NPSR(-/-) mice are reported to be less reactive in the acoustic startle response test by Zhu *et al.* and Fendt *et al.* [37-38], but no differences were recorded by Duangdao *et al.* [36] in this paradigm.

No differences were reported between NPSR(+/+) and NPSR(-/-) mice in terms of spontaneous LA [36-38], furthermore the sensitivity of NPSR(-/-) mice to the stimulant action of metamphetamine [38] and cocaine [37] was superimposable to that of NPSR(+/+) mice. These results clearly indicate that the endogenous NPS does not control mice locomotion and is not involved in the mechanisms by which cocaine and metamphetamine elicit stimulation. All the research groups demonstrated that NPS is able to increase mice locomotion in NPSR(+/+) but not in NPSR(-/-) mice, thus the NPS stimulant effects are selectively due to the NPSR activation [36-38]. NPSR(-/-) appear to have improved motor coordination in the accelerating rotarod test when compared to NPSR(+/+) mice, instead body weight and body length appeared equal between both groups of mice [36].

A very recent study by Okamura *et al.* [69] shown that mice lacking the functional NPSR receptor display deficits in inhibitory avoidance memory, NOR and novel place or context recognition, suggesting that activity of the endogenous NPS system is required for memory formation. Instead the performance of NPSR(-/-) mice in the Morris water maze test was similar to NPSR(+/+) mice, indicating that the NPS/NPSR system is not involved in spatial learning and memory mechanisms [38].

Finally, the plasma corticosterone levels after a stressful event (FST or immobilization) were found superimposable in NPSR(+/+) and NPSR(-/-) mice [38,50], instead after 1 hour of immobilization the gene expression levels of some anxiety and stress-related genes was found altered in NPSR(-/-) mice. In particular these mice presented an upregulation of JunB and interleukin 1 β in the cortex and hypothalamus and a downregulation of the neurotrophic factor neurotrophin 3 in cortex and striatum [50].

1.7 Aims

The main objectives of this work have been to:

- perform SAR studies aimed at the identification of novel and useful ligands selective for the NPSR receptor; these activities allow us to identified the first generation of NPSR peptide antagonists.
- investigate the *in vivo* action of NPS in mice in different behavioural assays, including LA, RR, EPM, OF and SIH tests.
- characterize the *in vitro* and *in vivo* pharmacological profile of peptide and non peptide NPSR antagonists.
- deeply evaluate the phenotype and NPS sensitivity of mice lacking the NPSR receptor in a battery of behavioural assays.

2. MATERIALS AND METHODS

2.1 Drugs and reagents

NPS and all related peptides described in this study were synthesized in the laboratory of Prof Guerrini (Department of Pharmaceutical Sciences and Biotechnology Centre, University of Ferrara) using standard solid-phase synthesis techniques and purified using High Pressure Liquid Chromatography, according to previously published methods [76]. Amino acids, protected amino acids, and chemicals were purchased from Bachem, Novabiochem, Fluka (Switzerland) or Chem-Impex International (U.S.A).

The compound SHA 68 was synthesized using the procedures described by Okamura *et al.* [33].

Caffeine and diazepam were purchased from Sigma Chemical Co (St Louis, MO, USA). All cell culture media and supplements were from Invitrogen (Paisley, U.K.). GoTaq DNA Polymerase and all PCR reagents were from Promega (Milan, IT). All the consumables and reagents were of the highest purity available.

For *in vitro* experiments, the peptides were solubilized in bidistilled water and stock solutions (1 mM) were stored at -20 °C until use; SHA 68 was solubilized in dimethyl sulfoxide at a final concentration of 10 mM and stock solutions were kept at -20 °C until use. The successive dilutions were made in HBSS/HEPES (20mM) buffer (containing 0.02% bovine serum albumin fraction V).

For *in vivo* studies, all peptides were dissolved in saline; SHA 68 was dissolved in saline containing 10% cremophor EL (Sigma) just before performing the experiment. Caffeine, morphine and amphetamine were dissolved in bidistilled water; diazepam was dissolved in water containing 0.5% Tween 80 (Sigma).

2.2 Buffer composition

Calcium mobilization assay buffers:

HBSS (Hanks' Balanced Salt Solution): KCl (5.4 mM), KH_2PO_4 (0.44 mM), NaCl (137 mM), $NaHCO_3$ (4.2 mM), Na_2HPO_4 x 7 H_2O (0.25 mM), $CaCl_2$ (1.3 mM), $MgSO_4$ x 7 H_2O (1 mM), glucose (5 mM).

Loading solution: cell culture medium, HEPES (20 mM), probenecid (2.5 mM), calcium-sensitive fluorescent dye Fluo 4AM (3 μM), pluronic acid (0.01%).

Dye loading solution: HBSS, HEPES (20 mM), probenecid (2.5 mM), Brilliant Black (500 µM).

2.3 *In vitro* studies

2.3.1 Calcium mobilization assay

Human Embryonic Kidney (HEK) 293 cells stably expressing the mouse recombinant NPSR receptor ($HEK293_{mNPSR}$) were generated as described in Reinscheid *et al.* [17], maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM, L-glutamine and Hygromycin (100 mg/L) and cultured at 37 °C in 5% CO_2 humidified air.

$HEK293_{mNPSR}$ was seeded at a density of 50000 cells/well into poly D-lysine coated, 96-well, black, clear-bottom plates. The following day the cells were incubated with the loading solution for 30 min at 37 °C (figure 5). Afterwards the loading solution was aspirated and 100 µl/well of dye loading solution was added. After placing cell culture plate and compound plate into the FlexStation II (Molecular Device, Union City, CA 94587, US), fluorescence changes were measured at real time or at 37°C. On-line additions were carried out in a volume of 50 µl/well.

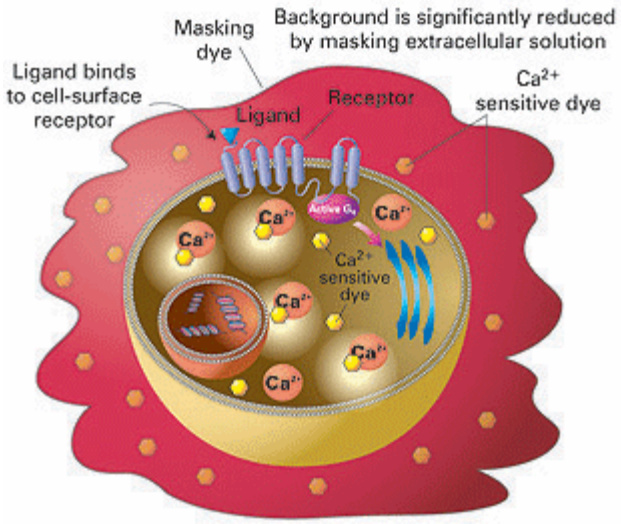

Figure 5 Diagram depicting the experimental protocol of the calcium mobilization assay. Cells are incubated with Fluo-4 AM, de-esterification of the ester group (AM) traps the dye in the cells and further leakage of the dye is prevented by blockage of organic anion-transport inhibitors using probenecid. Background fluorescence is reduced by addition of Brilliant Black dye which blocks extracellular signalling from any leaked Fluo-4.

Cell counting

Accurate cell number in a cell suspension can be efficiently calculated using a cell counting chamber (Burker's chamber, figure 6). A small volume of the cell suspension (10 µl) was pipetted onto the chamber, the capillary action under the cover slip will draw the suspension into the counting chamber. The space between the cover slip and the counting chamber ensures a specific volume of cell suspension is present.

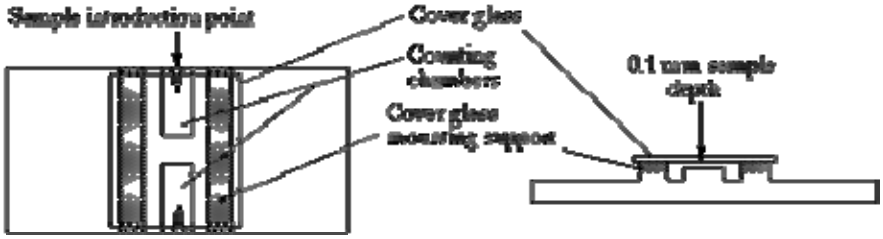

Figure 6 Burker's chamber.

Under a microscope the number of cells in diagonally opposite counting areas were counted, (figure 7).

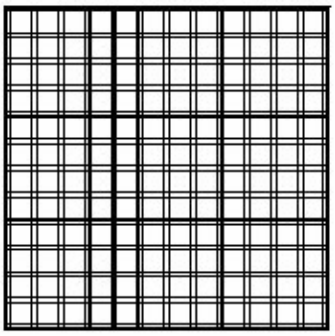

Figure 7. Schematic representation of the counting grid of the Burker's chamber.

The Burker's chamber is formed of 3x3 major squares, each of these major squares is subdivided into a grid of 4x4 squares. The number of cells present in three major cells are counted, cells in contact with two of the squares sides are included and the average taken. The volume of a major square is 0.1 mm^3 which is equal to 0.0001 ml. To determine the number of cells per ml the average number of cells determined is increased by a factor of 10^4.

Instruments

$[Ca^{2+}]_i$ levels were monitored using a FlexStation II fluorimeter (figure 8). The FlexStation II system includes:

1 Xenon-lamp light source

2 Automatic eight-channel pipettor

3 Tip rack drawer

4 Compound plate drawer

5 Reading chamber drawer

The Xenon-lamp light source and dual monochromators permit the use of essentially all dual-wavelength dyes for functional cellular assays.

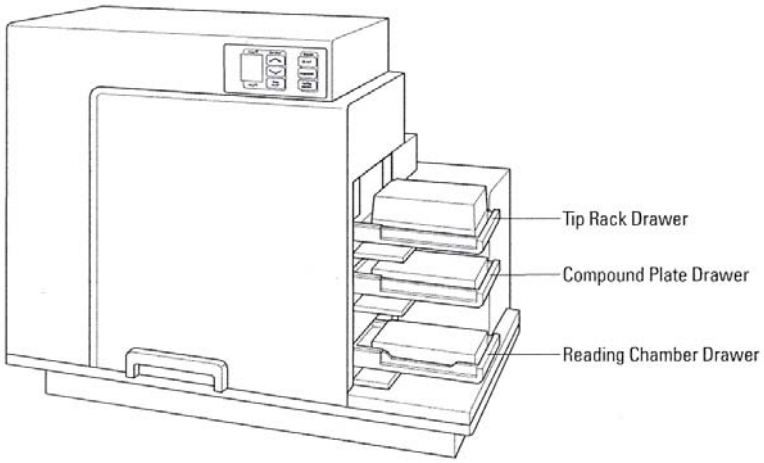

Figure 8. Diagram of FlexStation II used for calcium mobilization assay.

2.4 *Ex vivo* studies

2.4.1 DNA extraction and PCR

DNAs were prepared from tail biopsies using Eazy Nucleic Acid Isolation Tissue DNA Kit (Omega Biotek). One µl of genomic DNA was added to a PCR reaction mix containing 3 mM $MgCl_2$, deoxribonucleotide triphosphates (dNTPs) and primers in a final concentration of 4 µg/ml. Three oligonucleotide primers were used. The first one was a forward primer specific to the endogenous NPSR locus (5')CCTTATCCTCAAACCACGAAGTAT(3'). The second one was a common reverse primer (5')GTGGGTACATGAGAAGGTTAGGAG(3') and the third one was a forward primer (5')AAATGCCTGCTCTTTACTGAAGG(3') specific to the targeting plasmid. The reagents were mixed in a Green GoTaq ThermoPol Reaction Buffer. 2.5 units/reactions of GoTaq DNA Polymerase and an aliquot of water were added to bring the reaction mix to 50 µl total volume. The reaction was placed in a thermal cycler and heated to 94°C for 1 minute. The reaction was allowed to proceed for 39 cycles as follows: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute. Finally the reaction was heated to 72°C for 2 minutes and then stored at 4°C. The reaction products were separated in 1% agarose by horizontal gel electrophoresis in Tris-acetate-EDTA buffer, stained with ethidium bromide, and photographed under UV light (figure 9).

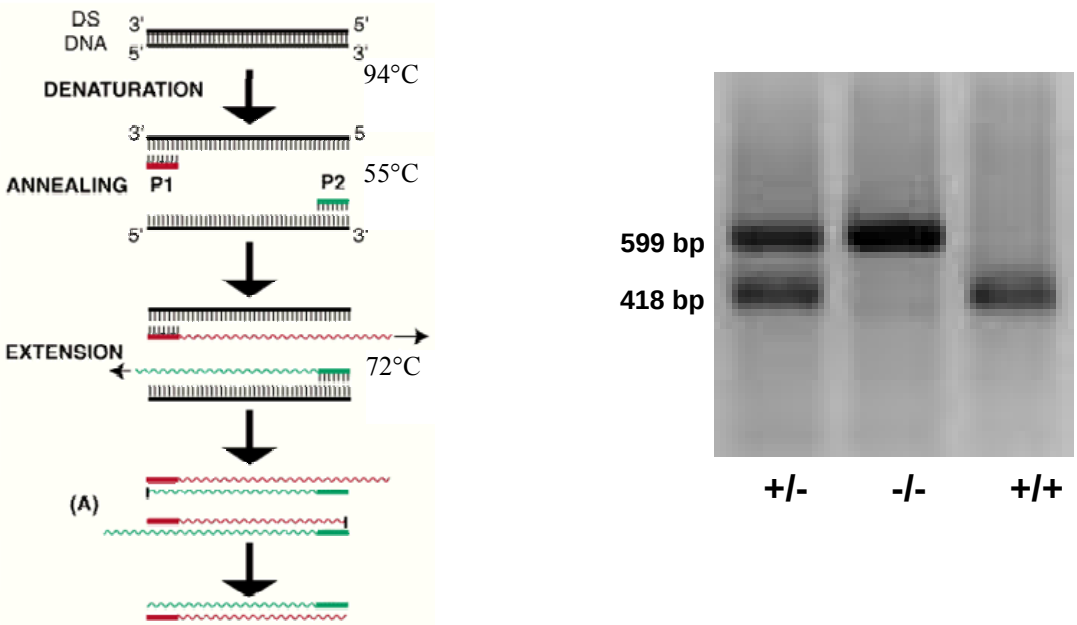

Figure 9 PCR protocol and products.

2.5 *In vivo* studies

2.5.1 Animals

All experimental procedures for in vivo studies complied with the standards of the European Communities Council directives (86/609/EEC) and National regulations (DL 116/ 92). Male CD-1 mice (2–3 months old, 28–35 g) were used. They were housed in 425x266x155 mm cages (Tecniplast, MN, Italy), eight animals per cage, under standard conditions (24°C, 55% humidity, 12-h light–dark cycle, lights on 07:00 hours) with food (MIL, standard diet Morini RE, Italy) and water ad libitum for at least 10 days before experiments began. All experiments were performed between 9:00 and 13:00 and the day before each experiment mice were moved to the testing room to acclimate. Each animal was used only once. All non peptide compounds were administered to the animals intraperitoneally (i.p., 100 µl/mouse) whereas NPS and related peptides were given to the lateral cerebral ventricle (intracerebroventricularly; i.c.v.). I.c.v. injections were performed either free hand or using a stainless-steel guide cannula (22 GA) (Plastic One, Roanoke, VA, USA) stereotaxically implanted. For each experiment at least four mice were randomly assigned to each experimental group, and the experiment was repeated at least three times: therefore each experimental point is the mean of the results obtained in $\geq$12 mice. All procedures were randomized across test groups and, in all the experiments, food was not available during testing.

Free hand i.c.v. injections (2 µl/mouse) were given, under light isofluorane anaesthesia (just sufficient to produce a loss of the righting reflex), in the left ventricule according to the procedure described by Laursen and Belknap [85]. Briefly, the syringe was held at an approximate 45° angle to the skull. Bregma was found by lightly rubbing the point of the needle over the skull until the suture was felt. Once found, care was taken to maintain the approximate 45° angle and the needle was inserted about 2 mm lateral to the midline. The skull is relatively thin at this point, so only mild pressure was required to insert the needle. The solutions of drugs were then injected slowly (2 µl in about 20 s).

Implantation of cannula into lateral ventricle: Mice (25–30 g) were anesthetized with isofluorane 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 was fixed with 3 skull screws and dental cement. Coordinates toward the bregma were L -1.1 mm, A - 0.5 mm, V + 3 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, and during this period, mice were gently handled daily to minimize the stress associated with manipulation of the animals throughout the experiments. For i.c.v. injection, awake mice were gently restrained by hand and the solutions of drugs (2 µl/mouse) were then injected slowly. After completion of testing, mice were i.c.v. injected with trypan blue dye (2 µl) that was allowed to diffuse for 10 min. Then mice were decapitated, and their brains were removed and rapidly frozen. Gross dissection of the brain was used to verify the placement of the cannula. Only the data from those animals with dispersion of the dye throughout the ventricles were used.

NPSR(+/+) and NPSR(-/-) mice

129/SvEv NPSR(+/+) and NPSR(-/-) mice, generated as described in Allen *et al.* [83], were obtained from Taconic Farms (Germantown, NY, USA). Knock out of the NPSR locus was achieved by replacing a 744-bp region containing the majority of exon 4 with a neomycin resistant cassette. Exon 4 includes regions of the gene encoding the third transmembrane domain and regions of the 2° intracellular loop. For a first series of experiments, NPSR(+/+) and NPSR(-/-) littermates were obtained by mating heterozygous NPSR(+/-) 129/SvEv mice. In parallel, mice with the genetic background of 129/SvEv were backcrossed for 8 generations to CD-1 mice, to obtain NPSR(+/+) and NPSR(-/-) CD-1 congenic colonies (figure 10). All mice were genotyped using PCR to determine the target disruption of the NPSR gene.

Experiments were conducted using adult male NPSR(+/+) and NPSR(-/-) mice 8 - 10 weaks old (weighing 28–35 g). For phenotype studies mice were moved to the test chamber at least one weak before the experiments to acclimate. Little is known about the potential carry over effect of experience of one behavioral test to another [86]. However, mice repeatedly exposed to the same test display altered responses when reassessed in that given animal model of anxiety [87]. Taking into account these considerations, our experiments were performed by subjecting two different groups of NPSR(+/+) and NPSR(-/-) mice to the procedure described in table 2.

Table 2 Experimental design used to develop the present study

Group 1	EPM	OF	LA	NOR	FST	RR
Group 2	OF	EPM	LA	NOR	SIH	FT

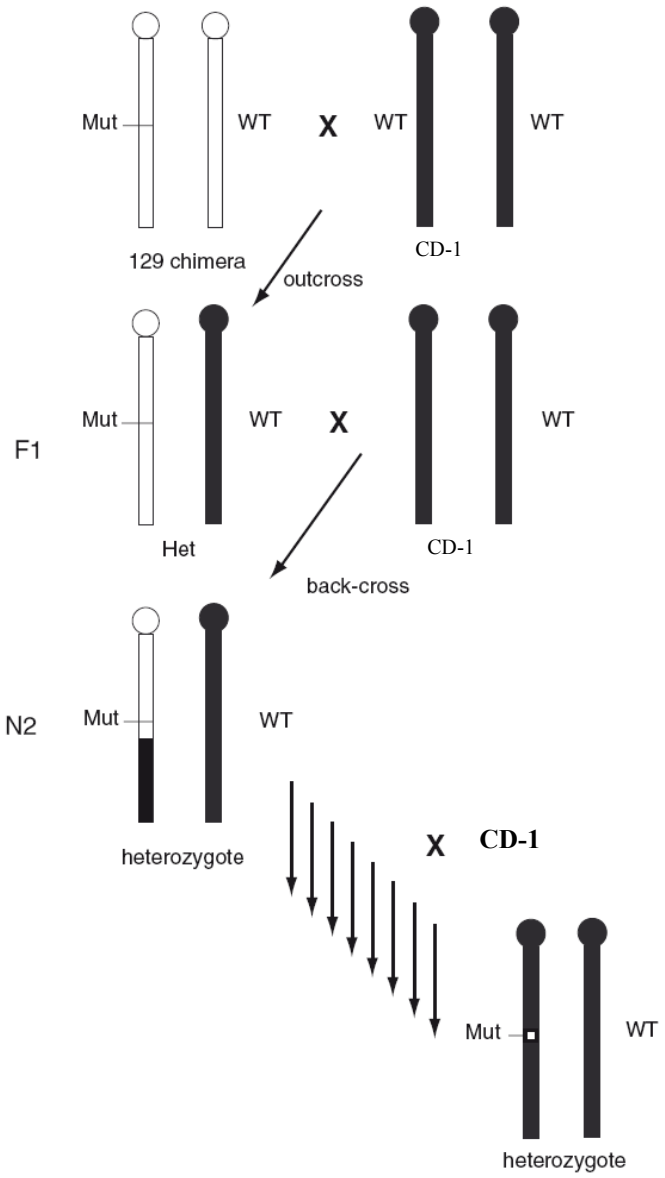

Figure 10. Generation of a CD-1 congenic strain. Congenic lines are generated by an outcross of 129Sv/Ev NPSR(+/-) to CD-1 strain followed by successive back-crosses of eterozigous to CD-1 strain. The strain is considered congenic after the 8 backcross generation. Most loci, except those tightly linked to the mutant locus, are derived from the host strain. At this stage it is possible to split the colony in NPSR(+/+) and NPSR(-/-) colonies and generate experimental animals.

2.5.2 Locomotor activity

For LA experiments the ANY-maze video tracking system was used (Ugo Basile, application version 4.52c Beta). Mice were positioned in square plastic cages (40 cm × 40 cm), one mouse per cage. Four mice were monitored in parallel. Mouse horizontal activity was monitored by a camera while vertical activity was measured by an infrared beam array. The parameters measured were cumulative distance travelled (total distance in m that the animal travelled during the test), total time immobile (the amount of seconds the animal stays immobile during the test; the animal is considered immobile when 90% of his image remains in the same place for at least 2.5 s), and the number of animal rearings (the number of beam breaks due to vertical movements). Naïve mice (that is, animals not habituated to the test cage before the experiment) were injected with NPS i.c.v. 5 min before the beginning of the test. Habituated mice were acclimatised to the test cage for 60 min before the i.c.v. injection of NPS and then returned to the test cage. Diazepam-treated mice received an i.p. injection (100 ml) of diazepam (5 mg/kg) in Tween 80 (0.5%) 15 min before the i.c.v. injection of NPS or the i.p. injection of caffeine. NPS (0.01, 0.1, 1 nmol) was given i.c.v. 5 min before the beginning of the test, [D-Val^5]NPS (10 nmol, i.c.v.) was co-injected with NPS, instead [^{t}Bu-D-Gly^5]NPS (1, 3 and 10 nmol) was administered 15 min before i.c.v. injections of NPS. SHA 68 (10 and 50 mg/kg) was given i.p. 10 min before NPS or caffeine injection. Caffeine (20 mg/kg) was given i.p. 15 min before recording LA.

2.5.3 Recovery of the righting reflex

The RR assay was performed according to the procedures described by [88]. Mice were given an i.p. injection of diazepam 15mg/kg .When the animals lost RR, they were placed in a plastic cage and the time was recorded by an expert observer, blind to drug treatments and/or genotype. Animals were judged to have regained the RR response when they could right themselves three times within 30 s. Sleeping time is defined as the amount of time between the loss and regaining of the RR; it was rounded to the nearest minute. Caffeine (20 mg/kg, i.p.) and NPS (0.01, 0.1, 1 nmol, i.c.v.) were administered 15 and 5 min before the injection of diazepam, respectively. [D-Cys(^{t}Bu)^5]NPS (10 nmol, i.c.v.) was co-injected with NPS, instead [^{t}Bu-D-Gly^5]NPS (0.1, 1, 3 and 10 nmol, i.c.v.) was administered 15 min before i.c.v. injections of NPS. SHA 68 (10 and 50 mg/kg) was given i.p. 10 min before NPS. In a separate series of experiments SHA 68 (50mg/kg, i.p.) was injected 10 min before the administration of different doses (5, 10, and 15mg/kg) of diazepam.

2.5.4 Elevated plus maze

The EPM assay was carried out essentially as previously described by Pellow *et al.* [89]. The EPM apparatus (Hamilton–Kinder, Poway, CA, USA) consists of two open arms (30x5x0.6 cm), which are facing two opposite wall-enclosed arms (30x5x20 cm) connected by a central platform (5x5 cm) elevated 50 cm from the floor. A red light was focused on the central platform (100 lux). Animals were placed at the centre of the maze, with the head facing an open arm. The number of entries and the time spent in both closed and open arms and some ethological variables (rearing, head dipping and stretch attend postures) were recorded during a 5min period by an experienced observer. An entry was scored as such only when the animal placed all four limbs into any given arm. The ratio of 'time spent in the open arms divided by time spent in all (open and closed) arms' and 'number of entries into open arms divided by total entries into all arms' was calculated and multiplied by 100, to yield the percentage of time spent in and the frequency of entries into open arms, respectively. NPS (0.001, 0.01, 0.1, 1 nmol) was given i.cv. 15 min before the beginning of the test, caffeine (20 mg/kg) and diazepam (1 mg/kg, i.p.) were administered i.p. 15 and 30 before starting the test respectively.

2.5.5 Stress induced hyperthermia

The SIH test was performed according to the method previously reported by Olivier *et al.* [90]. Rectal temperatures were measured to the nearest 0.1°C using an ELLAB instruments thermometer (Copenhagen, Denmark) using a lubricated thermistor probe designed for mice (2 mm diameter) inserted 20 mm into the rectum, while the mouse was handheld near the base of the tail. The probe was left in place until steady readings were obtained (approximately 10 s). Rectal temperatures were measured twice in each mouse, at t= 0min (T_1) and t= 10min (T_2). The first rectal body temperature measurement (T_1) induces a mild stress that causes an increase in the second value (T_2). The difference in temperature (T_2-T_1) was considered to reflect SIH. NPS (0.01, 0.1 and 1 nmol, i.c.v.) and diazepam (1, 3 and 5mg/kg, i.p.) were administered 60 min before the test. This relatively long period of pretreatment is needed because the injection procedure (handling plus injection) evokes a similar increase in core body temperature as the rectal temperature measurement procedure. This stress effect had waned after 60min and the basal body temperature returned to the undisturbed baseline [90].

2.5.6 Open field

The OF test was performed using the ANY-maze video tracking system (Ugo Basile, application version 4.52c Beta). Briefly the mouse was placed in a square plastic cage (40×40 cm) and ambulatory behaviour was monitored for 10 min. The central zone of the open field was defined as the central 20×20 cm square. Horizontal activity was monitored by a camera. Four mice were monitored in parallel in each experiment. The parameters measured were the same considered in the LA assay. The number of entries in the central zone and the time spent by the animal in the central area of the field were also measured. An entry in the central zone occurred when the entire area of the animal was in the central square and the time in the central zone is defined as the amount of time in seconds that the animal spent in the central square. Diazepam (0.3, and 1 mg/kg) was administer i.p. 30 min before the beginning of the experiment. NPS (0.01, 0.1 and 1 nmol) was given i.c.v. 15 min before the beginning of the test, evaluated. SHA 68 (50 mg/kg, i.p.) was administered 10 min before NPS injections.

2.5.7 Novel object recognition

The NOR was performed according to the method reported by Ennaceur *et al.* [91]. The test was conducted in three phases: acclimation, acquisition and test. For acclimation, the mouse was placed into the NOR chamber (a square open field 40×40 cm) and allowed to explore freely for 30 min. No objects were placed in the box during the habituation trial. Immediatly after habituation, the acquisition trial was conducted by placing the mouse in the field, in which two identical objects were positioned on the corners of the arena approximately 5 cm from the walls. Mice were allowed to explore the two objects for 10 min, and exploratory activity (i.e., the time spent exploring each object) was recorded. After 3 h, mice were re-exposed to the same object of the acquisition phase, together with a novel object (not used in acquisition phase). Once again, the time that each animal spent exploring each object was measured. A mouse was considered to be involved in exploratory behaviour when its head was oriented directly towards the object and within approximately 1-2 cm from it. For test data, the percentage of exploration time spent at the novel object was determined. The choice of object for novel or familiar was counterbalanced, and the position of each object was also alternated between trials to avoid any misinterpretation of data. After each exposure, the objects and test chamber were cleaned with 10% ethanol to eliminate odour cues. These experiments were performed using the ANY-maze video tracking system (Ugo Basile, application version 4.52c Beta).

2.5.8 Forced swimming test

The FST was performed as described by Porsolt *et al.* [92]. Mice were placed individually in polyethylene cylinders (18.5 cm high, 12.5 cm diameter), containing water (25 ± 1 °C, 13.5 cm deep), for two swimming sessions: an initial 15 min training session on day 1, which was followed, 24 h later (day 2), by a 5 min test session. Results were relative to this 5 min test session. The immobility time (i.e. the time spent floating in the water without struggling) was recorded. Scoring was performed by an observer blind to the animal genotype. At the end of the swimming sessions, the animal was removed from the cylinder, dried with paper towels, placed in an individual cage to rest and recover, and then it was returned to its collective home cage.

2.5.9 Formalin test

The procedure used was essentially the same as reported by Hunskaar *et al.* [93]. Approximately 30 min before testing, mice were individually placed in transparent observations chambers (32 cm high, 24 cm diameter) for adaptation. Then the animal was taken out of the chamber, and 20 µl of 1.5% formalin solution was injected into the dorsal surface of the right hind paw. Immediately after formalin injection, each mouse was returned to the observation chamber, and time (s) spent by the animal displaying pain-related behaviours was measured with a hand-held stopwatch for each 5-min block for 45 min after formalin injection. The nociceptive behaviours consisted of licking, biting and lifting of the injected paw. Time spent by the animal showing all these pain-related behaviour was cumulatively measured and expressed as seconds of nociceptive behaviour/min. The cumulative response times during 0-10 min and during 15-45 min were regarded as the first phase and second phase responses, respectively.

2.6 Data analysis and terminology

All data are expressed as means ± standard error of the mean (s.e.m.) of *n* experiments. For potency values 95 % confidence limits were indicated. Data have been statistically analyzed with the Student's *t* test for unpaired data, one way ANOVA followed by the Dunnett's test, or two-way ANOVA followed by the Bonferroni post hoc test, as specified in table and figure legends; p values less than 0.05 were considered to be significant. The pharmacological terminology adopted in this manuscript is consistent with the IUPHAR recommendations [94].

Calcium mobilization data are expressed as fluorescence intensity units (FIU) in percent over the baseline.

Agonist potencies are given as pEC_{50} = the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist. Concentration response curve to agonists were fitted with the following equation:

$$\text{Effect} = \text{baseline} + (E_{max}\text{-baseline})/(1+10\text{^}((\text{Log}EC_{50} - X)*\text{HillSlope}))$$

where X is the agonist concentration.

The E_{max} is the maximal effect that an agonist can elicit in a given tissue.

Antagonist properties were first evaluated in inhibition response experiments, antagonist potency was expressed as pK_B values. pK_B values were derived from inhibition response curves using the following equation:

$$K_B = IC_{50}/([2 +([A]/EC_{50})^n]^{1/n} - 1)$$

where IC_{50} is the concentration of antagonist that produces 50% inhibition of the agonist response, [A] is the concentration of agonist, EC_{50} is the concentration of agonist producing a 50% maximal response and n is the Hill coefficient of the concentration response curve to the agonist [95].

Moreover to investigate the type of antagonism exerted by NPSR ligands the classical Schild protocol was performed. In case of a competitive antagonist potency was expressed as pA_2. pA_2 is the negative logarithm to base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original response. The pA_2 values are calculated using Schild's linear regression, that correlates the log of concentrations of antagonists (x axis) to the log of (CR-1) (y axis), where CR is the ratio between the EC_{50} (nM) values of agonist, in the presence and in absence of antagonist. The value of x for y=0 represents the pA_2 value, and the slope not

significantly different from the unity means that the antagonist is competitive. In case of a non competitive antagonist potency was expressed as pK_B. pK_B values were derived from Shild protocol experiments using the following equation:

$$pK_B = \log_{10}[(slope-1)/[B]]$$

where slope is calculated from a double-reciprocal plot of equieffective concentrations of agonist in the absence and presence of antagonist [B] [95].

3. RESULTS AND DISCUSSION

3.1 In vitro pharmacological characterization of NPS analogues modified in position 2, 3 and 4

Pivotal structure and conformational–activity relationship studies consistently demonstrated that the N-terminal part of NPS is crucial for biological activity [17,76-77]. In particular, based on findings obtained from Ala- and D-aminoacid-scan studies, Phe^2-Arg^3-Asn^4 [18,76] were identified as the most important residues in NPS sequence. Thus, we decided to deeply investigate the chemical features of NPS position 2, 3 and 4 for biological activity.

In a first study Phe^2 was replaced with 10 different coded amino acids, with aromatic, lipophilic, and hydrophilic side chains. Subsequently Phe^2 was replaced with 21 non coded Phe analogues. These Phe analogues were used with the aim of examining in detail the effects on the NPS biological activity of (i) modifications of the aromaticity, length, size, and position of the amino acid side chain, (ii) conformational restrictions, and (iii) insertion of different chemical groups in the phenyl ring. All the compounds were tested for intracellular calcium mobilization using $HEK293_{mNPSR}$ cells in the fluorometric imaging plate reader FlexStation II. In the calcium mobilization assay NPS increased the initial calcium concentrations in a concentration dependent manner with pEC_{50} and E_{max} values of 8.96 and 270% over the basal ones, respectively (figure 11).

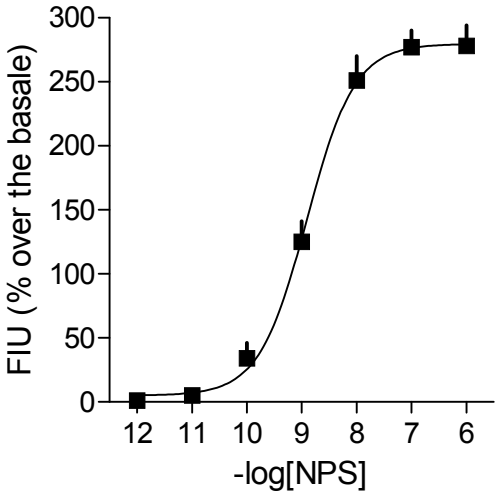

Figure 11 Concentration-response curves to NPS in $HEK293_{mNPSR}$. Data are mean±s.e.m of 4 experiments made in duplicate.

Table 3 summarizes data obtained investigating the SAR requirements of NPS positions 2 with natural amino acids.

Table 3 Effects of NPS and [X^2]NPS analogues substituted with coded residues in $HEK293_{mNPSR}$ cells

		pEC_{50} ($CL_{95\%}$)	**E_{max} ± sem**
	NPS	8.96 (8.81-9.11)	270 ± 12 %
1	[His^2]hNPS	6.88 (6.79-6.97)	288 ± 5 %
2	[Tyr^2]hNPS	8.63 (8.38-8.88)	312 ± 5 %
3	[Trp^2]hNPS	8.65 (8.45-8.85)	323 ± 4 %
4	[Leu^2]hNPS	8.08 (7.92-8.24)	282 ± 15 %
5	[Lys^2]hNPS	Crc incomplete: at 10 μM 103 ± 15 %	
6	[Asp^2]hNPS	Crc incomplete: at 10 μM 39 ± 3 %	
7	[Glu^2]hNPS	Crc incomplete: at 10 μM 170 ± 20 %	
8	[Asn^2]hNPS	Crc incomplete: at 10 μM 49 ± 2 %	
9	[Gln^2]hNPS	Crc incomplete: at 10 μM 54 ± 6 %	
10	[Thr^2]hNPS	Crc incomplete: at 10 μM 114 ± 5 %	

Data are means of at least 4 separate experiments, performed in duplicate.

The replacement of Phe^2 with His (compound 1) produced a 100-fold reduction in potency while that with Tyr (compound 2) and Trp (compound 3) generated only a slight decrease in biological activity. The nonaromatic lipophilic residue Leu produced a peptide (compound 4) about 10-fold less potent than NPS. The series of hydrophilic residues with basic (compound 5), acidic (compounds 6 and 7), and neutral (compounds 8–10) side chains produced a drastic (3 log unit) loss of potency. These results suggested that the lipophilicity of the side chain of the residue in position 2 is indeed a crucial requirement for the NPSR interaction. However, steric hindrance is also important since Ala/Phe^2 substitution has been consistently reported to be highly detrimental for biological activity. Although aromaticity is not strictly required, none of the investigated NPS analogues displayed higher potency than the natural sequence, thus indicating that the benzyl moiety of Phe^2 is the most effective chemical structure in promoting receptor binding. On the aforementioned bases, a second round of synthesis was planned to investigate in details the SAR requirements of the Phe^2 benzyl moiety. The biological results of this study are summarized in table 4. The elimination of aromaticity (compound 11) is well tolerated and generates an NPS analogue equipotent to the natural ligand. Data obtained with compound 11 confirmed those relative to compound 4 indicating that aromaticity in position 2 is not crucial for the NPSR interaction. However, cyclic lipophilicity seems to be favored, since [Cha^2]NPS is equipotent to the reference peptide while 6-fold more potent than [Leu^2]NPS. The introduction, into the para position of the phenyl ring, of an atom of the halogen series (compounds 12–15) as well as of chemical groups

with electron donor (compound 16) or withdrawing (compounds 17–19) properties seemed to be well tolerated, and it generated NPS analogues with similar potency to that of the natural sequence. Similarly, the introduction of a methyl group into the ortho position of the phenyl ring (compound 20) did not affect the biological activity at NPSR. Collectively, these data indicate that the modulation of the electronic asset of the benzyl side chain of Phe^2 is not important for the NPSR interaction. Changes of the distance between the Phe^2 phenyl ring and the peptide backbone obtained by eliminating (compound 21) or adding (compound 22) a methylene group produced very different results. In fact, compound 21 was almost inactive up to 10 μM while compound 22 was only 3-fold less potent than NPS. The shift of the Phe^2 benzyl moiety with respect to the peptide backbone produced a 10-fold less active derivative in the case of N-shift (compound 23) while substituting Phe with β-Phe (compound 24) generated an inactive derivative. These results together with the detrimental effect obtained by changing the chirality of Phe^2 demonstrated that the spatial disposition of the phenyl moiety relative to the peptide backbone was indeed very important for biological activity.

Table 4 Effects of NPS and [X^2]NPS analogues substituted with non coded residues in $HEK293_{mNPSR}$ cells

N°	Compound	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem
	NPS	8.93 (8.81-9.05)	277 ± 11 %
11	[Cha^2]NPS	8.87 (8.29-9.45)	221 ± 16 %
12	[(pI)Phe^2]NPS	8.75 (8.54-8.96)	243 ± 25 %
13	[(pF)Phe^2]NPS	8.96 (8.72-9.20)	291 ± 21 %
14	[(pCl)Phe^2]NPS	9.15 (8.81-9.49)	298 ± 27 %
15	[(pBr)Phe^2]NPS	9.19 (8.93-9.45)	309 ± 26 %
16	[(pCH_3)Phe^2]NPS	9.17 (8.77-9.57)	317 ± 18 %
17	[(pCF_3)Phe^2]NPS	8.59 (8.34-8.84)	286 ± 12 %
18	[(pCN)Phe^2]NPS	8.48 (8.19-8.77)	296 ± 14 %
19	[p(NO_2)Phe^2]NPS	8.44 (8.24-8.64)	290 ± 10 %
20	[(oCH_3)Phe^2]NPS	8.73 (7.89-9.57)	222 ± 40 %
21	[Phg^2]NPS	crc incomplete: at 10 µM:141 ± 24 %	
22	[hPhe^2]NPS	8.38 (7.43-9.33)	196 ± 23 %
23	[NPhe^2]NPS	7.81 (7.25-8.37)	246 ± 13 %
24	[βPhe^2]NPS	crc incomplete: at 10 µM:177 ± 24 %	
25	[Tic^2]NPS	8.09 (8.01-8.17)	244 ± 19 %
26	[Atc^2]NPS	7.95 (7.30-8.60)	257 ± 27 %
27	[Aic^2]NPS	7.95 (7.30-8.60)	257 ± 27 %
28	[1Nal^2]NPS	8.71 (8.24-9.18)	232 ± 6 %
29	[2Nal^2]NPS	8.66 (8.44-8.88)	203 ± 14 %
30	[Bip^2]NPS	7.70 (6.98-8.42)	170 ± 19 %*
31	[Dip^2]NPS	crc incomplete: at 10 µM:130 ± 24 %	

Data are means of at least 4 separate experiments, performed in duplicate.. *p < 0.05 vs NPS *E*max according to one-way ANOVA followed by the Dunnett test.

This was further corroborated by the results obtained with the introduction in position 2 of constrained Phe analogues. In fact, the cyclization of the benzyl side chain on the nitrogen (compound 25) or on the Phe chiral carbon (compounds 26 and 27) produced NPS derivatives approximately 10-fold less potent than NPS. The addition of a further phenyl ring on the Phe^2 side chain was well tolerated in the case of Nal isomers (compounds 28 and 29) but not in the case of [Bip^2]NPS (compound 30) or [Dip^2]NPS (compound 31), which were 10- and 1000-fold less potent than the parent peptide, respectively. Collectively, these results suggest that the NPSR ligand binding pocket allocating the side chain of Phe^2 does not show particularly stringent hindrance requirements. In fact, among the enlarged aromatic side chain analogues tested, only compound 31 completely lost biological activity. Interestingly, compound 30 displays a statistically significant reduction of efficacy, with its maximal effect being only 62% of those elicited by the natural peptide (figure 12). This suggests that the correct position of the phenyl ring of Phe^2 into the NPSR

ligand-binding pocket is important not only for binding but also for receptor activation. Other NPSR ligands with reduced efficacy have been described by substituting Arg^3 with Ala [76] and Gly^5 with 2-aminoisobutyric acid (Aib) or D-Ala [77]. Collectively, these findings corroborate the proposal [18,76] that the N-terminal part of NPS represents the message domain of this peptide.

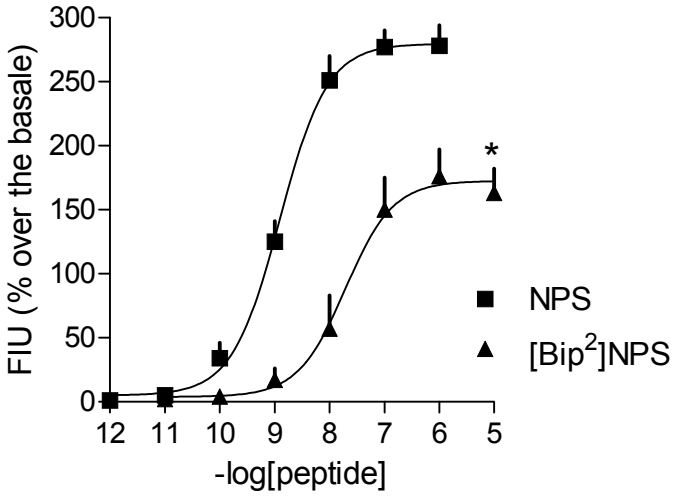

Figure 12 Concentration-response curves to NPS and [Bip^2]NPS obtained in the same plates of $HEK293_{mNPSR}$. Data are mean±s.e.m of 4 experiments made in duplicate. *$p < 0.05$ *vs* NPS E_{max} according to the Student t test for unpaired data

A subsequent study was performed by replacing Arg^3 and Asn^4 with several coded and non coded amino acid residues. 38 novel NPS analogues were synthesised and pharmacologically evaluated in a calcium mobilization assay using $HEK293_{mNPSR}$ and the fluorometric imaging plate reader FlexStation II. All data obtained investigating the SAR requirements of NPS positions 3 and 4 are summarized in tables 5 and 6, respectively. The substitution of Arg^3 with aromatic residues (compounds 32–34) produced a drastic loss of biological activity; the best compound ([His^3]NPS) being more than 100-fold less potent than the natural peptide. Similar results were obtained with non-aromatic lipophilic residues (compounds 35–36). The introduction in position 3 of residues with hydrophilic neutral (compounds 37–42), basic (compound 43) or acidic (compounds 44–45) character produced low potency or inactive NPS analogues. Among these, only [Lys^3]NPS displayed a potency value similar to that of native NPS. These results, obtained by exchanging Arg^3 with coded amino acids, suggest that position 3 does not tolerate substitution with aromatic and aliphatic branched residues. The Arg^3 replacement with hydrophilic residues with short side chain produced in general low potency peptides (compounds 37-41). Interestingly, while the substitution of Arg^3 with Gln (compound 42) reduced peptide potency only by 10-fold, exchanges to Asn or Glu lead to inactive analogs. These results, together with the inactivity of [Val^3]NPS, indicate that a linear 3-carbon atom moiety of the amino acid side chain, which is similar in both Arg and Gln, but

not in Val or Ala [18,76], is important for binding the NPSR pocket that harbors the Arg^3 side chain. However, this same pocked does not tolerate an acidic side chain as demonstrated by the lack of activity of [Glu^3]NPS. Moreover, the data obtained by the replacement Arg/Gln suggested that basicity in position 3 is not crucial for bioactivity. The shortening of the Lys side chain by one carbon atom (compound 46) appears to be well tolerated producing a peptide only 2-fold less potent. In addition, the elimination of the primary amino function (compound 47) produced a modest reduction of biological activity corroborating the hypothesis that basicity in position 3 is not a stringent requirement for NPSR interaction. Further shortening of the side chain (compound 48) reduced peptide potency while the addition on the ethyl moiety of an amino function, as in Dab (compound 49), produced an NPS analog with similar potency as compound 47. Finally, the elimination of a methylene group from the side chain of compound 49 (compound 50) produced negligible changes in biological activity. Collectively, these data indicate that the length of the side chain of the amino acid in position 3 and its linear shape seem to be more important for biological activity than its basic character. Surprisingly, the transformation of the guanidine function into urea (compound 51) generated a peptide 10-fold less potent than NPS. Since compound 51 is also less potent than compound 43 or 45, it is probable that the reduction of NPSR binding is due to the urea carbonyl (C=O), rather than to its non basic character. An increase in length of the side chain of Arg^3 (compound 52) or its methylation (compounds 53 and 54) are completely tolerated. The elimination of the basic character of the Arg^3 side chain obtained with compound 55 was tolerated while that obtained with compound 56 was not. Collectively these results corroborate the hypothesis that the guanidine moiety and its basicity are not particularly relevant for biological activity. In addition, the steric hindrance of the guanidine moiety does not seem to represent a particularly stringent chemical requirement since it can be enlarged (as in compounds 53–55) without loss of potency. However, this does not apply for moieties as bulky as tosyl since compound 56 was found inactive. The elimination of the Lys side chain basicity (compound 57) produced a 3-fold decrease of activity compared to 43; this could be due, at least in part, to the increase of the side chain steric hindrance, similar to what observed with compound 56. Finally we inserted in position 3 amino acids with a pyridine nucleus as side chain, which combines in the same moiety aromatic character and a nitrogen atom able to accept hydrogen bond (compounds 58–60). In line with previous findings (see compounds 32–34) this chemical substitution was not tolerated, corroborating the evidence that aromaticity in position 3 is detrimental for NPSR binding.

Table 5 Effects of NPS and [X^3]NPS analogues in HEK293$_{mNPSR}$ cells

N°	Compound	pEC$_{50}$ (CL$_{95\%}$)	E$_{max}$ ± sem
	NPS	7.92 (7.83-8.01)	267 ± 10 %
32	[Phe^3]NPS	Crc incomplete: at 10 µM: 46 ± 6 %	
33	[Tyr^3]NPS	Crc incomplete: at 10 µM: 73 ± 34 %	
34	[His^3]NPS	5.81 (5.20-6.42)	273 ± 18 %
35	[Leu^3]NPS	Crc incomplete: at 10 µM: 36 ± 9 %	
36	[Val^3]NPS	Crc incomplete: at 10 µM: 129 ± 30 %	
37	[Thr^3]NPS	6.06 (5.97-6.15)	272 ± 20 %
38	[Ser^3]NPS	Crc incomplete 10 µM: 175 ± 19 %	
39	[Cys^3]NPS	6.43 (5.97-6.89)	256 ± 26 %
40	[Gly^3]NPS	5.90 (5.71-6.09)	264 ± 7 %
41	[Asn^3]NPS	Crc incomplete 10 µM: 161 ± 33 %	
42	[Gln^3]NPS	6.89 (6.76-7.02)	295 ± 21 %
43	[Lys^3]NPS	7.74 (7.70-7.78)	290 ± 17 %
44	[Asp^3]NPS	Crc incomplete 10 µM: 73 ± 24 %	
45	[Glu^3]NPS	Crc incomplete 10 µM: 89 ± 3 %	
46	[Orn^3]NPS	7.50 (7.24-7.76)	318 ± 11 %
47	[Nva^3]NPS	6.87 (5.95-7.79)	207 ± 23 %
48	[Abu^3]NPS	6.29 (5.43-7.15)	218 ± 17 %
49	[Dab^3]NPS	6.69 (6.12-7.26)	207 ± 32 %
50	[Dap^3]NPS	6.62 (6.32-6.87)	196 ± 17 %
51	[Cit^3]NPS	6.78 (6.45-7.11)	285 ± 11 %
52	[hArg^3]NPS	7.97 (7.70-8.24)	288 ± 16 %
53	[Arg(Me)$_2$^{3}(Asym)]NPS	7.65 (7.52-7.78)	281 ± 15 %
54	[Arg(Me)$_2$^{3}(Sym)]NPS	7.50 (7.40-7.60)	272 ± 10 %
55	[Arg(NO$_2$)^3]NPS	7.66 (7.49-7.83)	288 ± 17 %
56	[Arg(Tos)^3]NPS	Crc incomplete 10 µM: 104 ± 16 %	
57	[Lys(Tfa)^3]NPS	7.07 (6.60-7.54)	288 ± 17 %
58	[2'-Pal^3]NPS	Crc incomplete 10 µM: 19 ± 4 %	
59	[3'-Pal^3]NPS	Crc incomplete 10 µM: 42 ± 11 %	
60	[4'-Pal^3]NPS	Crc incomplete 10 µM: 154 ± 17 %	

Data are means of at least 4 separate experiments, made in duplicate.

The replacement of position 4 with amino acids with acidic (compound 61) or aromatic (compound 62) side chains produced inactive derivatives. Previous findings indicated that aromaticity in position 4 is detrimental for biological activity since [His^4]NPS was found more than 100 fold less potent than NPS. A 10-fold reduction of potency has been obtained by replacing Asn with Thr (compound 63) while the Asn related amino acid Gln (compound 64) produced a larger decrease of biological activity (55 fold). To investigate in details the contribution of the primary

amide function of Asn^4 to biological activity, we substituted either alone (compound 65 and 66) or in combination (compound 67) the C=O and $-NH_2$ groups with $-CH_2$ and $-CH_3$, respectively. A profound loss of biological activity (>1000-fold) was observed with all these analogs suggesting a pivotal role of the amide function of the Asn^4 side chain for biological activity. In compounds 68 and 69 this moiety was mono or dimethylated generating, also in this case, inactive peptides. These latter modifications reduced or eliminated the hydrogen bond donor properties of the amide function and at the same time progressively increased the steric hindrance of position 4. The relative importance of these factors in the loss of NPSR binding can not be clearly unravelled. Collectively, these results, together with previous Ala- and D-amino acid scan indications [18,76] underline the importance of Asn^4 for the activity of NPS and suggest strict chemical requirements of the agonist binding pocket in NPSR allocating the side chain of this residue.

.**Table 6** Effects of NPS and [X^4]NPS analogues in $HEK293_{mNPSR}$ cells

N°	Compound	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem
	NPS	7.99 (7.65-8.33)	234 ± 12 %
61	[Asp^4]NPS	Crc incomplete 10 µM: 146 ± 6 %	
62	[Phe^4]NPS	Crc incomplete 10 µM: 118 ± 19 %	
63	[Thr^4]NPS	6.99 (6.86-7.12)	253 ± 10 %
64	[Gln^4]NPS	6.25 (5.65-6.85)	210 ± 19 %
65	[Dab^4]NPS	Crc incomplete 10 µM: 169 ± 18 %	
66	[$NVal^4$]NPS	Crc incomplete 10 µM: 240 ± 39 %	
67	[Ala^4]NPS	Crc incomplete 10 µM: 175 ± 27 %	
68	[Nmm^4]NPS	Crc incomplete 10 µM: 89 ± 12 %	
69	[Ndm^4]NPS	Crc incomplete 10 µM: 136 ± 17 %	

Data are means of at least 4 separate experiments.

In conclusion, the SAR studies performed on position 2 of NPS demonstrated the following: (i) lipophilicity but not aromaticity is crucial, and a cyclic lipophilic side chain seems to be favored. (ii) both the size of the chemical moiety and its distance or position from the peptide backbone are important for biological activity. In particular, the size can be enlarged up to a naphthyl or reduced down to an isobutyl moiety while the distance can only be increased by one carbon atom without major changes of biological activity. (iii) finally, position 2 plays a role in both receptor binding and activation, as demonstrated by the reduction in efficacy displayed by [Bip^2]NPS.

Results related to position 3 suggest that (i) the guanidine moiety and its basic character are not crucial requirements, (ii) an aliphatic amino acid with a linear three carbon atom long side chain is sufficient to bind and fully activate NPSR, (iii) the receptor pocket allocating the side chain of

position 3 can accommodate slightly larger side chains at least to a certain degree [hArg, Arg(NO_2) or Arg($Me)_2$ but not Arg(Tos)].

Position 4 seems to be more sensitive to amino acids replacement compared to position 3; in fact, all the amino acid replacements investigated produced an important decrease of biological activity or generated inactive derivatives suggesting a pivotal role of the Asn^4 side chain for NPS bioactivity.

3.2 Biological activity of NPS analogues modified in position 5

In the context of a conformation-activity study, [Aib^5]NPS and [D-Ala^5]NPS were identified as NPSR partial agonists [77]. These results indicate that conformational changes induced by substituting Gly^5 with the achiral α helix promoting amino acid Aib or with D-Ala are capable of reducing agonist efficacy. On these basis, we planned a SAR study on Gly^5 and replaced it with a series of L and D amino acids characterized by hydrophobic aromatic and aliphatic side chains, including some Cys derivatives protected on the sulfhydryl group. Fifteen novel NPS analogues were synthesized and pharmacologically evaluated in the calcium mobilization assay using HEK293$_{mNPSR}$ and the fluorometric imaging plate reader FlexStation II.

All data obtained investigating the SAR requirements of NPS positions 5 are summarized in table 7. In the calcium mobilization assay NPS increased the intracellular calcium leavels in a concentration-dependent manner with pEC_{50} and E_{max} values of 8.65 and 295% over basal, respectively. This result is in line with those previously reported. The substitution of Gly^5 with natural amino acids containing hydrophobic aromatic side chains (compound 70 and 71) produced a drastic decrease (>300-fold) in peptide potency and, in the case of [Phe^5]NPS, also an important reduction of efficacy. On the other hand, the replacement of Gly^5 with natural amino acids with hydrophobic aliphatic side chains (compounds 72-75) generated NPSR full agonists with moderate to high potency. In particular, [Cys^5]NPS was found to be only 6-fold less potent than the natural peptide, while increasing the size of the amino acid side chain produced a progressive decrease in potency with [Leu^5]NPS being 100 fold less potent than NPS. These results suggest that position 5 can tolerate substitutions with amino acids characterized by small side chains while larger side chains reduce agonist potency. Previous results obtained with [Ala^5]NPS which behaves as a high potency NPSR full agonist [76-77] corroborate this suggestion. It should be emphasized that these analogues differ from the natural peptide not only by their position 5 side chain but also by the insertion of an L chiral centre, which substitutes the achiral C^{α} of Gly^5. This chiral insertion, however, does not seem to significantly affect the pharmacological activity of the peptide analogues. Interestingly, this modification is compatible with the nascent helix spanning residues 74 through 82 recently proposed as the bioactive conformation of NPS [18]. To further investigate the possible role of chirality in this position, the D enantiomers of the same amino acids were used to generate compounds 76-81. The substitution of Gly^5 with D amino acids with hydrophobic aromatic side chains (compound 76 and 77) produced a complete elimination of efficacy and, as in the case

of their L enantiomers, an important reduction (approximately 100 fold) of potency. The replacement of Gly^5 with D amino acids with hydrophobic aliphatic side chains (compound 78-81) generated NPSR partial agonists ([D-Leu^5]NPS and [D-Cys^5]NPS) or pure antagonists ([D-Val^5]NPS and [D-Met^5]NPS) with moderate to high potency. These results clearly indicate that the insertion of a C^{α} chiral carbon with relative D-configuration in NPS position 5 produces, depending on the chemical features of the side chain, an important reduction of efficacy or its total elimination. Interestingly and corroborating this evidence, [D-Ala^5]NPS behaves as an NPSR partial agonist while, as mentioned before, [Ala^5]NPS is a full agonist [77]. As far as peptide potency is concerned, the rank order of agonist potency obtained with L amino acid substitutions (Cys > Val > Met > Leu > Phe > Trp) is very similar to the rank order of antagonist potency obtained with the D enantiomers (Cys > Val > Leu > Met > Trp > Phe). On this basis, it can be proposed for position 5 that the amino acid side chain size is very important for NPSR binding and inversely related to peptide potency, while the amino acid chirality has a crucial impact on the ability of the peptide to activate the receptor with L residues acting as partial/full agonists and D residues acting as low efficacy partial agonists or pure antagonists. Among [D-Xaa^5]NPS derivatives, the partial agonist [D-Cys^5]NPS was the most potent. In an attempt to increase peptide potency and particularly to reduce efficacy, some D-Cys side chain protected derivatives were used to substitute NPS Gly^5 (compound 82-84). These peptides behaved as pure antagonists.

Table 7 Effects of NPS and [X^5]NPS analogues in $HEK293_{mNPSR}$ cells

		agonist		antagonist
N°	**compound**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± sem**	**pK_B ($CL_{95\%}$)**
	NPS	8.65 (8.55-8.75)	295 ± 22%	ND
70	[Phe^5]NPS	6.11 (5.35-6.90)	69 ± 8%*	< 6
71	[Trp^5]NPS	Crc incomplete 10 µM: 30 ± 9 %		< 6
72	[Leu^5]NPS	6.64 (6.47-6.81)	214 ± 26%	ND
73	[Val^5]NPS	7.18 (6.57-7.79)	231 ± 68%	ND
74	[Met^5]NPS	7.06 (6.69-7.43)	240 ± 19%	ND
75	[Cys^5]NPS	7.86 (7.25-8.47)	280 ± 37%	ND
76	[D-Phe^5]NPS	Inactive up to 10 µM		6.27 (5.88-6.66)
77	[D-Trp^5]NPS	Inactive up to 10 µM		6.79 (6.21-7.37)
78	[D-Leu^5]NPS	7.05 (6.53-7.57)	118 ± 33%*	7.44 (6.96 – 7.94)
79	[D-Val^5]NPS	Inactive up to 10 µM		7.56 (7.12-8.00)
80	[D-Met^5]NPS	Inactive up to 10 µM		7.09 (6.31-7.87)
81	[D-Cys^5]NPS	7.15 (6.39-7.91)	59 ± 13%*	7.84 (7.52-8.16)
82	[D-Cys(Acm)^5]NPS	Inactive up to 10 µM		6.47 (5.27-7.67)
83	[D-Cys(Bzl)^5]NPS	Inactive up to 10 µM		7.22 (7.01-7.43)
84	[D-Cys(tBu)^5]NPS	Inactive up to 10 µM		6.62 (6.40-6.84)

Data are means of at least 4 separate experiments, made in duplicate. *p < 0.05 vs NPS E_{max} according to one-way ANOVA followed by the Dunnett test.

In conclusion, the present study demonstrated a crucial role of the chirality of the amino acid residues of position 5 of NPS for peptide efficacy with L aminoacids favoring agonist and D aminoacids antagonist bioactive conformations. Moreover, in the frame of the present study we identified a series of NPS analogues devoid of efficacy and able to block agonist activity at NPSR with moderate to high potency: thus the first series of NPSR peptide antagonists has been discovered.

The finding that the replacement of Gly^5 with a D aminoacid promotes an antagonist bioactive conformations prompted us to further investigate NPS position 5 with the aim of understanding the chemical requirements of the D aminoacid side chains that are instrumental for

generating NPSR antagonism. In the context of this study eleven novel peptides were synthesized and pharmacologically evaluated in the calcium mobilization assay.

All data obtained from this study are summarized in table 8. Here [D-Val^5]NPS was used as reference NPSR antagonist. It is worth of mention that, to facilitate drug diffusion into the wells in antagonist type experiments, the present studies were performed at 37 °C and three cycles of mixing were performed immediately after antagonist injection to the wells. This is different from previous calcium mobilization experiments, performed at room temperature and without mixing. NPS increased the intracellular calcium concentrations in a concentration-dependent manner with pEC_{50} and E_{max} values of 8.32 and 295% over basal, respectively. Confirming previous findings, [D-Val^5]NPS did not evoke any effect *per se* but inhibited in a concentration dependent manner the stimulatory effect of 30 nM NPS, thus behaving as an NPSR antagonist. A pK_B value of 6.54 was derived from these experiments. The replacement of the isopropyl group (as in Val) with a sec-butyl group (85 and 86) produced a similar moderate reduction of potency independently from the side chain chiral centre. Similar results were obtained substituting a methyl of the isopropyl group with an oxidril function (87 and 88). In this latter case, the side chain chiral centre seems to exert an effect on peptide efficacy, since [D-allo-Thr^5]NPS behaves as a pure NPSR antagonist while [D-Thr^5]NPS as a low efficacy partial agonist. However, the difference in efficacy between 87 and 88 ([D-Thr^5]NPS $\alpha = 0.08$; [D-allo-Thr^5]NPS $\alpha = 0$) is too little to be meaningful. A linear three-carbon side chain (89) produced an analogue that behaved as a NPSR low efficacy partial agonist 10 fold less potent than [D-Val^5]NPS. These findings indicated that the isopropyl moiety is highly important for NPSR antagonist binding and that the replacement of one of its methyl groups with ethyl or oxidril functions produced a reduction of potency. Moreover, the three carbon atoms of the D-Val side chain must have a ramified (isopropyl) rather than linear (n-propyl, as in 89) shape; in fact, the latter generates a clear reduction of peptide potency. The introduction in position 5 of a cyclohexyl or methylcyclohexyl moiety (90 and 91) generated inactive derivatives, while the introduction of a phenyl ring (92) produced only a 3 fold reduction in potency compared to the isopropyl moiety of [D-Val^5]NPS. These data suggest that the increase in the side chain size (as in 90 and 91) decreases peptide potency. This is further suggested by the results obtained with 92. In fact, the aromaticity of the phenyl ring of 92 reduced the side chain size and changed its shape compared with the cyclohexyl moiety of 90, and this may explain the moderate potency of [D-Phg^5]NPS compared to the inactivity of [cyclohexyl-D-Gly^5]NPS. Next, the effect of the insertion in the D-Val^5 isopropyl moiety of a CH_3 (93) or SH (94) group was evaluated. In both cases the chemical change did not modify the pharmacological activity of the peptides; i.e., they behaved as

pure antagonists, with a 3 fold increase in potency. Finally, the insertion of a carbon atom between the ^{t}Bu moiety and the peptide backbone (95) caused an important reduction of peptide potency associated with a clear increase in efficacy ([^{t}Bu-D-Gly^{5}]NPS α = 0, pK_B 7.06; [^{t}Bu-D-Ala^{5}]NPS α = 0.35, pK_B 6.32).

Table 8 Effects of NPS and [X^{5}]NPS analogues residues in HEK293$_{mNPSR}$ cells

		agonist		**antagonist**
N°	**compound**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± sem**	**pK_B ($CL_{95\%}$)**
	NPS	8.65 (8.55-8.75)	295 ± 22%	ND
	[D-Val^{5}]NPS	Inactive up to 10 μM		6.54 (5.99-6.99)
85	[D-Ile^{5}]NPS	Inactive up to 10 μM		5.72 (5.10-6.34)
86	[D-allo-Ile^{5}]NPS	Inactive up to 10 μM		6.04 (5.52-6.56)
87	[D-Thr^{5}]NPS	6.10 (5.79-6.41)	25 ± 5%*	6.09 (5.79-6.36)
88	[D-allo-Thr^{5}]NPS	Inactive up to 10 μM		6.04 (5.51-6.57)
89	[D-Nva^{5}]NPS	5.57 (5.02-6.12)	24 ± 7%*	5.68 (5.38-5.98)
90	[cyclohexyl-D-Gly^{5}]NPS	Inactive up to 10 μM		< 5
91	[D-Cha^{5}]NPS	Inactive up to 10 μM		< 5
92	[D-Phg^{5}]NPS	6.18 (5.81-6.55)	15 ± 2%*	6.79 (6.21-7.37)
93	[^{t}Bu-D-Gly^{5}]NPS	Inactive up to 10 μM		7.06 (6.48-7.64)
94	[D-Pen^{5}]NPS	Inactive up to 10 μM		7.08 (6.46-7.70)
95	[^{t}Bu-D-Ala^{5}]NPS	5.78 (5.06-6.50)	104 ± 2%*	6.32 (5.63-7.01)

Data are means of at least 4 separate experiments, made in duplicate. *p < 0.05 vs NPS *E*max according to one-way ANOVA followed by the Dunnett test.

In conclusion, the present study confirmed previous indications that the D relative configuration of amino acid residues at position 5 of NPS promotes antagonist activity, indicated that the peptide antagonist potency is inversely related to the D-Xaa^{5} side chain size, and demonstrated that the ^{t}Bu (and its sulfhydryl derivative) directly linked to the Cα carbon atom is the best chemical moiety for increasing antagonist potency. [^{t}Bu-D-Gly^{5}]NPS and [D-Pen^{5}]NPS identified in the context of the present study represent the most potent NPSR peptide antagonists available up to now.

3.3 *In vivo* neuropeptide S actions in mice

In the pivotal study by Xu *et al.* [9] it has been reported that the supraspinal administration of NPS (0.01- 1 nmol) stimulated locomotor activity, reduced all stages of sleep and promoted wakefulness. The same range of doses, supraspinal administration of NPS evoked clear anxiolytic-like effects in mice subjected to the EPM, the light-dark box, the OF and the marble burying test [9]. Thus, NPS was proposed as an unique neuropeptidergic signal: an activating anxiolytic. The observations by Xu [9] were subsequently confirmed and extended by Leonard [43], which reported NPS anxiolytic activity in three preclinical mouse models predictive of anxiolytic action: the four-plate test, elevated zero maze, and SIH.

In our laboratories we set up the experimental conditions to further evaluate the supraspinal NPS actions *in vivo* in a battery of mouse behavioural assay, including LA with naïve mice, mice habituated to the test cage and mice sedated with 5 mg/kg diazepam, recovery of RR following treatment with a hypnotic dose of diazepam (15 mg/kg), EPM, OF and SIH. The effects elicited by NPS in these behavioural assays were compared to those evoked under the same experimental conditions by reference drugs, such as caffeine and diazepam.

Locomotor activity

A first series of experiments to evaluate mouse LA activity was performed using Basile activity cages which consist of a four-channel resistance detector circuit, which converts the bridges 'broken' by the animals paws into pulses that are summed by an electronic counter every 5 min. In these experiments NPS effects were compared to those evoked by caffeine (reference stimulant drug). Naïve mice injected with saline displayed a progressive reduction in spontaneous LA over the time course of the experiment. Their cumulative LA in 60 min was >1000 impulses. Mice habituated to the test cage for 60 min before the experiment showed an important reduction in LA with cumulative impulses <500. An even greater inhibitory effect was produced by i.p. administration of a sedative dose of diazepam (5 mg/kg), such that mice treated with the benzodiazepine displayed a cumulative LA of approx 250 impulses in 60 min. Caffeine administration promoted a robust and consistent increase in locomotion in naïve, habituated and diazepam treated mice (figure 13). The stimulatory effect of caffeine was more pronounced in habituated (327% of controls) and diazepam treated (310% of controls) animals than that in naïve mice (167% of controls). Similar results were obtained following i.c.v. injection of NPS in naïve, habituated and diazepam-treated mice (figure 14). NPS in the range of 0.01–1 nmol per mouse dose

dependently stimulated LA. At 0.01 nmol, the peptide was inactive whereas at 0.1 and 1 nmol, the peptide produced a robust stimulatory effect. However, in naïve and habituated animals, the effect produced by 0.1 nmol was higher than that produced by the 1 nmol dose. This was due to the fact that the onset of action of the latter dose of NPS was delayed (figure 14, top and middle panels). The maximal stimulatory effect of NPS was 162, 276 and 488% of controls in naïve, habituated and diazepam treated mice, respectively.

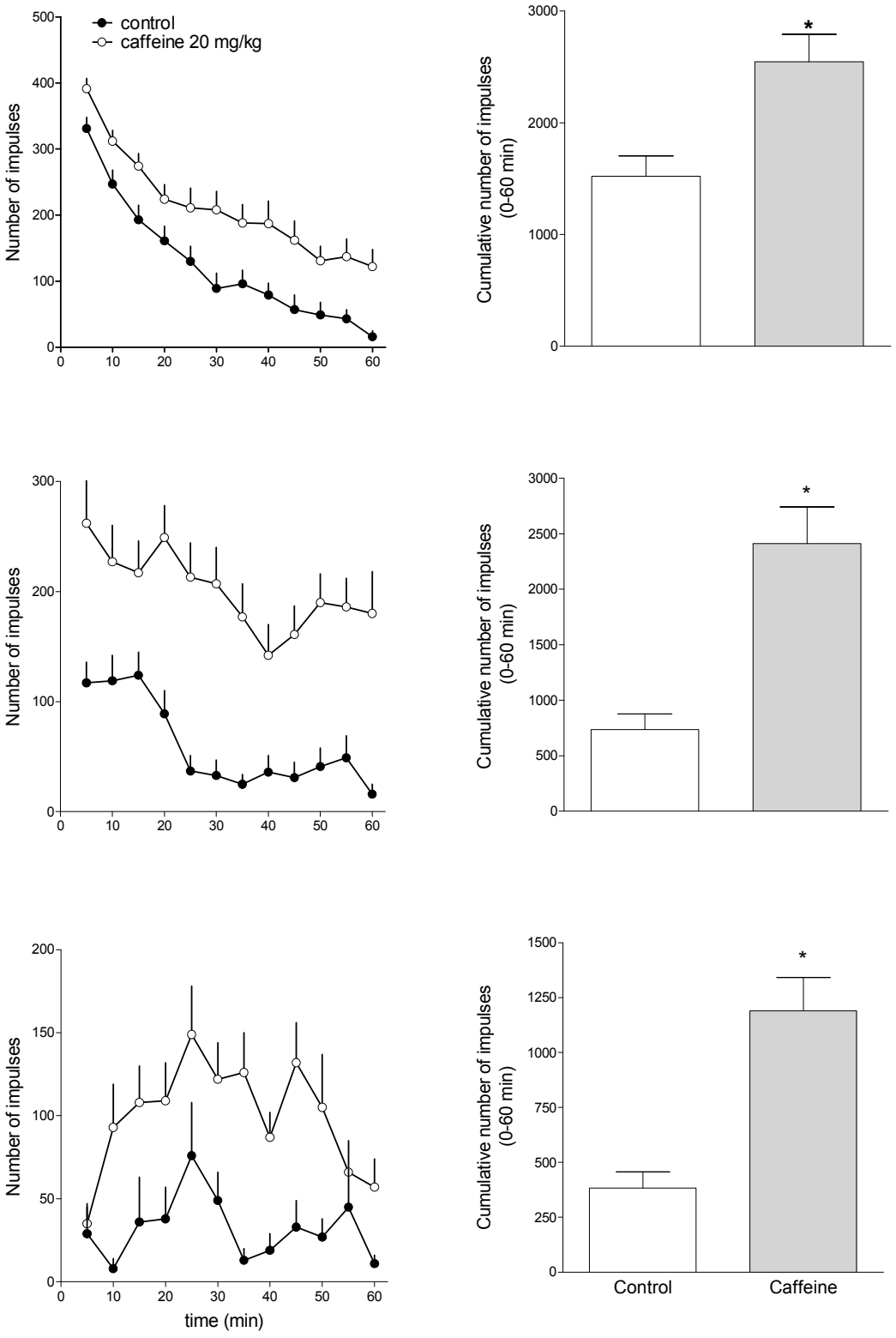

Figure 13 Locomotor activity assay. Effect of caffeine (20 mg/kg) in naïve (top panels), habituated (middle panels) and in diazepam treated (bottom panels) mice. Locomotor activity of mice is displayed over the time course of the experiment in the left panels and as cumulative impulses over the 60 min observation period in the right panels. Data are mean ± s.e.m. of 16 mice per group. *P<0.05 vs control, Student's t-test for unpaired data.

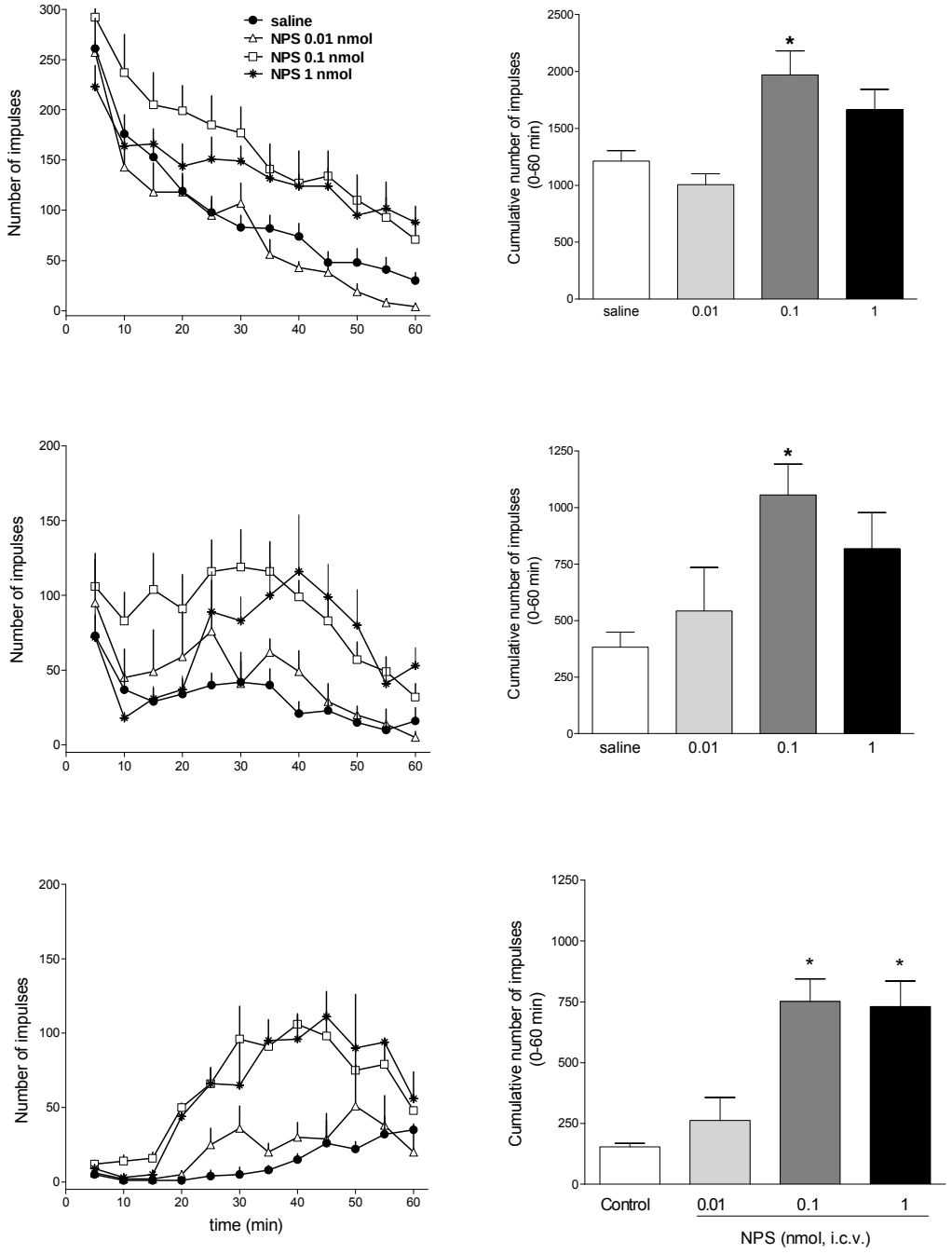

Figure 14 Locomotor activity assay. Dose response curve to NPS in naïve (top panels), habituated (middle panels) and in diazepam treated (bottom panels) mice. Locomotor activity of mice is displayed over the time course of the experiment in the left panels and as cumulative impulses over the 60 min observation period in the right panels. Data are mean ± s.e.m. of 20 mice per group. *P<0.05 vs control, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison

In a second series of experiments NPS dose response cure was assessed using the video tracking system ANY-maze. These experiments were performed only in naïve mice. Results obtained with this methods were superimposable to the previous findings. Naïve mice injected with saline displayed a progressive reduction in spontaneous LA over the time course of the experiment. Their cumulative distance travelled in 60 min was approximately 60 m, their immobility time was around 1500 s and they reared 200 times. NPS in the range of 0.01–1 nmol per mouse dose dependently stimulated LA. At 0.01 nmol, the peptide was inactive whereas at 0.1 and 1 nmol, the peptide produced a robust stimulatory effect, increasing cumulative distance travelled by the animals, the number of rearings, and reducing the total immobility time in a statistically significant manner. The effect produced by 0.1 nmol was similar to that produced by the 1 nmol dose. Distance travelled by NPS 0.1 nmol treated mice was 100 m (167% of controls), their immobility time was 700 s (47% of controls) and their number of rearing was 600 (300% of controls) (figure 15).

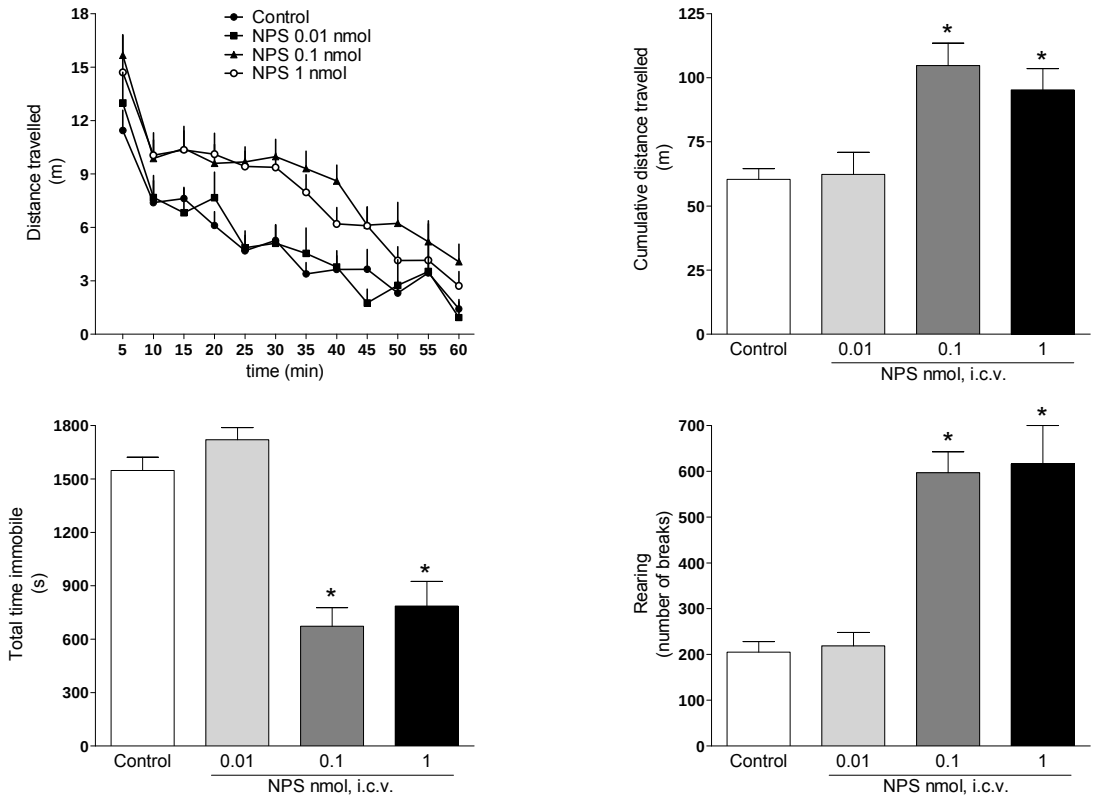

Figure 15 Locomotor activity assay. Dose response curve of NPS in naïve mice. The time course of NPS effects is shown in the top left panel, while the other panels display the cumulative effects exerted by the peptide over the 60 min observation period. Data are mean ± s.e.m. of 16 mice per group. *P<0.05 vs control, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

Recovery of righting reflex

In RR test, the injection of diazepam at the hypnotic dose of 15 mg/kg produced loss of RR in 100% of the mice injected with saline. These mice needed around 80 min to regain the RR. Pre-treatment with caffeine (20 mg/kg i.p.) decreased both the percentage of mice losing RR in response to the benzodiazepine and their sleeping time. Only 33% of caffeine treated mice lost RR and the sleeping time of those animals responding to diazepam was reduced to 28 min (35% of controls, figure 16, top panels). This arousal promoting effect of caffeine was mimicked by i.c.v. injection of NPS. Over the 0.01–1 nmol range, the peptide dose dependently reduced the proportion of animals responding to diazepam and their sleeping time. The maximal arousal promoting effect of NPS was obtained with the dose of 1 nmol, which reduced the percentage of animals showing loss of RR to 50% and their sleeping time to 22 min (28% of controls, figure 16, bottom panels).

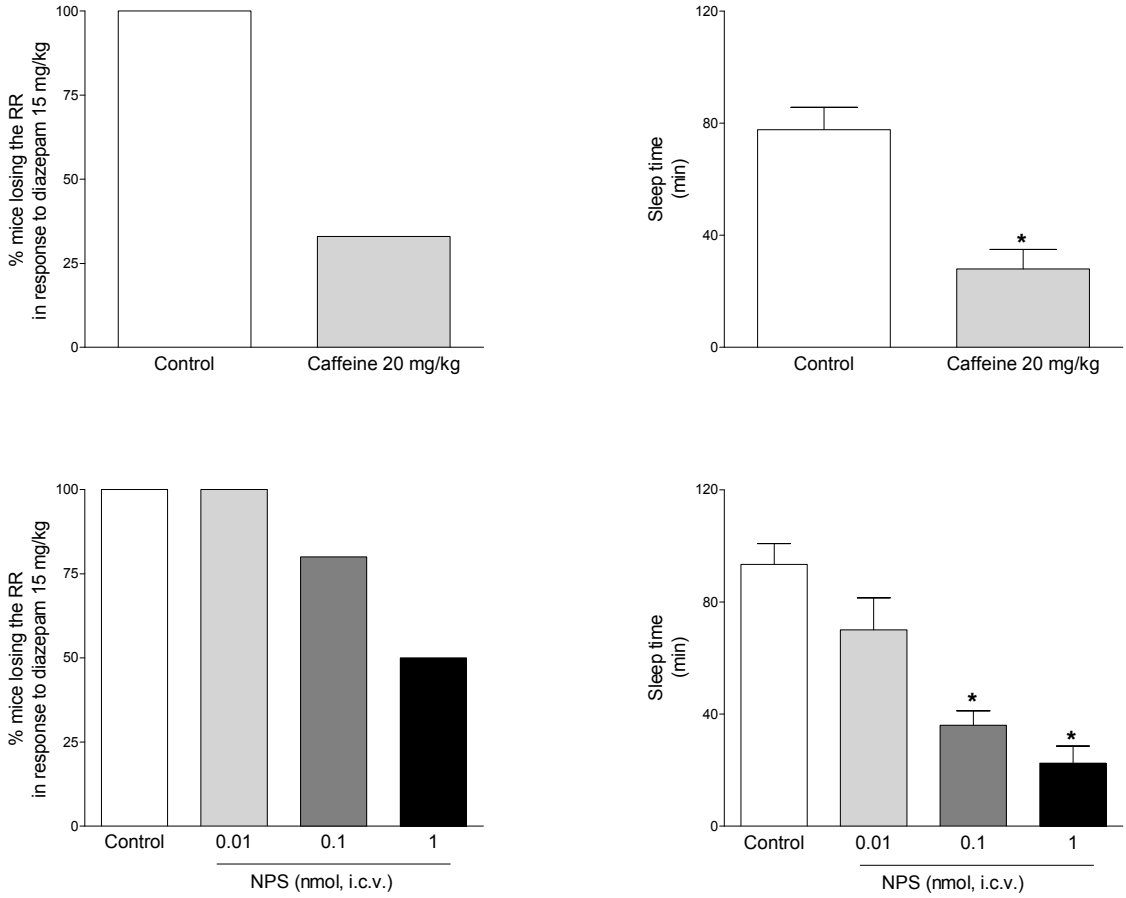

Figure 16 Recovery of righting reflex in mice. Effect of caffeine (20 mg/kg, i.p., top panels) and of NPS (0.01–1 nmol, bottom panels) on the per cent of animals losing the righting reflex in response to diazepam 15 mg/kg (left panels) and on their sleeping time (right panels). Sleeping time is defined as the amount of time between the loss and regaining of the righting reflex. Data are mean ± s.e.m. of 14 mice per group. *P<0.05 vs control, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

Elevated plus maze test

In EPM, NPS effects were compared to those evoked by diazepam (reference anxiolytic drug) and caffeine (reference anxiogenic drug). In this test, saline injected mice spent about 35 s in the open arms (corresponding to 19% of the total time spent in open and closed arms) and their entries into open arms were approximately four (corresponding to 27% of the total entries into open and closed arms). Diazepam (1 mg/kg; i.p.) significantly increased both the percentage of time spent in and the number of entries into open arms (figure 17, top panels). Similar anxiolytic-like effects were promoted by i.c.v. injection of NPS (figure 17, bottom panels). In the range 0.001–1 nmol, NPS dose dependently increased the time spent by animals in the open arms with a maximal effect, evoked at 1 nmol, equal to 157 s (corresponding to 68% of the total time spent in open and closed arms). This effect was associated with a statistically significant increase in entries into the open arms, which was, however, dose independent. Under the same experimental conditions, caffeine produced the opposite result reducing to 15 s, the time spent by animals in the open arms (corresponding to 11% of the total time spent in open and closed arms) and the number of entries into open arms to three (corresponding to 21% of the total entries into open and closed arms) (table 9). All the behavioural parameters measured in the EPM in saline as well as drug-treated animals are summarised in table 9.

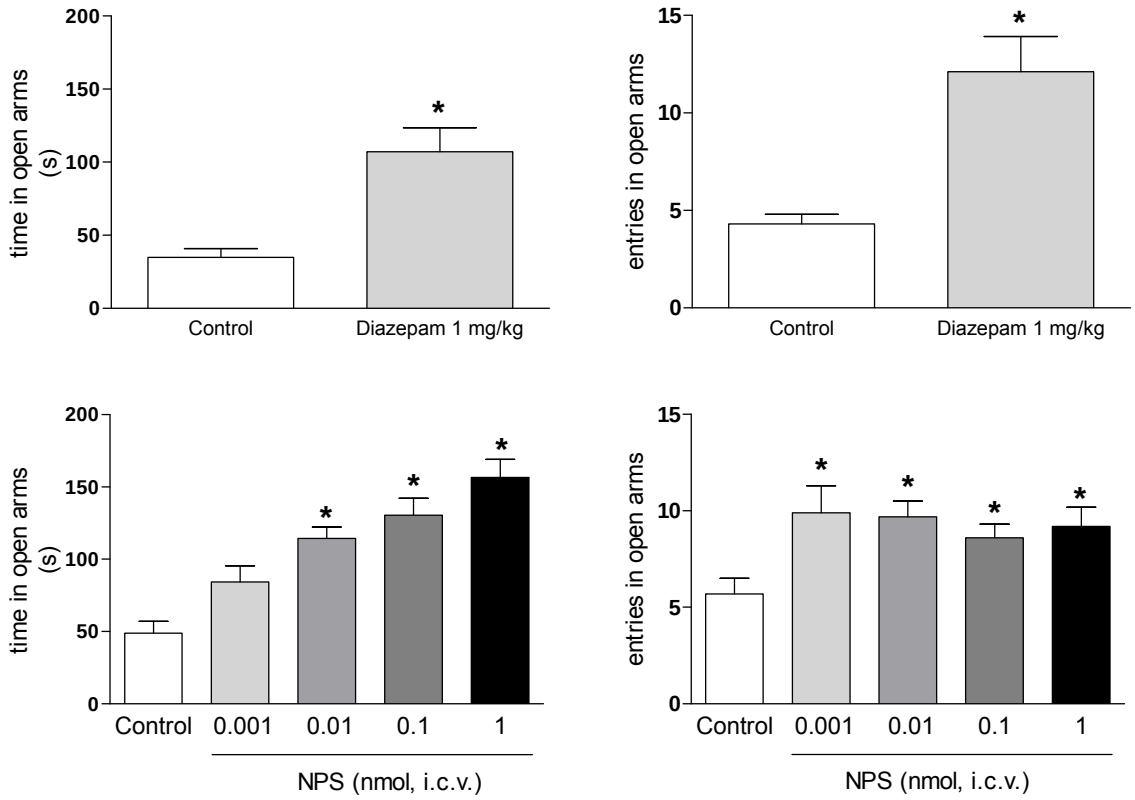

Figure 17 Elevated plus maze assay. Effects of diazepam (1 mg/kg i.p., top panels) and of i.c.v. injected NPS (0.001–1 nmol, bottom panels) on the time spent in (left panel) and on the number of entries into the open arms (right panel). Data are mean ± s.e.m. of 16 mice per group. *P<0.05 vs control, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

Table 9 Effects of diazepam, caffeine and NPS on various behavioural parameters displayed by mice subjected to the EPM

Treatment	*Time in open arms (s)*	*Entries in open arms*	*Time in closed arms (s)*	*Entries in closed arms*	*Stretch attend posture*	*Head-dipping*	*Rearing*
Control	34.9± 6.0	4.3± 0.5	161.6± 8.4	11.1± 1.3	8.1± 0.7	3.8± 0.7	11.1± 1.4
Diazepam 1 mg/kg	107± 16.6*	12.1± 1.8*	111.9± 17.4*	13.3± 1.9	4.9± 1.2*	8.9± 1.0*	12.5± 1.4
Control	39.8± 6.9	4.8± 0.9	129.7± 6.5	8.8± 0.8	4.1 ± 0.6	5.7± 0.9	11.8± 1.6
Caffeine 20 mg/kg	14.8± 3.5*	2.7± 0.4*	125.4± 10.2	10.1± 0.7	7.5± 0.8*	4.5± 0.7	12.3± 1.6
Control	48.9± 8.2	5.7± 0.8	133.6± 10.1	10.3± 0.7	5.3± 0.9	8.2± 1.2	10.3± 1.6
NPS 0.001 nmol	84.2 ± 11.2	9.9± 1.4	111.8± 6.6	10.8± 0.7	3.2± 1.2	11.1± 1.7	12.0± 1.8
NPS 0.01 nmol	114.4± 7.9*	9.7± 0.8*	107.2± 7.1	8.8± 0.8	1.8± 0.4*	14.9± 2.3	11.6± 1.9
NPS 0.1 nmol	130.4± 11.6*	8.6± 0.7*	89.2± 10.6*	7.1± 0.5*	1.3± 0.3*	16.3± 2.8	8.6± 1.7
NPS 1 nmol	156.6± 12.4*	9.2± 1.0*	68.2± 4.9*	6.1± 0.7*	0.5± 0.1*	18.8±4.4*	10.2± 1.5

All values are expressed as mean ± s.e.m. of 12–16 mice per group.
*P<0.05 vs control, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

Open field

A first series of experiments were made to set up the experimental conditions of the OF test. In these experiments the effects of diazepam were assessed. Saline injected mice spent about 15 s in the central zone of the field (corresponding to 2.5% of the total time of the experiments) and their entries into the central zone were approximately 7. Diazepam (0.3 and1 mg/kg; i.p.) significantly increased both the time spent in and the number of entries into the central zone (figure 18). Similar anxiolytic-like effects were promoted by i.c.v. injection of NPS (figure 19). In the range 0.01–1 nmol, NPS dose dependently increased the time spent by animals in the central zone with a maximal effect, evoked at 1 nmol, equal to 41 s (corresponding to 7% of the total duration of the experiment). This effect was associated with a statistically significant and dose dependent increase in entries into the central zone, with a maximal effect, evoked at 1 nmol, equal to 28. All the behavioural parameters measured in the OF in saline as well as drug-treated animals are summarised in table 10.

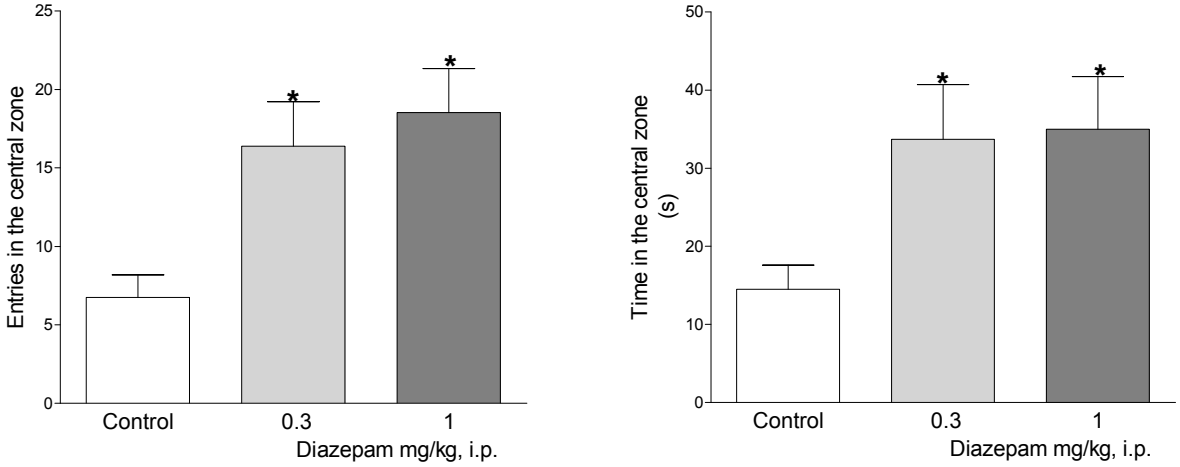

Figure 18 Open field assay. Effects of diazepam (1 and 3 mg/kg i.p.) on the number of entries into the central zone (left panel) and on the time spent in this area (right panel). Data are mean ± s.e.m. of 20 mice per group. *P<0.05 vs control, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

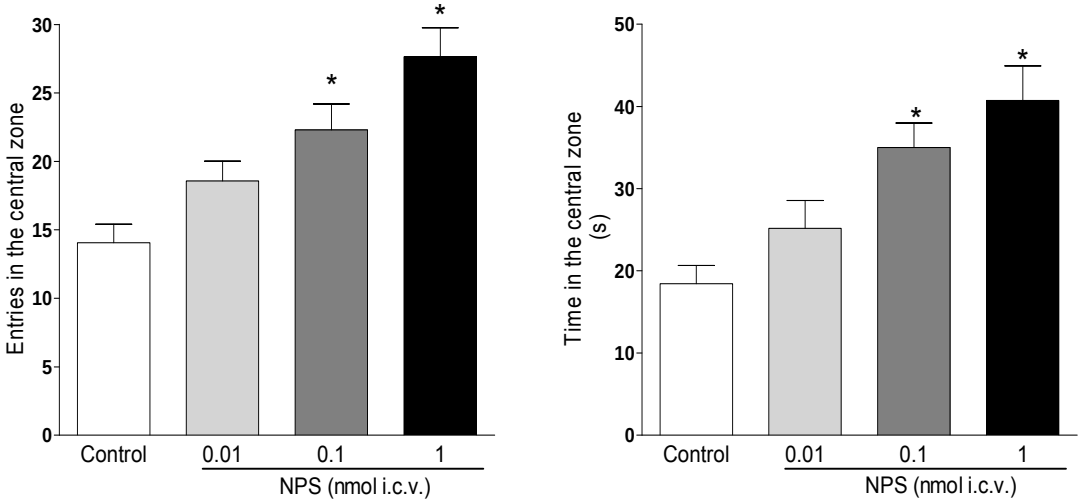

Figure 19 Open field assay. Effects of NPS (0.01- 1 nmol, i.c.v.) on the number of entries into the central zone (left panel) and on the time spent in this area (right panel). Data are mean ± s.e.m. of 20 mice per group. *P<0.05 vs control, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

Table 10 Effects of diazepam and NPS on various behavioural parameters displayed by mice subjected to the OF

Treatment	*Total distance travelled (m)*	*Time immobile (s)*	*Rearings (number of breaks)*	*Entries in the central zone*	*Time in the central zone (s)*
Control	16.79 ± 1.42	72.45 ± 14.28	71.40 ± 8.27	6.75 ± 1.44	14.50 ± 3.10
Diazepam 0.3 mg/kg	22.83 ± 1.46	41.28 ± 9.20	88.94 ± 10.32	16.38 ± 2.85*	33.68 ± 7.01*
Diazepam 1 mg/kg	27.20 ± 1.89*	69.05 ± 11.98	80.68 ± 10.35	18.53 ± 2.80*	34.99 ± 6.74*
Control	24.47 ± 1.32	101.44 ± 12.17	85.58 ± 9.38	14.06 ± 1.35	18.45 ± 2.19
NPS 0.01 nmol	27.92 ± 1.59	67.77± 9.68*	106.10 ± 8.36	18.57 ± 1.44	25.18 ± 3.39
NPS 0.1 nmol	32.76 ± 2.78*	36.90± 5.54*	129.63 ± 9.78*	22.30 ± 1.90*	35.00 ± 2.98*
NPS 1 nmol	30.96 ± 2.24	26.74± 6.69*	127.78 ± 11.39*	27.65 ± 2.11*	40.71 ± 4.21*

All values are expressed as mean ± s.e.m. of 20 mice per group.
*P<0.05 vs control, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison

Stress-induced hyperthermia test

In control mice, stress induced by the measurement of body temperature evoked a significant increase in T_2 compared with T_1 corresponding to a ΔT of about 0.6 °C. Diazepam (1–5mg/kg, i.p.) produced a dose-dependent reduction in baseline temperature, although this effect did not reach statistical significance (figure 8, top left panel). In the same dose range, the benzodiazepine reduced ΔT to negative values (figure 20, top right panel). These effects of diazepam were statistically significant at the doses of 3 and 5mg/kg. NPS (0.01-1 nmol, i.c.v.) mimicked the anxiolytic-like action of the benzodiazepine. It did not modify baseline temperature but produced a dose-related reduction in ΔT, which reached statistical significance at doses of 0.1 and 1 nmol (figure 20, bottom panels).

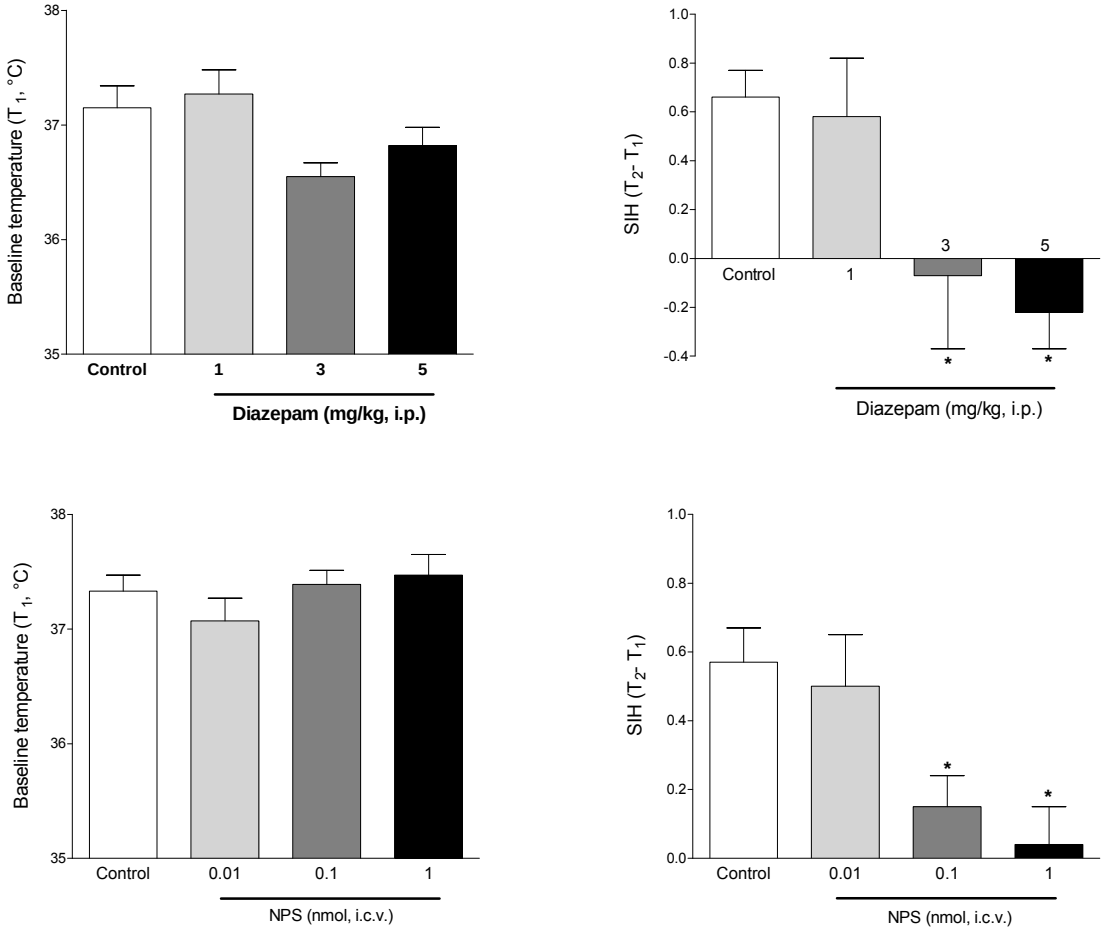

Figure 20 Stress-induced hyperthermia test. Effects of diazepam (1, 3 and 5mg/kg, i.p., top panels) and NPS (0.01–1 nmol. i.c.v., bottom panels) on baseline temperature (T_1, left panels) and SIH (T_2–T_1, right panels) in the mouse SIH test. Data are mean±s.e.m. of 14 mice per group. *P<0.05 vs control, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

Collectively, the present findings demonstrate that NPS was able to evoke a robust and consistent arousal response in mice. This stimulant effect was associated with a clear anxiolytic-like action. Thus, the present findings corroborate the proposal of NPS as a unique neuropeptidergic signal: an activating anxiolytic [9]. In LA assays, NPS mimicked the stimulatory effect of caffeine and this effect is highly consistent across different experimental conditions, that is, naïve and habituated mice, and animals sedated with diazepam. Similar results in terms of degree of stimulation, NPS potency, onset and duration of action were previously reported both in mice [9] and in rats [35]. This suggests that the locomotor stimulant effect of NPS is highly consistent among experimental conditions and animal species. The activating properties of NPS were further confirmed in the RR assay, where NPS mimicked the arousal-promoting action of caffeine in mice by reducing the number of animals losing RR in response to a hypnotic dose of diazepam and markedly decreasing the sleep time in those animals responding to the benzodiazepine. These findings parallel the results obtained by Xu *et al.* [9] in rats in electroencephalographic studies, where NPS given i.c.v. in the same dose range (0.1 and 1 nmol) increases the amount of wakefulness and decreases SWS1, SWS2 and REM sleep. These data demonstrated that the arousal-promoting action of NPS is a robust effect, which can be easily replicated in different laboratories and animal species. Collectively, our findings are in line with those already published, indicating that NPS behaves as an important signal in the brain to stimulate LA and wakefulness. In this respect, the effects of NPS are similar to those elicited by caffeine (and other psychostimulant drugs) and opposite to those of diazepam.

In order to investigate NPS effects on anxiety, EPM and OF assays were performed. EPM is based on natural aversion of rodents to open and unprotected spaces. In this experiments, 1mg/kg diazepam produced clear anxiolytic like effects, increasing the time spent by mice in the open arms. In contrast, caffeine promoted anxiogenic like effects. These results validate our EPM experimental conditions. NPS induced a dose-dependent reduction in anxiety-like behaviour in this paradigms, increasing the number of entries and the time spent in open arms of the maze. OF is a paradigm of free exploratory behaviour in a novel environment. In this model, the supraspinal administration of NPS mimicked the effects of diazepam. It was found that NPS, in a dose dependent manner, significantly increased the number of entries and the time spent in central zone, which could indicate an anxiolytic-like effect. These results are similar to those obtained by Xu *et al.* [9] not only in the same assays but also in the light–dark test. Moreover, an anxiolytic like action of NPS was also reported in mice subjected to the four plate test and elevated zero maze [43]. Thus, similar to the stimulant effects of NPS, its anxiolytic like action also seems to be a robust effect easily reproduced in different laboratories and in different species, using different assays. However, the stimulatory

action of NPS can bias the interpretation of results obtained in the above mentioned assays, in which anxiety levels are measured as inhibited behaviours.

This prompted us to investigate NPS effects in the SIH assay, a model of anxiety, which is not sensitive to LA [90]. As expected, in this assay, diazepam produced anxiolytic-like effects, that is, it counteracted the SIH response in a dose-dependent manner. However, higher benzodiazepine doses were required in this assay compared to EPM to elicit statistically significant effects. In addition, the effect of diazepam on body temperature (T_1), although not statistically significant, may represent a bias for the analysis of its effect on SIH. However, this is unlikely because it has been clearly demonstrated using different mouse strains and several benzodiazepines (diazepam, oxazepam, alprazolam and chlodiazepoxide) that the decrease in ΔT is indeed independent of the effect of the drug on temperature T_1 [96]. The action of caffeine was not evaluated in this assay because of the reported hyperthermic/hypothermic response of this drug [97] and because this assay failed to detect anxiogenic-like effects of several drugs, such as pentylenetetrazol, the beta-carboline FG7142, meta-chlorophenylpiperazine [96]. Thus, anxiogenic-like actions are extremely difficult to assess using this test, probably due to a ceiling effect of ΔT. Under the same experimental conditions, NPS did not produce any modification of T_1 but dose dependently prevented SIH. This same result has been independently replicated in a different laboratory [43]. Thus the ability of NPS to counteract SIH, a physiological parameter insensitive to locomotion, indicates that the action of NPS should be considered as a genuine (that is, not merely dependent on experimental bias) anxiolytic-like effect. This proposal is corroborated by the anxiolytic-like effects measured in mice in response to NPS in the mouse marble burying assay [9] and in rat defensive burying test [44], two model where anxiety levels are measured as an active (marble and electric probe burying) behaviour. Collectively, the effects promoted by NPS in the EPM, OF and SIH assays are similar to those elicited by diazepam and opposite to those of caffeine. Collectively, the present study confirmed and extended previous findings [9] demonstrating that NPS produced a unique behavioural profile: stimulation associated with anxiolysis.

3.4 *In vitro* and *in vivo* pharmacological characterization of peptide NPSR antagonists

The previously reported SAR studies focussed on NPS position 5 led to the identification of the first series of NPSR peptide antagonists. Based on the data reported in tables 7 and 8 the NPSR pure antagonists [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS were selected for further *in vitro* and *in vivo* pharmacological characterization.

In vitro, the antagonistic properties of these peptides were investigated in HEK239$_{mNPSR}$ cells by performing inhibition response curves against the effect elicited by 10 and 100 nM NPS, corresponding to submaximal and maximal concentrations, respectively, as well as by testing the peptides with the classical Schild protocol. *In vivo*, their NPSR antagonistic activity was assessed in the RR ([D-Cys(^{t}Bu)^5]NPS and [^{t}Bu-D-Gly^5]NPS) and LA ([D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS) assays. For the *in vivo* studies the NPS dose of 0.1 nmol was selected, based on previous results, as the lower dose producing statistically significant effects, to be challenged with the NPSR antagonists.

Calcium mobilization assay

In the calcium mobilization assai performed in HEK239$_{mNPSR}$ cells [D-Cys(^{t}Bu)^5]NPS concentration-dependently inhibited 10 and 100 nM NPS effects, with pIC_{50} values of 5.75 and 4.64 (for the latter assuming a complete inhibition at higher concentrations), respectively. A [D-Cys(^{t}Bu)^5]NPS pK_B value of 6.62 ($CL_{95\%}$, 6.40–6.84) was derived from these experiments (figure 21).

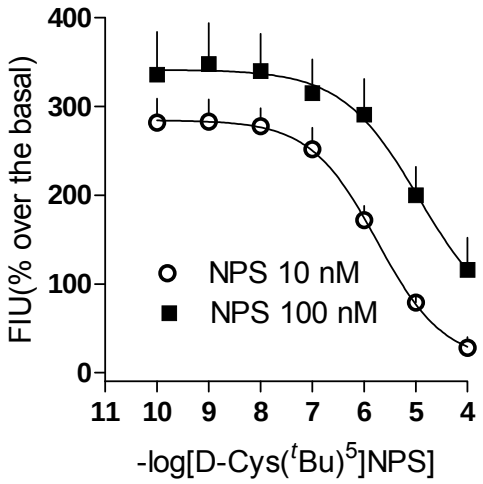

Figura 21 Calcium mobilization assay performed on HEK293$_{mNPSR}$ cells. Inhibition response curve to [D-Cys(^{t}Bu)^5]NPS (0.1 nM–100 μM) against the stimulatory effect of 10 and 100 nM NPS. Data are mean±s.e.m of 4 experiments made in duplicate.

Next, to get information on the nature of the antagonist action exerted by [D-Cys(^{t}Bu)^5]NPS, the classical Schild analysis was performed. As depicted in figure 22, left, [D-Cys(^{t}Bu)^5]NPS, in the range 0.1 to 100 μM, did not have any effect *per se* but produced a rightward shift of the concentration response curve to NPS in a concentration-dependent manner, whereas the curves remained parallel to the control and reached similar maximal effects. The corresponding Schild plot, which was linear (r^2 = 0.99) with a slope of 0.97 ± 0.07, is shown in figure 22, right. The extrapolated pA_2 value was 6.44. Finally, [D-Cys(^{t}Bu)^5]NPS selectivity of action was investigated by challenging the peptide against a panel of G protein-coupled receptors (table 11). These include native muscarinic receptors expressed in HEK293 cells, native PAR-2 receptors expressed in A549 cells, and recombinant human NK-1, UT, and opioid receptors expressed in CHO cells. In these experiments, [D-Cys(^{t}Bu)^5]NPS did not stimulate calcium mobilization up to 10 μM and did not modify the concentration response curves to receptor agonists (table 11).

Table 11 Selectivity profile of [D-Cys(tBu)^5]NPS at seven different G-protein coupled receptors.

			Control		**[D-Cys(^{t}Bu)^5]NPS 10 μM**	
Cell lines	Receptor	Agonist	pEC_{50}	E_{max} ± sem	pEC_{50}	E_{max} ± sem
HEK293	native muscarinic	carbachol	5.60 (5.21-5.69)	309 ± 31%	5.44 (4.82-6.06)	309 ± 33%
CHO	recombinant hNK-1	substance P	10.26 (9.88-10.64	122 ± 15%	10.10 (9.5-10.7)	110 ± 20%
CHO	recombinant hUT	urotensin-II	8.31 (7.62-9.00)	224 ± 21%	8.37 (7.54-9.20)	225 ± 23%
CHO-α_{qi5}	recombinant hMOP	dermorphin	7.98 (7.85-8.11)	186 ± 11%	7.80 (7.16-8.44)	194 ± 13%
CHO-α_{qi5}	recombinant hDOP	DPDPE	8.63 (8.41-8.84)	169 ± 26%	8.65 (8.52-8.78)	155 ± 22%
CHO-α_{qi5}	recombinant hKOP	dynorphin A	8.11 (7.72-8.50)	143 ± 12%	8.53 (8.00-9.06)	144 ± 12%
A549	native hPAR2	SLIGKV-NH_2	4.66 (4.43-4.89)	449 ± 25%	4.77 (4.31-5.23)	433 ± 37%

Data are mean ± sem of three separate experiments performed in duplicate. The chimeric protein α_{qi5} [98] was used to force opioid receptors to couple with the calcium pathway.

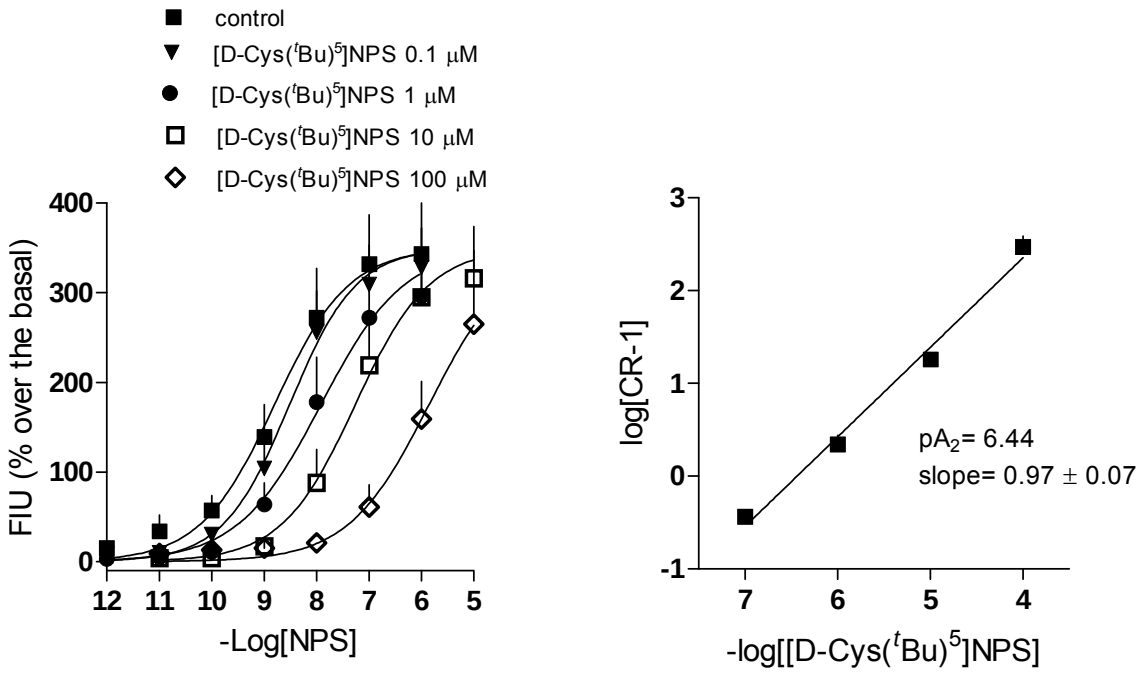

Figure 22 . Calcium mobilization assay performed on $HEK293_{mNPSR}$ cells. Concentration-response curve to NPS obtained in the absence (control) and in presence of increasing concentrations of [D-Cys(^{t}Bu)^5]NPS (0.1-100 μM) (left panel); the corresponding Schild plot is shown in the right panel. Data are mean ± sem of 4 experiments made in duplicate.

Figure 23 left panel displays the results obtained by performing inhibition response curve to [D-Val^5]NPS (0.01 nM - 10 μM) against the stimulatory effect of NPS 10 and 100 nM. As mentioned before, [D-Val^5]NPS was completely inactive *per se* up to 10 μM concentrations, however it produced a concentration dependent inhibition of NPS effects, with pIC_{50} values of 6.59 vs NPS 10 nM and 5.96 (assuming full inhibition at concentration higher than 10 μM) vs NPS 100 nM. A [D-Val^5]NPS pK_B value of 7.50 ($CL_{95\%}$ 7.18– 7.82) was derived from these experiments. In order to get further information on the nature of the antagonist action exerted by [D-Val^5]NPS the classical Schild analysis was also performed. As depicted in figure 23 right panel, [D-Val^5]NPS in the range 0.1–10 μM did not produced any effect *per se* but displaced to the right the concentration response curve to NPS in a concentration dependent manner; however this was associated to a slight but statistically significant decrease of the maximal effect elicited by NPS at the higher concentrations of antagonist (i.e. 1 and 10 μM). The extrapolated pK_B values was 7.02. To investigate [D-Val^5]NPS selectivity of action, this peptide was challenged against the effect produced by carbachol via stimulation of endogenously expressed muscarinic receptors in $HEK293_{mNPSR}$ cells. Carbachol produced a concentration dependent stimulation of calcium levels with E_{max} and pEC_{50} values of 305 ± 31% over the basal, and 5.58, respectively. The concentration response curve to carbachol was not significantly modified in the presence of [D-Val^5]NPS 10 μM both in terms of efficacy (E_{max} 309 ± 33% over the basal) and potency (pEC_{50} 5.69).

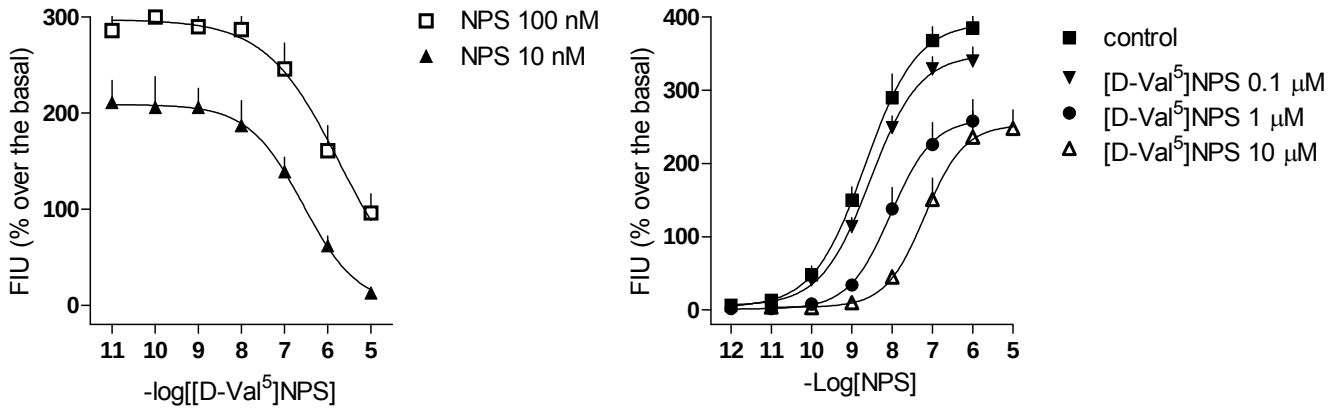

Figure 23 Calcium mobilization assay performed on $HEK293_{mNPSR}$ cells. Inhibition response curve to [D-Val^5]NPS (0.1 nM–100 μM) against the stimulatory effect of 10 and 100 nM NPS (left panel). Concentration-response curve to NPS obtained in the absence (control) and in presence of increasing concentrations of [D-Val^5]NPS (0.1-10 μM) (right panel). Data are mean ± sem of 4 experiments made in duplicate.

Finally the *in vitro* pharmacological profile of [^{t}Bu-D-Gly^5]NPS was investigated. In order to compare [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS antagonist potencies, the two peptides were tested in parallel, in the same series of experiments and under the same experimental conditions (37 °C; three cycles of mixing). Figure 24 displays the results obtained by performing inhibition response curve to [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS (0.01 nM - 10 μM) against the stimulatory effect of NPS 30 nM. As mentioned before, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS were completely inactive *per se* up to 10 μM concentrations, however they produced a concentration dependent inhibition of NPS effects, with pK_B values of 6.02 and 6.67 respectively. These data demonstrated that [^{t}Bu-D-Gly^5]NPS acted as a pure NPSR antagonist, 3 fold more potent than [D-Val^5]NPS.

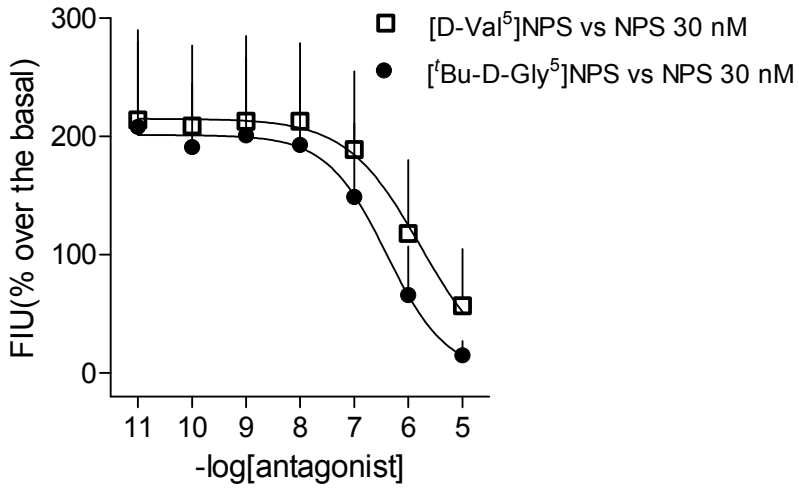

Figure 24 Calcium mobilization assay performed on HEK293$_{mNPSR}$ cells. Inhibition response curve to [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS (0.1 nM–100 μM) against the stimulatory effect of 30 nM NPS. Data are mean ± sem of 3 experiments made in duplicate.

To get further information on the nature of the antagonist action exerted by [^{t}Bu-D-Gly^5]NPS the classical Schild analysis was performed. Figure 25 summarizes the data obtained by determining concentration response curves to NPS in the presence of increasing concentrations (0.1 - 10 μM) of [^{t}Bu-D-Gly^5]NPS. This peptide produced a concentration dependent rightward shift of the concentration response curve to NPS which was associated with a slight but significant depression of NPS maximal effects. From these experiments a pK_B of 6.78 ($CL_{95\%}$ = 6.33 - 7.23) was derived using the equation described in section 2.6. To get information about the selectivity of action of [^{t}Bu-D-Gly^5]NPS, the peptide was evaluated as agonist and antagonist in calcium mobilization experiments performed using the same unrelated human G protein-coupled receptors (table 12) used to determine the selectivity of [D-Cys(^{t}Bu)^5]NPS. Moreover, this investigation was extended to the bradykinin B2 receptor and to nociceptin/orphanin FQ peptide receptor (NOP) forced to couple with the calcium pathway by the chimeric protein α_{qi5} [98]. [^{t}Bu-D-Gly^5]NPS up to

10 μM neither stimulated calcium mobilization in these cells nor affected the stimulatory effects elicited by the reference receptor agonists (table 12).

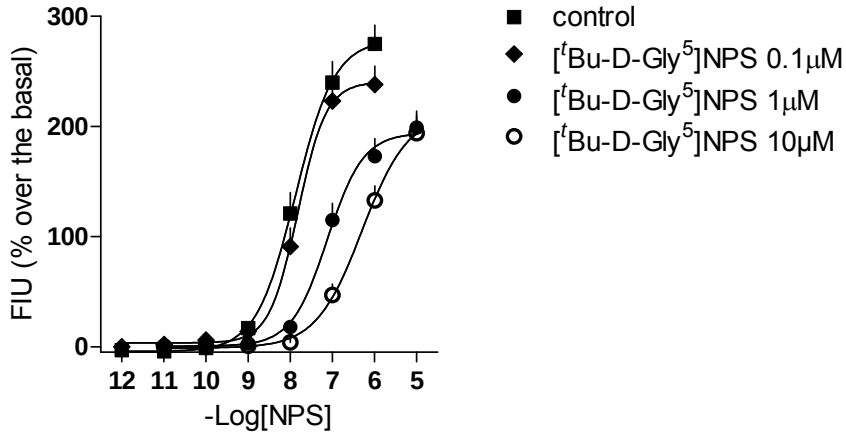

Figure 25 Concentration-response curve to NPS obtained in the absence (control) and presence of increasing concentrations of [^{t}Bu-D-Gly^5]NPS in calcium mobilization experiments performed in HEK293$_{mNPSR}$ cells. Data are mean ± sem of 4 experiments made in duplicate.

Table 12 Selectivity profile of [^{t}Bu-D-Gly5]NPS at eight different G-protein coupled receptors.

			Control		**[^{t}Bu-D-Gly^5]NPS 10 μM**	
Cell lines	Receptor	Agonist	pEC_{50}	E_{max} ± sem	pEC_{50}	E_{max} ± sem
CHO	recombinant hNK-1	substance P	10.24 (9.86-10.6)	122 ± 15%	10.31 (9.9-10.7)	113 ± 11%
CHO	recombinant B_2	bradykinin	10.31 (10.2-10.3)	125 ± 2%	10.47 (9.9-11.0)	115 ± 11%
CHO	recombinant hUT	urotensin-II	8.39 (7.50-8.88)	224 ± 21%	8.46 (8.19-8.73)	234 ± 19%
CHO-α_{qi5}	recombinant hMOP	dermorphin	7.98 (7.85-8.11)	186 ± 11%	7.96 (7.71-8.21)	186 ± 15%
CHO-α_{qi5}	recombinant hDOP	DPDPE	8.53 (8.23-8.73)	169 ± 26%	8.71 (8.65-8.77)	177 ± 11%
CHO-α_{qi5}	recombinant hKOP	dynorphin A	8.20 (7.81-8.59)	143 ± 12%	8.63 (8.33-8.99)	139 ± 13%
CHO-α_{qi5}	recombinant hNOP	N/OFQ	9.41 (9.26-9.56)	231 ± 12%	9.55 (9.11-9.99)	207 ± 30%
A549	native hPAR2	SLIGKV-NH_2	4.55 (4.06-5.04)	449 ± 25%	4.50 (3.96-5.04)	445 ± 30%

Data are mean ± sem of three separate experiments performed in duplicate. The chimeric protein α_{qi5} [98] was used to force opioid receptors to couple with the calcium pathway.

Recovery of righting reflex

[D-Cys(^{t}Bu)^5]NPS effects were evaluated *in vivo* in RR test. As shown in figure 26, i.p. injection of diazepam at the hypnotic dose of 15 mg/kg produced loss of the RR in 92% of the mice and approx 105 min were needed to regain this reflex. NPS injected i.c.v. at 0.1 nmol reduced the percentage of animals responding to diazepam to 58% and their sleep time to approx 50 min. On the contrary, the administration of [D-Cys(^{t}Bu)^5]NPS at 1 and 10 nmol did not significantly modify the hypnotic effect of diazepam either in terms of percent of animals losing the RR or sleeping time. When [D-Cys(^{t}Bu)^5]NPS 1 nmol was coinjected with NPS 0.1 nmol it did not significantly modify the action of the natural peptide, however when the higher dose of 10 nmol was used [D-Cys(^{t}Bu)^5]NPS prevented the arousal promoting effect of NPS (figure 26).

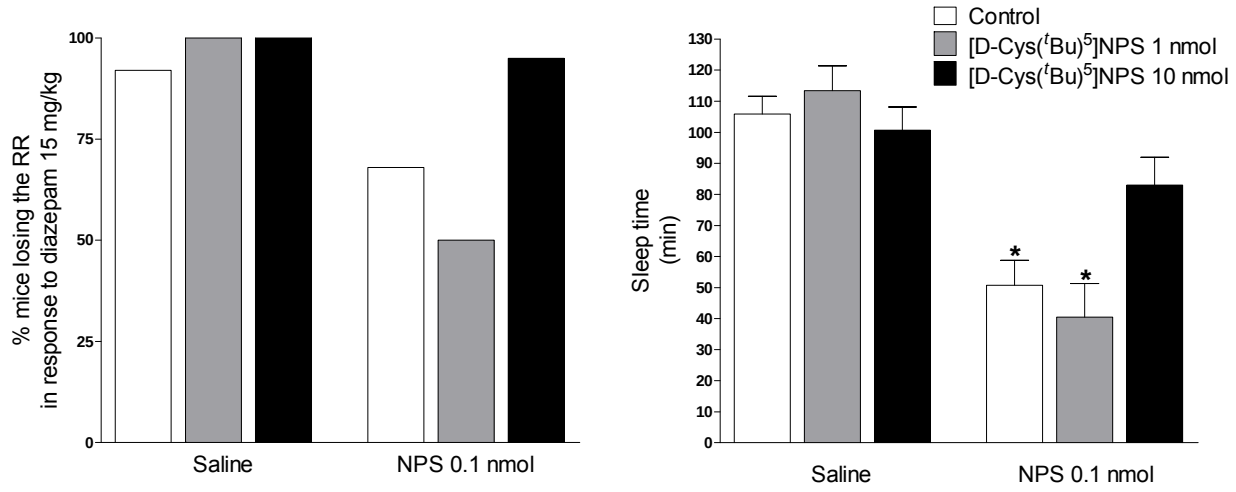

Figure 26 Recovery of righting reflex in mice. Effects elicited by i.c.v. injected NPS (0.1 nmol) and [D-Cys(^{t}Bu)^5]NPS (1–10 nmol) alone or coinjected on the percentage of animals losing the righting reflex in response to 15 mg/kg i.p. diazepam (left) and on their sleep time (right). Sleep time is defined as the amount of time between the loss and regaining of the righting reflex. Data are mean ± sem of 16 mice per group. *P<0.05 vs saline, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

With a second series of experiments the *in vivo* activity of [^{t}Bu-D-Gly^5]NPS was assessed in the RR test. In line with previous findings, i.p. injection of diazepam at the hypnotic dose of 15 mg/kg produced loss of the RR in 100% of the mice and approx 120 min were needed to regain this reflex. NPS injected i.c.v. at 0.1 nmol reduced the sleep time of mice responding to diazepam to approx 70 min. [^{t}Bu-D-Gly^5]NPS up to 10 nmol did not significantly modify the hypnotic effect of diazepam either in terms of percent of animals losing the RR or sleep time. When [^{t}Bu-D-Gly^5]NPS 0.1 nmol was challenged with NPS it did not block the action of the natural peptide, however when the higher doses of 1 and 10 nmol were used [^{t}Bu-D-Gly^5]NPS completely prevented the arousal promoting effect of NPS (figure 27).

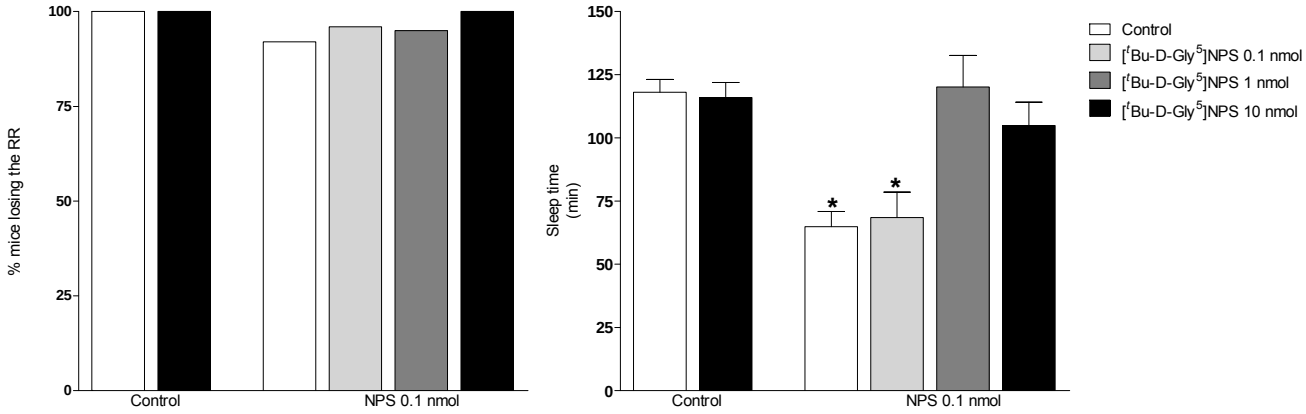

Figure 27 Recovery of righting reflex in mice. Effects elicited by i.c.v. injected NPS (0.1 nmol) and [^{t}Bu-D-Gly5]NPS (0.1–10 nmol) on the percentage of animals losing the righting reflex in response to 15 mg/kg i.p. diazepam (left) and on their sleep time (right). Sleep time is defined as the amount of time between the loss and regaining of the righting reflex. Data are mean ± sem of 16 mice per group. *P<0.05 vs saline, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

Locomotor activity

[D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS effects were investigated *in vivo* in LA test. In line with previous findings, NPS injected i.c.v. at 0.1 nmol evoked a stimulatory effect on mouse LA by increasing mice cumulative distance travelled and number of rearings, and reducing the total immobility time, in a statistically significant manner. [D-Val^5]NPS injected i.c.v. at 10 nmol did not statistically modify the animal locomotor behaviour (figure 28). However, it is worthy of mention (although this effect did not reach the statistical level of significance) that there was a clear tendency of animals treated with 10 nmol [D-Val^5]NPS to reduce their rearing behaviour. More importantly, [D-Val^5]NPS completely prevented the locomotor stimulatory effect of 0.1 nmol NPS on all parameters detected (figure 28).

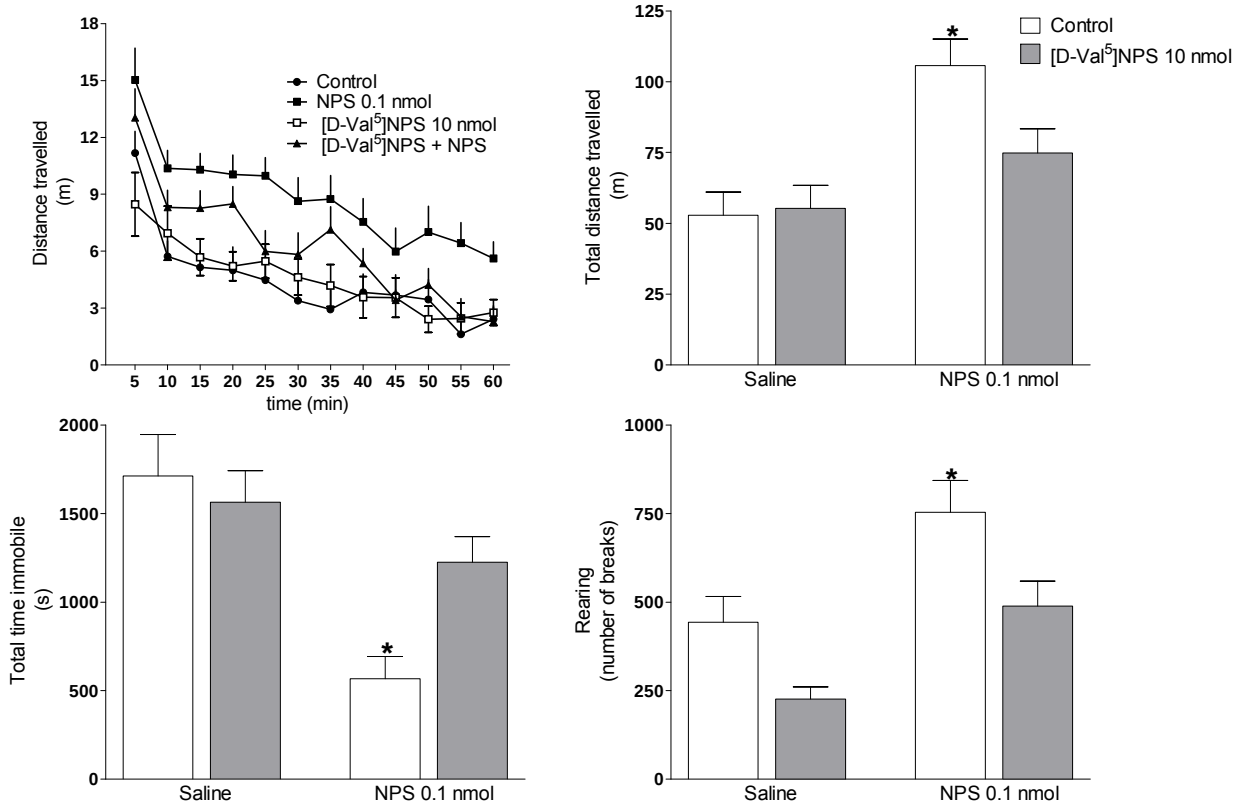

Figure 28 Locomotor activity assay. Effects of 0.1 nmol NPS, 10 nmol [D-Val^5]NPS and their coapplication. The time course of peptide effects is shown in the top left panel, while the other panels display the cumulative effects exerted by the peptides over the 60 min observation period. Data are mean ± sem of 16 mice per group. *P<0.05 vs saline, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

[^{t}Bu-D-Gly^5]NPS at 10 nmol did not affect *per se* the animal locomotion. When [^{t}Bu-D-Gly^5]NPS 1 nmol was challenged with NPS it did not significantly modify the action of the natural peptide, however when the higher doses of 3 and 10 nmol were given 15 min before NPS administration, [^{t}Bu-D-Gly^5]NPS completely prevented, in a dose dependent manner, the stimulatory effects evoked by NPS (figure 29).

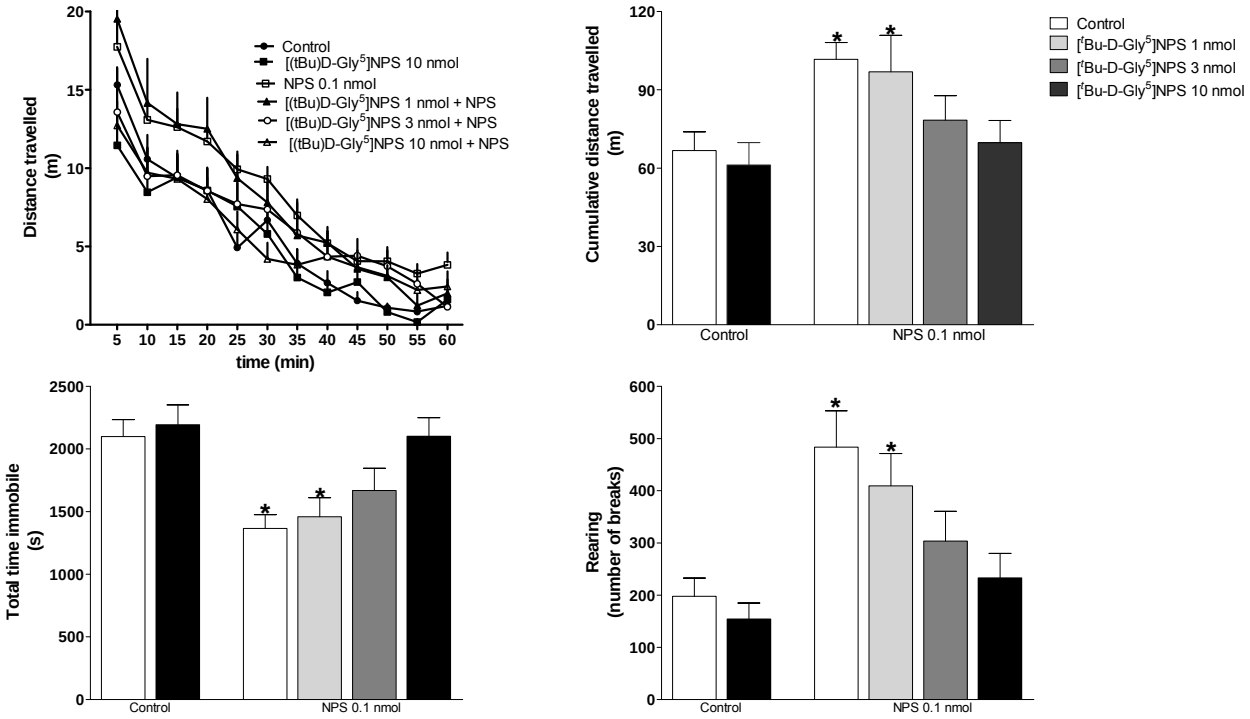

Figure 29 Locomotor activity assay. Effects elicited by i.c.v. injected NPS (0.1 nmol) and [^{t}Bu-D-Gly^{5}]NPS (1–10 nmol). The time course of the distance travelled is shown in the top left panel, while the other panels display the cumulative effects exerted by the peptides on distance travelled (top right panel), total time immobile (bottom left panel) and number of rearings (bottom right panel) over the 60 min observation period. Data are mean ± sem of 16 mice per group. *P<0.05 vs saline, analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparison.

[D-Cys(^{t}Bu)^5]NPS at concentrations as high as 100 μM did not stimulate calcium mobilization in HEK293$_{mNPSR}$ cells but was able to completely inhibit, in a concentration dependent manner, the stimulatory effect of NPS. These results demonstrate that [D-Cys(^{t}Bu)^5]NPS lacks efficacy and behaves as a pure NPSR antagonist. In addition, in inhibition response curve experiments [D-Cys(^{t}Bu)^5]NPS antagonized NPS effects with pIC_{50} values (5.75 vs NPS 10 nM, 4.64 vs NPS 100 nM) clearly influenced by the concentration of agonist, thus suggesting a competitive type of interaction [95]. This was confirmed by classical Schild analysis where the peptide produced a concentration dependent rightward shift of the concentration-response curves to NPS without modifying its maximal effects. The estimated potency of [D-Cys(^{t}Bu)^5]NPS in the two series of experiments, namely Schild plot (pA_2 6.44) and inhibition experiments (pK_B 6.62), is virtually superimposable and allows to classify this ligand as a moderate potency competitive antagonist. Under the same experimental conditions, [D-Cys(^{t}Bu)^5]NPS 10 μM was found to be inactive both as agonist and antagonist at different G protein coupled receptors including muscarinic, opioid, NK-1, UT, and PAR2 receptors. These results certainly allow to exclude the possibility that the antagonist action of [D-Cys(^{t}Bu)^5]NPS vs NPS can be due to a non-specific inhibitory effect on calcium signalling. However the panel of receptors used is probably not enough for firmly classifying [D-Cys(^{t}Bu)^5]NPS as a selective NPSR antagonist. On the other hand, it is worthy of mention that [D-Cys(^{t}Bu)^5]NPS was generated by substituting a single residue into a 20 aminoacid long peptide characterized by high selectivity of action [9] and whose primary sequence is highly conserved among animal species [11]. These considerations make the possibility that [D-Cys(^{t}Bu)^5]NPS maintains the same high selectivity of action as the natural peptide extremely likely.

In calcium mobilization assay [D-Val^5]NPS was completely inactive *per se* up to 10 μM, however it produced a concentration dependent inhibition of NPS effects. Thus [D-Val^5]NPS clearly behaves in these experiments as a pure NPSR antagonist. As far as the type of antagonism exerted by [D-Val^5]NPS vs NPS, it should be keep in mind that in inhibition curves experiments competitive antagonists are expected to display different pIC_{50} values depending on the concentration of agonist used while for non competitive antagonist similar pIC_{50} values are expected against different concentrations of agonist and $pIC_{50} \approx pK_B$. [95]. In the present experiments [D-Val^5]NPS displayed different pIC_{50} values dependent on agonist concentrations, i.e. 6.59 *vs* NPS 10 nM and 5.96 (assuming full inhibition at concentration higher than 10 μM) *vs* NPS 100 nM. However, the increase of 1 log unit of agonist concentration produced a decrease of only 0.63 log unit in antagonist pIC_{50}. Therefore, inhibition response data are not compatible with a simple competitive type of interaction between [D-Val^5]NPS and NPS. A [D-Val^5]NPS pK_B value of 7.50 ($CL_{95\%}$ 7.18– 7.82) was derived from these experiments. In the classical Schild analysis [D-

Val^5]NPS displaced to the right the concentration response curve to NPS in a concentration dependent manner; however this was associated to a slight but statistically significant decrease of the maximal effect elicited by NPS at the higher concentrations of antagonist (i.e. 1 and 10 μM). The pattern of antagonist effect of [D-Val^5]NPS vs NPS can be described as dextral displacement of the agonist concentration response curve with depression of maximum at high concentrations. Thus, in line with inhibition response data, the results obtained by Schild analysis are not compatible with competitive type of antagonism. A pK_B values of 7.02 was derived from Schild analysis which is not too far from that obtained in inhibition experiments (7.50). To investigate [D-Val^5]NPS selectivity of action, this peptide was challenged against the effect produced by carbachol via stimulation of endogenously expressed muscarinic receptors in HEK293$_{mNPSR}$ cells. [D-Val^5]NPS at 10 μM did not modify the stimulatory effect of carbachol. These results demonstrated that the antagonist effect of [D-Val^5]NPS is selective for NPSR over muscarinic receptors and certainly does not derive from a non specific inhibitory effect of the peptide on calcium signalling.

In order to compare [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS antagonist potencies, the two peptides were tested in parallel, under the same experimental conditions. In this series of experiments, performed at 37°C and with three cycles of mixing, [D-Val^5]NPS showed a pK_B of 6.02, so it was 10 fold less potent than in the previous study performed at room temperature and without mixing (pK_B: 7.5). From these inhibition experiments a pK_B of 6.67 was derived for [^{t}Bu-D-Gly^5]NPS. These data demonstrated that [^{t}Bu-D-Gly^5]NPS represents the more potent pure peptide NPSR antagonist available up to now, being 3 fold more potent than [D-Val^5]NPS. To get information on the nature of the antagonist action exerted by [^{t}Bu-D-Gly^5]NPS the classical Schild analysis was performed. This peptide produced a concentration dependent rightward shift of the concentration response curve to NPS which was associated with a slight but significant depression of NPS maximal effects. From these experiments a pK_B of 6.78 was derived. This value is close to that obtained in inhibition response curve studies (7.06, table 1). Thus, these experiments confirmed the pure and potent antagonist properties of [^{t}Bu-D-Gly^5]NPS. However, these results are not compatible with a simple competitive interaction between [^{t}Bu-D-Gly^5]NPS and NPS. Similar results (rightward shift associated with significant depression of agonist maximal effects) were previously reported for [D-Val^5]NPS, which was tested at room temperature and without the three cycles of mixing. Under these same experimental conditions [^{t}Bu-D-Gly^5]NPS caused a profound depression of NPS maximal effects (down to less than 50%) in a concentration dependent manner (data not shown). This depression of E_{max} was strongly reduced even if not completely eliminated performing the experiments at 37 °C and introducing the three cycles of mixing. Collectively these findings suggest that the depression of NPS E_{max} caused by NPSR antagonists in the calcium

mobilization assay may likely come from hemiequilibrium conditions due to lack of stirring rather than from a real insurmountable type of antagonism [95]. Under the same experimental conditions, [^{t}Bu-D-Gly^5]NPS 10 μM was found to be inactive both as agonist and antagonist at different G protein coupled receptors including muscarinic, opioid, NK-1, UT, B_2 and PAR2 receptors. These results suggest that [^{t}Bu-D-Gly^5]NPS behaves as a selective NPSR antagonist. However, the panel of receptors investigated is probably too limited to draw final conclusions about compound selectivity at this time.

NPS produces a clear arousal promoting effect and a strong locomotion stimulant effect when administered supraspinally in rodents. These findings are robust and were independently replicated in different laboratories (see sections 3.3) [9]. Thus, we used the mouse RR and LA assays for investigating the *in vivo* action of [D-Cys(^{t}Bu)^5]NPS (RR), [D-Val^5]NPS (LA) and [^{t}Bu-D-Gly^5]NPS (RR and LA). For these studies, the NPS dose of 0.1 nmol was selected based on previous dose-response studies [99] as the lower dose producing statistically significant effects, to be challenged with the NPSR antagonists.

In line with previous findings, NPS 0.1 nmol produced a clear arousal promoting effect in the mouse RR assay. [D-Cys(^{t}Bu)^5]NPS tested at 1 nmol was found inactive *per se* and *vs* the effect elicited by NPS. At 10 fold higher doses, [D-Cys(^{t}Bu)^5]NPS did not modify *per se* the hypnotic effect of diazepam but fully prevented the arousal promoting action of NPS. This result confirmed *in vivo* the antagonistic properties of [D-Cys(^{t}Bu)^5]NPS. The *in vivo* dose range of activity of [D-Cys(^{t}Bu)^5]NPS perfectly matches its *in vitro* potency at NPSR. In fact the peptide was active when tested *vs* NPS in a 100/1 but not 10/1 dose ratio and its *in vitro* antagonist potency (≈ 6.5) is 100 fold lower than NPS agonist potency (≈ 8.5). Similarly, [^{t}Bu-D-Gly^5]NPS tested at 10 nmol did not modify *per se* the hypnotic effect of diazepam. However it was able to completely block the arousal promoting action of NPS from the dose of 1 nmol.

NPS 0.1 nmol had a strong stimulant action on mice LA, in all parameters detected. Because the *in vitro* ratio of potency NPS/[D-Val^5]NPS is approximately 1/30, [D-Val^5]NPS was tested *in vivo* at 10 nmol (agonist/antagonist ratio 1/100). [D-Val^5]NPS injected i.c.v at the doses of 10 nmol did not statistically modify the animal locomotor behaviour. However it is worthy of mention (although this effect did not reach the statistical level of significance) that there was a clear tendency of animals treated with [D-Val^5]NPS 10 nmol to reduce their rearing behaviour. Interestingly enough, this effect is opposite to that of NPS which produces a statistically significant increase in number of rearings. More importantly [D-Val^5]NPS completely prevented the locomotor stimulatory effect of 0.1 nmol NPS and this applies to all the parameters investigated (cumulative distance travelled, number of rearings, and total immobility time). These results confirmed and

extended to an *in vivo* assay the NPSR antagonist properties of [D-Val^5]NPS. [^{t}Bu-D-Gly^5]NPS injected i.c.v at the doses of 10 nmol did not statistically modify the animal locomotor behaviour. At the doses of 1 nmol it did not counteract the stimulant NPS action but at the higher doses of 3 and 10 nmol it was able to block, in a dose dependent manner the stimulant NPS actions. These results confirmed the NPSR antagonist proprieties of [^{t}Bu-D-Gly^5]NPS in two *in vivo* assays. From these studies [^{t}Bu-D-Gly^5]NPS was demonstrated to be the most potent peptide NPSR antagonist available up to now. In fact in RR test it was able to completely counteract the NPS arousal promoting action at the dose of 1 nmol, being 10 fold more potent than [D-Cys(^{t}Bu)^5]NPS (active at 10 nmol) and in LA test [^{t}Bu-D-Gly^5]NPS was able to block the NPS stimulant effects at the dose of 3 nmol, being 3 fold more potent than [D-Val^5]NPS (active at 10 nmol). The *in vivo* dose range of activity of [^{t}Bu-D-Gly^5]NPS perfectly matches its *in vitro* potency at NPSR and the *in vitro* ratio of potency [D-Val^5]NPS/[^{t}Bu-D-Gly^5]NPS.

The neurobiological implications of these experiments are twofold. First, with the use of [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS we demonstrated that the stimulant and arousal promoting effects of NPS are exclusively due to NPSR activation. These results are in line with previous reports about the stimulatory effect of NPS on LA using the non peptide antagonist SHA 68 [33] and the peptide partial agonist [Ala^3]NPS [100]. Second, the lack of effect of this NPSR antagonists *per se* at doses able to prevent the action of exogenously applied NPS suggests that endogenous NPS signalling is not activated under the present experimental conditions. It is worthy of mention (although this effect did not reach the statistical level of significance) that there was a clear tendency of animals treated with [D-Val^5]NPS 10 nmol to reduce their rearing behaviour. This effect is opposite to that of NPS which produces a statistically significant increase in number of rearings. Interestingly SHA 68 at doses able to counteract the stimulatory effect of exogenously applied NPS produced per se a selective reduction of mouse vertical activity [33]. These results together with the present findings obtained with [D-Val^5]NPS might implicate a tonic control of the endogenous NPS/NPSR system on this particular animal behaviour. Clearly further studies are needed to firmly understand the role of the endogenous NPS/NPSR system in the regulation of locomotion, wakefulness and sleep functions.

In conclusion, [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS were pharmacologically characterized *in vitro* and *in vivo*. These molecules were demonstrated to be pure and selective NPSR antagonists, with [D-Cys(^{t}Bu)^5]NPS and [D-Val^5]NPS characterized by a moderate potency, while [^{t}Bu-D-Gly^5]NPS represents the most potent NPSR peptide antagonist available up to now. Moreover it has been demonstrated that the stimulant and arousal promoting action of NPS is due to the selective activation of the NPSR protein. These peptide molecules

together with the recently discovered NPSR non peptide antagonist SHA 68 represent very important tools needed for understanding which and how biological functions are controlled by the NPS/NPSR system; in addition these NPSR ligands might be instrumental for identifying innovative strategies for treating neurological as well as psychiatric diseases.

3.5 In vitro and in vivo pharmacological characterization of the non peptide NPSR antagonist SHA 68

SHA 68, i.e. the racemic mixture (9R/S)-3-oxo-1,1-diphenytetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluorobenzylamide has been identified by Takeda researchers and characterized pharmacologically in vitro and in vivo by Okamura *et al.* [33]. In radioligand ([^{125}I][Tyr^{10}]NPS) binding experiments performed in HEK293$_{hNPSR}$ cells SHA 68 displayed high affinity (pK_i 7.3). In calcium mobilization experiments SHA 68 was inactive *per se* while antagonizing NPS stimulatory effects in a concentration-dependent and competitive manner. Similar high pA_2 values were obtained with SHA 68 at Ile^{107} (7.6) and Asn^{107} (7.8) hNPSR isoforms expressed in HEK293 cells [33]. SHA 68 appeared to be selective for NPSR since it did not affect signaling at 14 unrelated GPCR. In vivo in mice SHA 68 reached pharmacologically relevant levels in plasma and brain after i.p. administration [33]. Despite this, SHA 68 (50mg/kg i.p.) was only able to partially counteract NPS induced stimulation of LA [33]. In the present study the pharmacological profile of SHA 68 was further investigated in vitro in calcium mobilization experiments performed on HEK293$_{mNPSR}$ cells and in vivo in LA, RR and OF tests in mice.

Calcium mobilization assay

In the calcium mobilization assay performed on HEK293$_{mNPSR}$ cells, NPS increased the intracellular calcium concentrations in a concentration-dependent manner with pEC_{50} and E_{max} values of 8.30 and 236±15% over the basal values, respectively. Inhibition response curve to SHA 68 (0.01 nM–10 μM) were performed against the stimulatory effect of 30 nM NPS, approximately corresponding to the EC_{80} value for the agonist. As shown in figure 30, SHA 68 concentration-dependently inhibited 30 nM NPS stimulatory effects with a pIC_{50} value of 7.37. A pK_B value of 7.74 ($CL_{95\%}$ 7.44–8.04) was derived for SHA 68 from these experiments.

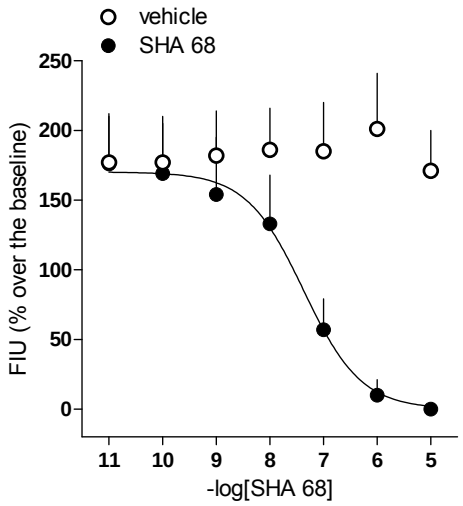

Figure 30 Calcium mobilization assay performed on $HEK293_{mNPSR}$ cells. Inhibition response curve to SHA 68 (0.01 nM–10 μM) against the stimulatory effect of 30 nM NPS. Data are mean±s.e.m of 4 experiments made in duplicate.

In order to investigate the nature of the antagonist action exerted by SHA 68 the classical Schild analysis was performed. As depicted in figure 31, left panel, SHA 68 in the range of 10–1000 nM did not produce any effect per se but displaced the concentration response curve to NPS to the right in a concentration-dependent and parallel manner. A slight but significant depression of NPS E_{max} was recorded in the presence of the highest concentrations of antagonist (i.e. 100 and 1000 nM). The corresponding Schild plot, which was linear ($r^2 = 0.97$) with a slope of 1.00±0.18, is shown in the right panel of figure x. The extrapolated pA_2 value was 8.06.

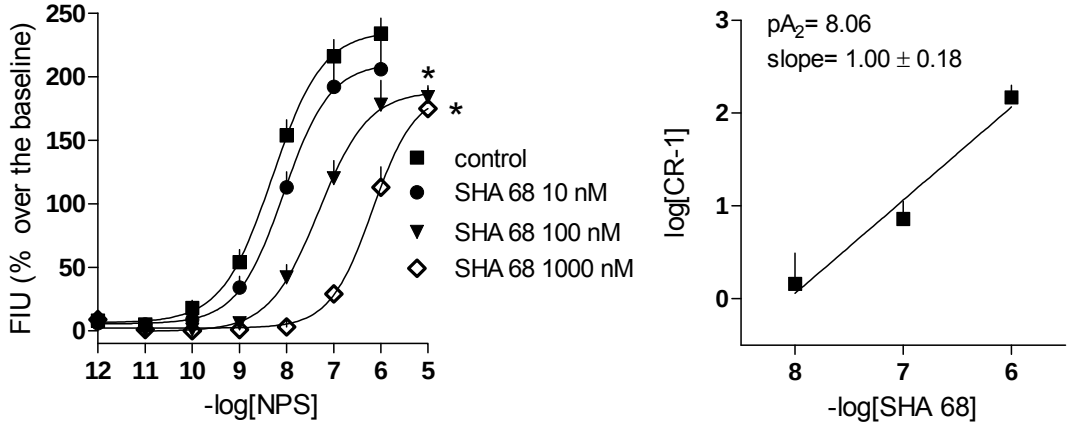

Figure 31. Calcium mobilization assay performed on $HEK293_{mNPSR}$ cells. Concentration-response curve to NPS obtained in the absence (control) and in presence of increasing concentrations of SHA 68 (10-100 nM) (left panel); the corresponding Schild plot is shown in the right panel. Data are mean ± sem of 4 experiments made in duplicate.

Locomotor activity

As shown in figure 32 and in line with previous findings, NPS injected i.c.v. at 0.1 nmol evoked a stimulatory effect on mouse LA by increasing cumulative distance travelled by the animals and their number of rearings, and reducing the total immobility time, in a statistically significant manner. SHA 68 at 50mg/kg did not modify *per se* the animal behaviour (figure 32).When challenged with NPS, SHA 68 at 10mg/kg displayed a trend toward inhibition of the stimulatory effects evoked by the peptide. Statistically significant effects were obtained with SHA 68 50mg/kg (figure 32) although the antagonist did not fully prevented the effects elicited by the peptide.

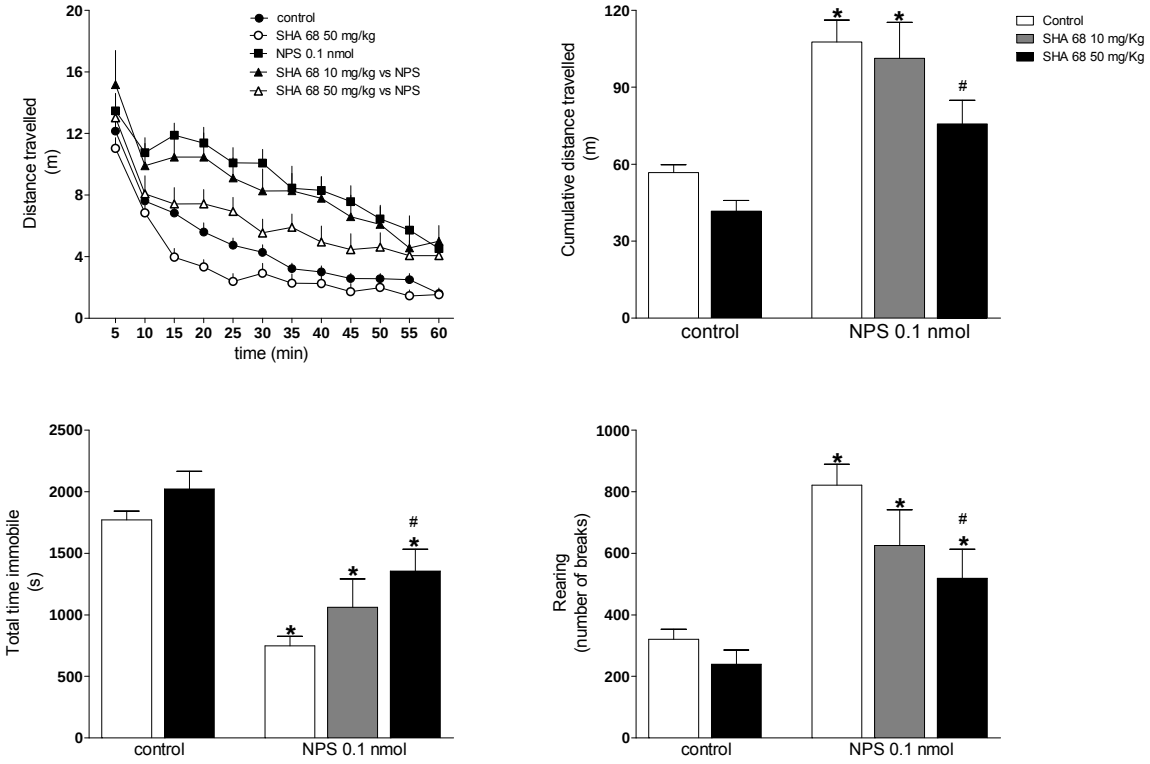

Figure 32 Locomotor activity assay. Effects of NPS and SHA 68. Time course of the distance traveled is shown in the top left panel, while the other panels display the cumulative effects exerted by the peptide and SHA 68 on distance traveled (top right panel), total time immobile (bottom left panel), and number of rearings (bottom right panel) over the 60 min observation period. Data are mean ± sem of 4 separate experiments (total 16 mice per group). *p<0.05 vs control, #p<0.05 vs NPS 0.1 nmol according to two-way ANOVA followed by the Bonferroni test for multiple comparisons.

In order to investigate the in vivo selectivity of action of SHA 68, the NPSR antagonist was tested against the effect of caffeine in LA experiments. Caffeine at 20 mg/kg evoked a clear stimulatory effect on mouse locomotor activity by increasing cumulative distance travelled by the

animals and reducing the total time immobile, in a statistically significant manner. SHA 68 at 50mg/kg did not affect the stimulatory effects of caffeine (figure 33).

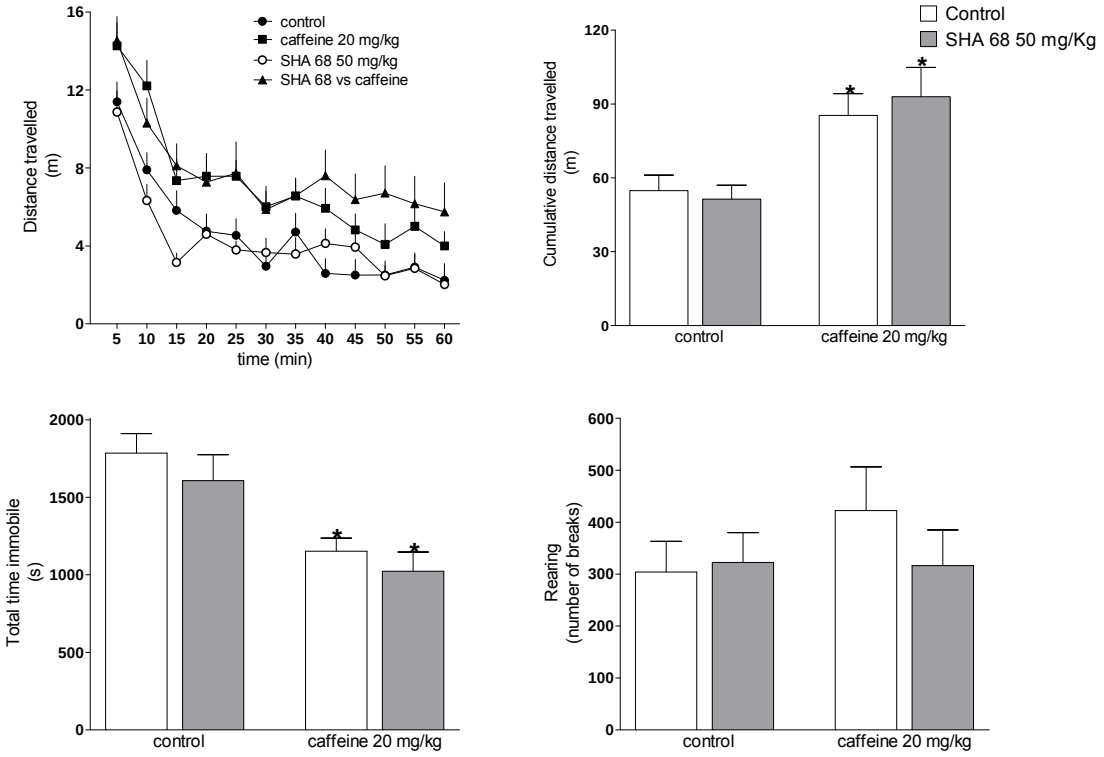

Figure 33 Locomotor activity assay. Effects of caffeine and SHA 68. Time course of the distance travelled is shown in the top left panel, while the other panels display the cumulative effects exerted by the peptide and SHA 68 on distance traveled (top right panel), total time immobile (bottom left panel), and number of rearings (bottom right panel) over the 60 min observation period. Data are mean ± sem of 4 separate experiments (total 16 mice per group). *p<0.05 vs control, #p<0.05 vs NPS 0.1 nmol according to two-way ANOVA followed by the Bonferroni test for multiple comparisons.

Recovery of righting reflex

As shown in figure 34 and in line with previous findings, i.p. injection of diazepam at the hypnotic dose of 15mg/kg produced loss of the RR in all treated mice and approximately 100 min were needed to regain this reflex. NPS injected i.c.v. at 0.1 nmol reduced both the percent of animals responding to diazepam below 75% and their sleep time to approximately 50 min. The administration of SHA 68 (10 and 50mg/kg) did not significantly modify the hypnotic effect of diazepam either in terms of percent of animals losing the RR or duration of their sleep time (figure 34).When SHA 68 (10 mg/kg) was co-administered with 0.1 nmol NPS, it prevented the NPS mediated reduction in the percent of mice responding to diazepam but did not modify their sleep time. On the contrary, SHA 68 50 mg/kg fully prevented the arousal promoting effect of NPS; in

fact results obtained in mice treated with NPS plus SHA68 50 mg/kg were superimposable to those of vehicle-treated animals (figure 34).

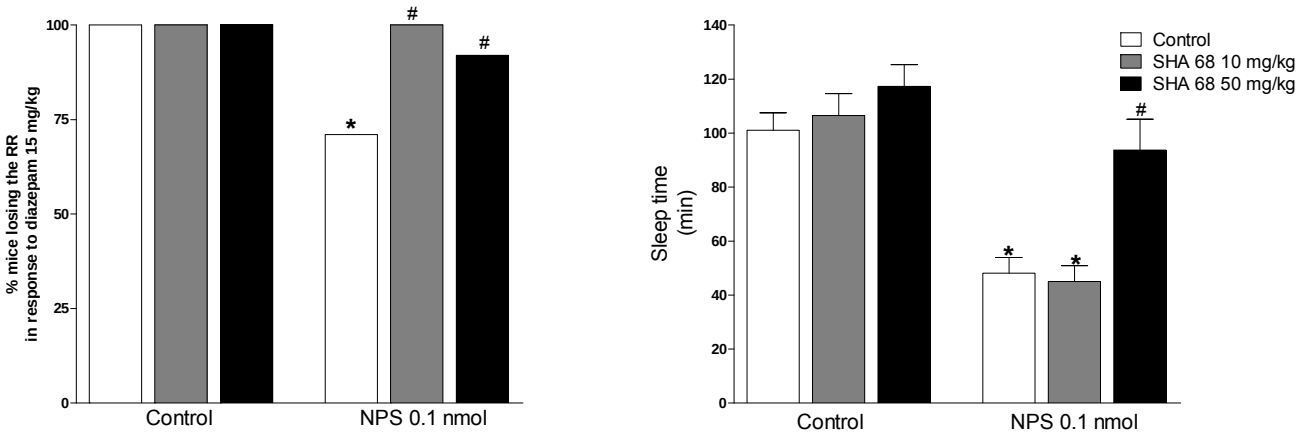

Figure 34 Recovery of righting reflex in mice. Effects elicited by NPS (0.1 nmol) and SHA 68 (10–50 mg/kg) on the percentage of animals losing the righting reflex in response to 15 mg/kg i.p. diazepam (left) and on their sleep time (right). Sleep time is defined as the amount of time between the loss and regaining of the righting reflex. Data are mean ± sem of 16 mice per group. *p<0.05 vs control, #p<0.05 vs NPS 0.1 nmol according to two-way ANOVA followed by the Bonferroni test for multiple comparisons.

In order to investigate the possible effects of the NPSR antagonist on the hypnotic action of diazepam, a separate series of experiments were performed by testing SHA 68 50 mg/kg against different doses of the benzodiazepine. As shown in figure 35, 5 mg/kg diazepam was inactive in the RR paradigm. At 10 mg/kg the benzodiazepine produced loss of the RR in 70% of the treated mice and approximately 25 min were needed to regain the reflex. Diazepam at 15 mg/kg produced loss of the RR in all treated mice and they sleep time was approximately 110 min. At 50 mg/kg SHA 68 did not modify the dose response curve to diazepam; in fact superimposable results were obtained in animals treated with saline or SHA 68 both in terms of percent of animals losing the RR and regarding their sleep time (figure 35).

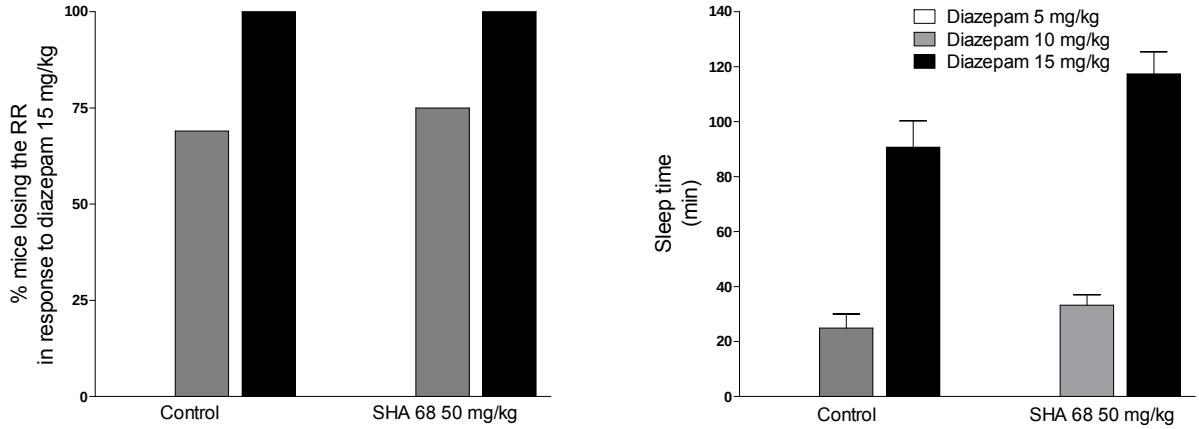

Figure 35 Recovery of righting reflex in mice. Effect of i.p. injection of SHA 68 (50mg/kg) on the percent of animals losing their righting reflex (left panel) and on their sleep time (right panel) in response to diazepam 5, 10, and 15mg/kg i.p. Sleep time is defined as the amount of time between the loss and regaining of the righting reflex. Data are mean ± sem of 16 mice per group.

Open field

As shown in figure 36 and in line with previous findings, NPS injected i.c.v. at 0.1 nmol evoked an anxiolytic like effect on mouse OF, by increasing number of entries in the central zone and the time spent by mice in this area of the field. SHA 68 50 mg/kg did not significantly modify *per se* the behaviour of mice subjected to the OF assay. Moreover, SHA 68 50mg/kg only counteracted the effects elicited by NPS on time in the central area but not on the number of entries (figure 36).

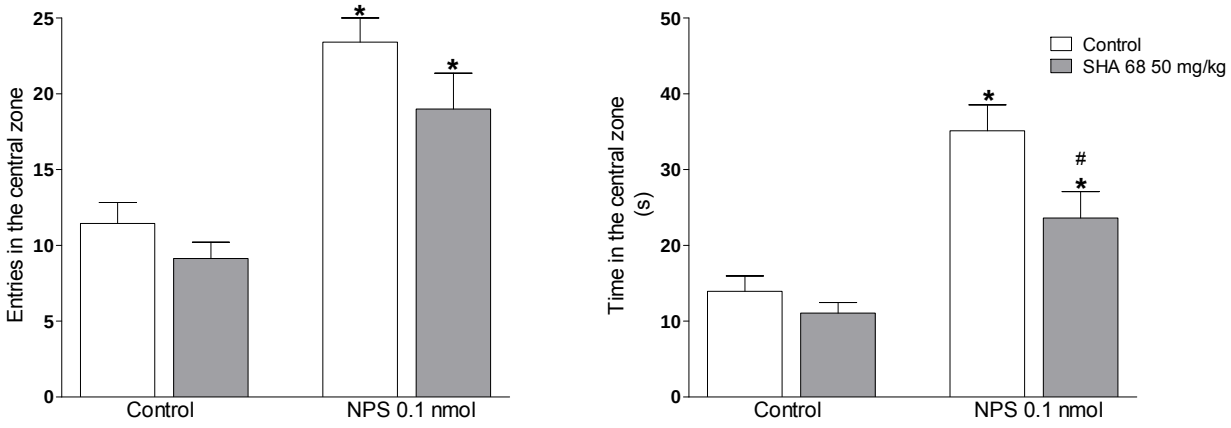

Figure 36 Open field assay in mice. Effects of NPS and SHA 68 on the number of entries in the central zone (left panel) and the time spent in this are of the field (right panel). Data are mean ± sem of 20 mice per group. *p<0.05 vs control, #p<0.05 vs NPS 0.1 nmol according to two-way ANOVA followed by the Bonferroni test for multiple comparisons.

In the present study the *in vitro* and *in vivo* pharmacological profile of the NPSR ligand SHA 68 was investigated. Our findings confirmed and extended previous results [33] demonstrating that SHA 68 behaves *in vitro* as a pure and potent NPSR antagonist. In vivo, in mice, SHA 68 was investigated in a panel of assays sensitive to the stimulatory and arousal promoting (LA and RR), and anxiolytic-like (OF) actions of NPS. Depending on the assay, SHA 68 displayed different effectiveness: it fully blocked NPS effects in the RR, partially counteracted the actions of the peptide in the LA and was barely active in the OF assay. The reasons for this different pattern of action of SHA 68 are not known but they may likely be attributed to its poor pharmacokinetic profile [33]. In $HEK293_{mNPSR}$ cells SHA 68 behaved as a pure antagonist. Similar high values of potency were obtained in inhibition curve experiments (pK_B 7.74) and by performing the classical Schild protocol (pA_2 8.06). Results obtained in the latter assay are however not compatible with a simple competitive interaction between SHA 68 and NPS since the antagonist at the highest concentration tested produced a slight but significant depression of NPS maximal effects. However, the following considerations suggest that the depression of NPS E_{max} caused by SHA 68 in the calcium mobilization assay probably derives from hemiequilibrium conditions rather than from a real insurmountable type of antagonism. In fact in a pilot series of experiments performed at room temperature and without three cycles of mixing, SHA 68 caused, in a concentration dependent manner, a profound depression of NPS maximal effects (down to less than 50% E_{max}). This depression of E_{max} was strongly reduced even if not completely eliminated (see figure 23) by performing the experiments at 37 °C and introducing three cycles of mixing. Similar results were obtained investigating the NPSR antagonist properties of the NPS related peptide [^{t}Bu-D-Gly^5]NPS. Thus, the fact that antagonist induced depression of agonist maximal effects is highly sensitive to procedures which facilitate drug diffusion (i.e. increase in temperature and cycles of mixing) strongly suggests that this phenomenon is due to hemiequilibrium conditions due to lack of stirring and not to a real insurmountable type of antagonism [95]. The value of SHA 68 potency at mouse NPSR obtained in the present study is superimposable to those measured at human NPSR isoforms Asn^{107} (pA_2 7.77) and Ile^{107} (pA_2 7.55) [33] indicating that this NPSR ligand does not discriminate between NPSR species-specific proteins and isoforms. This is in contrast to findings for NPS [17-18] and NPS-related peptides [101] that displayed significantly higher agonist potencies at hNPSR Ile^{107} and mNPSR (that contains Ile at position 107) than at the hNPSR Asn^{107} isoform. It would be interesting in future studies to analyze and compare the pharmacological profile of NPSR peptide antagonists (i.e. [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS) and non-peptide agonists (that are not yet described in the literature) at NPSR species-specific proteins and isoforms. Such studies may help to identify the specific binding site(s) recognized by peptide and non-peptide

ligands able of block or activate NPSR and thus facilitate molecular modeling studies and the rational design of novel potent NPSR ligands.

In vivo SHA 68 was first challenged against the LA stimulatory effect of NPS. This action of NPS has been independently replicated in several different laboratories (see for a review Guerrini *et al.* [100]). Data obtained in LA experiments with SHA 68 are virtually superimposable to those previously published by Okamura *et al.* [33]. SHA 68 at the higher dose tested (50 mg/kg) was inactive *per se* and only partially counteracted the stimulatory effect elicited by the supraspinal injection of NPS. The absence of a full block of NPS effects despite the relatively high dose of antagonist used may likely be linked to the poor pharmacokinetic properties of this molecule, in particular its very high lipophilicity [33] that may reduce the ability of this molecule to reach its target. Recent findings obtained by industrial investigators corroborate this view [102]. In fact, *ex vivo* binding studies demonstrated that only approximately 50% of [^{125}I]NPS binding to brain sections is inhibited by treating mice with SHA 68 50 mg/kg. The results obtained with the peptide NPSR antagonists [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS that fully blocked NPS stimulatory effects but being *per se* inactive parallel those of SHA 68. Thus findings obtained from different laboratories using chemically unrelated molecules demonstrated that the stimulatory effect of NPS on LA is solely due to NPSR activation. This has been recently confirmed by knockout studies that demonstrated that NPS can stimulate LA in NPSR(+/+) but not in NPSR(-/-) mice [36].

In line with *in vitro* findings (SHA 68 does not affect signaling at 14 unrelated G protein-coupled receptors, [33]), the present in vivo results confirmed that SHA 68 antagonist properties are selective for the NPSR since the stimulatory effect of caffeine on LA was not affected by this molecule.

The supraspinal administration of NPS elicits a robust arousal promoting action in rodents. SHA 68 was able to fully prevent this NPS effect in the mouse RR assay. This implies that NPSR regulating arousal might be more accessible to the antagonist than those stimulating LA. This result parallels previous data obtained under the same experimental conditions using the NPSR antagonist [D-Cys(^{t}Bu)^5]NPS and investigating the phenotype of NPSR(-/-) mice. [D-Cys(^{t}Bu)^5]NPS blocked the arousal promoting effect of NPS and NPS elicited its stimulatory effects in NPSR(+/+) but not in NPSR(-/-) mice. In addition, NPSR(+/+) and NPSR(-/-) animals were similarly sensitive to the hypnotic effect of diazepam and the peptide NPSR antagonist did not modify *per se* the effect of the benzodiazepine. This latter result has been confirmed and extended in the present study by challenging SHA 68 versus increasing doses of diazepam. Thus, collectively these results demonstrated that i) NPSR activation is involved in the arousal promoting action of NPS, and ii) endogenous NPSR is not tonically activated under the present experimental conditions. Obviously

this does not preclude the possibility that the endogenous NPS/NPSR system might be important for controlling circadian sleep/wakefulness cycles. In fact, NPSR(-/-) mice displayed reduced late peak wheel running (an index of activity of the internal clock) compared to NPSR(+/+) mice [36] and, more importantly, the functional hNPSR polymorphism Ile[107] has been associated with delayed average bedtime in humans [20].

In line with previous findings the present data confirmed that the supraspinal injection of NPS elicited dose-dependent anxiolytic-like actions in rodents. In fact, NPS 0.1 nmol increased the time spent in and the number of entries into the central area of the OF in mice. SHA 68 slightly and non-significantly reduced the effects of the peptide in the mouse OF. The reasons for these weak SHA 68 effectiveness are not completely known, however they may likely be attributed to the pharmacokinetic rather than pharmacodynamic properties of this molecule. In fact, SHA 68 was reported to be able to prevent the anxiolytic NPS actions in rat EPM and defensive burying assays [103] and these data support the hypothesis that the anxiolytic-like actions evoked by NPS in rodents are due to the selective activation of the NPSR protein. Probably the lack of effectiveness of SHA 68 given systemically in the mice OF test may derive from an incomplete occupation of brain NPSR controlling fear and anxiety levels which are most probably located in the amygdala [25-26,28]. Since SHA 68 50 mg/kg was not able to block the anxiolytic effect of exogenous given NPS, the present study did not provide any indication about the role of endogenous NPS/NPSR system in controlling anxiety behaviours. Clearly further studies performed with different NPSR selective antagonists and with NPSR(-/-) mice are needed before drawing firm conclusion on the role of the endogenous NPS/NPSR system in the control of fear and anxiety.

In conclusion, this study confirmed and extended previous findings demonstrating that the bicyclic piperazine derivative SHA 68 behaves as a selective NPSR antagonist *in vitro* and *in vivo*. However the usefulness of this research tool is limited by its poor pharmacokinetic properties. This may likely be one reason for the variable effectiveness of SHA 68 in the different NPS sensitive assays examined in the present study. Nevertheless SHA 68 can be successfully used as a template for the identification of non-peptide NPSR selective antagonists characterized by better pharmacokinetic features. These kind of molecules should be used together with the available NPSR peptide ligands [100] and genetic models [83] for performing detailed target validation studies that will hopefully allow to firmly identify the therapeutic potential of innovative drugs acting at NPSR.

3.6 Behavioural characterization of NPSR knock out mice

Up to now little information is available about the physiological functions modulated by the endogenous NPSergic pathways. For this reason, NPSR(-/-) mice represent a useful and powerful tool for the elucidation of the biological roles played by this new peptidergic system. Moreover time these mice can be used to investigate the involvement of the NPSR receptor in the well known activities promoted by exogenously given NPS and, when they will be available, by non peptide NPSR agonists. NPSR(-/-) mice were generated on a 129S6/SvEv genetic background as described in Allen *et al.* [83]. 129S6/SvEv derived embryonic stem cells were choose as they are the most useful cell type for gene modification purposes. However the 129S6/SvEv strain provide an unfavourable genetic background, as these mice are characterized by poor reproductive performance and behavioural abnormalities including low locomotor activity, high anxiety levels and depressive-like behaviours [104-107]. The need to avoid a strain with extreme traits, in this case very low locomotor activity associated to high anxiety levels, which could produce ceiling or floor effects that mask the outcome of the mutation, prompted us to change the genetic background of NPSR(-/-) mice from 129S6/SvEv to CD-1. This has been achieved by performing 8 cycles of backcross to the CD-1 strain.

Since NPS is reported to have stimulant and arousal promoting affects and at the same time a clear anxiolytic-like action [9,43] (see also section 3.3), we investigated the behaviour of NPSR(+/+) and NPSR(-/-) mice in LA, RR, EPM, OF and SIH assays. Furthermore, to understand if the NPS/NPSR system plays a role in controlling depressive-like behaviours we performed the FS test. NPS is also able to facilitate memory [67-69] and to reduce pain transmission [71], thus we studied NPSR(-/-) mice phenotype in the NOR test and in the formalin test.

Behavioural comparison of 129S6/SvEv and CD-1 mice

As mentioned before, we backcrossed for 8 generations 129S6/SvEv NPSR(+/-) mice to the CD-1 strain, that is the inbred strain we routinely use for our experiments. A congenic strain is a mouse strain that is isogenic to an inbred strain, but contains a chromosomal segment from another background. Thus after 8 backcross cycles we obtained a CD-1 congenic colony (mice which are 99.61% CD-1 pure) and then we split it into two separate colonies, NPSR(+/+) and NPSR(-/-) mice. As the two colonies differ only for the presence or the absence of a functional NPSR gene, they can be directly compared in phenotype studies. Figure 38 compares the behaviour of 129S6/SvEv NPSR(+/+) mice with that of CD-1 congenic NPSR(+/+) mice obtained at the end of backcross in the OF assay. Unpaired t-test revealed that CD-1 mice shown a significant increase in locomotor activity, both in terms of total distance travelled and number of rearing, compared to 129S6/SvEv mice. In addition, CD-1 mice resulted less anxious than 129S6/SvEv mice as they did significantly more entries in and spent more time into the central zone of the field.

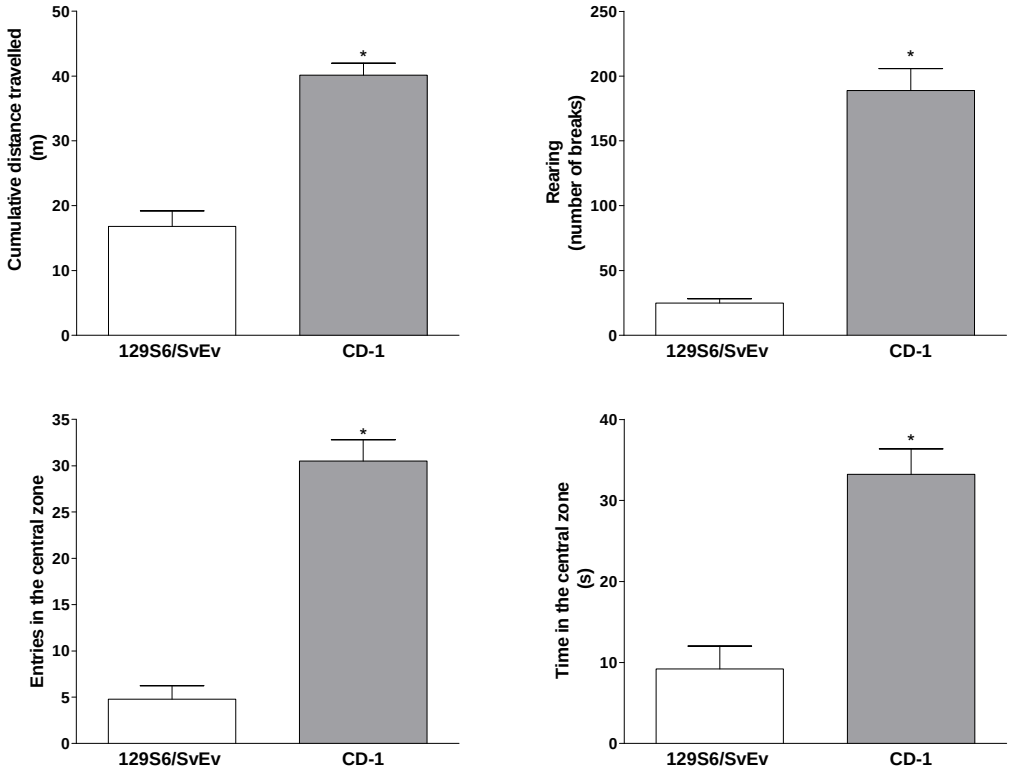

Figure 37 Open field assay in 129S6/SvEv and CD-1 mice. Effects of genetic background on the total distance travelled (top left panel), number of rearings (top right panel), number of entries in the central zone (bottom left panel) and the time spent in this are of the field (bottom right panel). Data are mean ± s.e.m. of 8 mice per group. *P<0.05 vs 129S6/SvEv, Student's t-test for unpaired data.

Locomotor activity

Locomotor activity test was performed both with naïve mice and with mice habituated for 60 min to the test chamber. In this test, naïve NPSR(-/-) mice did not show any difference in the total distance travelled, in the total time immobile and in the number of rearings compared to NPSR(+/+) mice (figure 38).

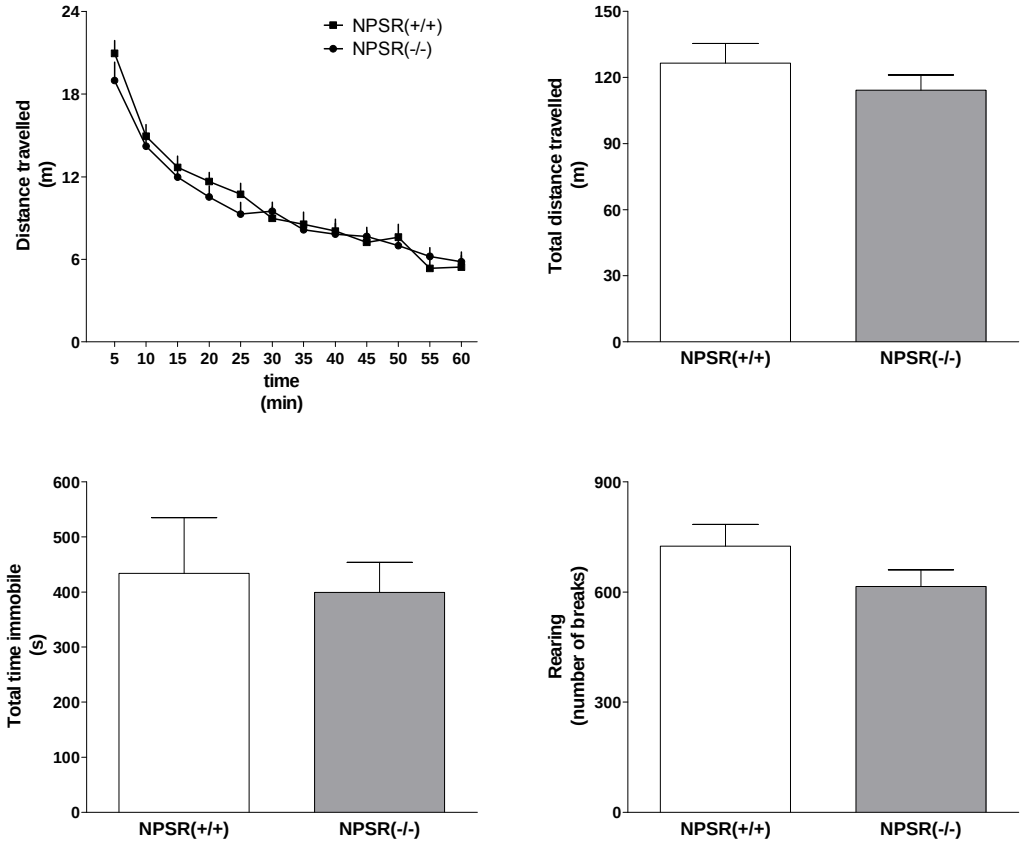

Figure 38 Locomotor activity assay performed in NPSR(+/+) and NPSR(-/-) mice. Effect of genotype on the time course of distance travelled (top left panel), on the cumulative total distance travelled (top right panel), immobility time (bottom left panel) and number of rearings (bottom right panel). Data are mean ± s.e.m. of 21 mice per group.

Similar results were obtained comparing the LA of NPSR(+/+) and NPSR(-/-) mice after 60 min of habituation to the test cage. A shown in figure 39, after the habituation period, NPSR(+/+) mice showed an important reduction in LA with total distance travelled < 50 m, total time immobile > 1500 s and number of rearing ~ 300. NPSR(-/-) did not display any alteration in locomotion compared to NPSR(+/+) mice (figure 39).

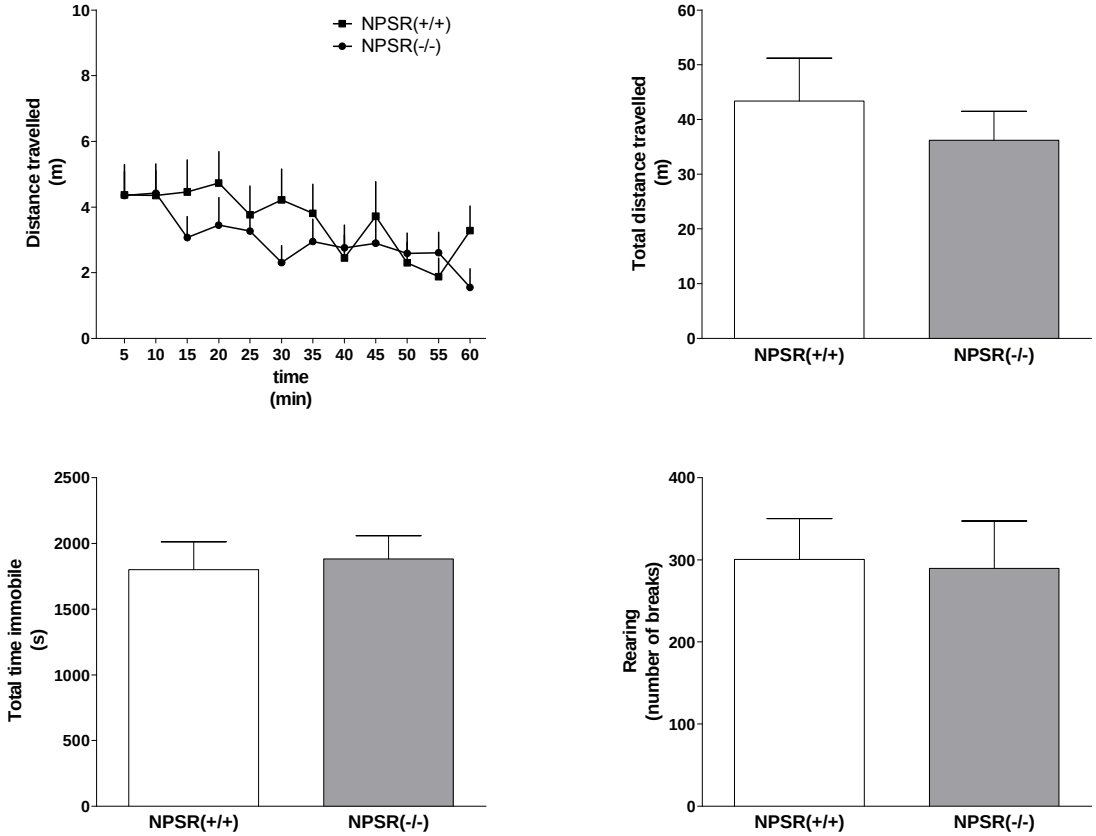

Figure 39 Locomotor activity assay performed in NPSR(+/+) and NPSR(-/-) mice habituated to the test cage. Effect of genotype on the time course of distance travelled (top left panel), on the cumulative total distance travelled (top right panel), immobility time (bottom left panel) and number of rearings (bottom right panel). Data are mean ± s.e.m. of 21 mice per group.

To investigate the involvement of the NPSR receptor in the NPS stimulant affects, NPSR(+/+) and NPSR(-/-) were treated with saline or NPS and their LA measured. No differences were found between NPSR(+/+) and NPSR(-/-) mice treated with saline. The i.c.v. injection of NPS 1 nmol caused a significant increase in mice horizontal and vertical activity and a decrease of mice immobility time in NPSR(+/+) but not NPSR(-/-) mice (figure 40).

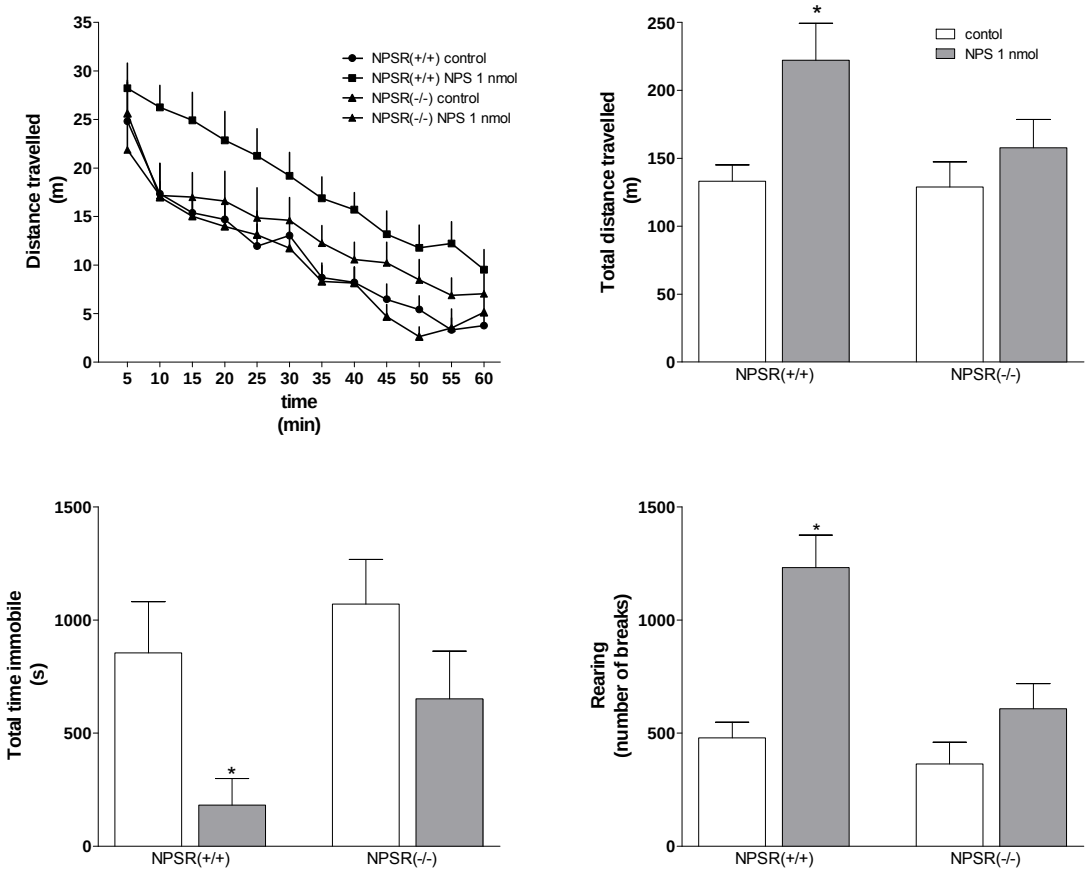

Figure 40 Locomotor activity assay performed in NPSR(+/+) and NPSR(-/-) mice. Effect of NPS 1 nmol on the time course of distance travelled (top left panel), on the cumulative total distance travelled (top right panel), immobility time (bottom left panel) and number of rearings (bottom right panel), Data are mean ± s.e.m. of 9 mice per group. *$p<0.05$ vs control according to two-way (treatment and genotype) ANOVA followed by the Bonferroni test for multiple comparisons.

Recovery of righting reflex

A first series of experiments was performed with NPSR(+/+) and NPSR(-/-) littermates obtained by mating heterozygous NPSR(+/-) 129S6/SvEv mice from Taconic. These mice were investigated for their phenotype and sensitivity to NPS in the RR assay. The i.p. injection of diazepam at 15 mg/kg produced loss of the RR in 100% of both NPSR(+/+) and NPSR(-/-) mice and 180 ± 18 and 211 ± 19 min were needed to regain this reflex in NPSR(+/+) and NPSR(-/-) mice, respectively (figure X). In NPSR(+/+) mice, NPS injected i.c.v. at the dose of 1 nmol did not modify the percent of animals responding to diazepam, however it clearly reduced their sleeping time to 67 ± 9 min. On the contrary, NPS 1 nmol failed to modify the hypnotic effect of diazepam in NPSR(-/-) mice (figure 41).

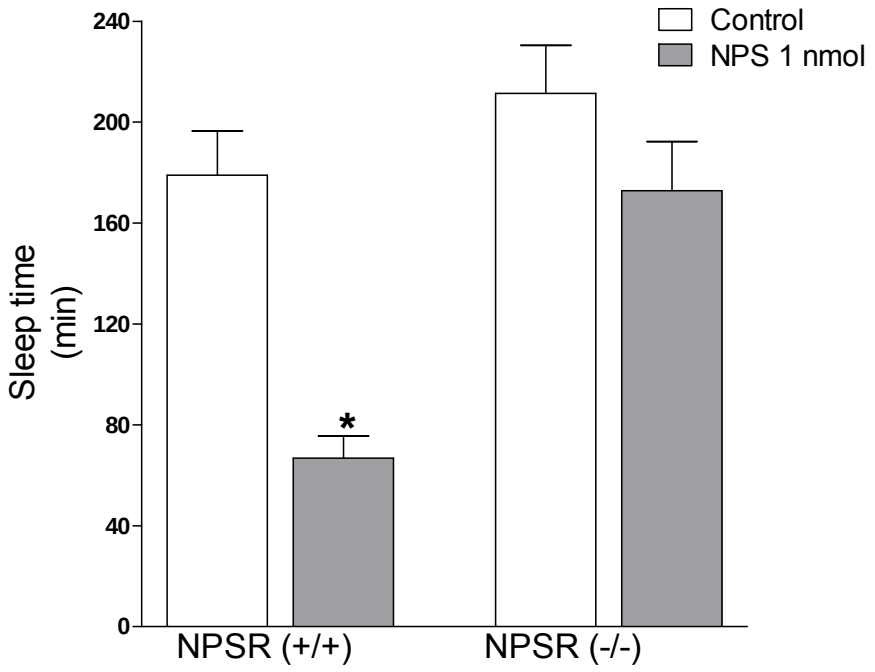

Figure 41 Recovery of righting reflex in NPSR(+/+) and NPSR(-/-) mice. Effect of 1 nmol i.c.v. injected NPS on the sleep time of animals losing of the righting reflex in response to diazepam 15 mg/kg i.p. Sleeping time is defined as the amount of time between the loss and regaining of the righting reflex. Data are mean ± sem of 10-12 mice per group. *p<0.05 *vs* control, according to two-way (treatment and genotype) ANOVA followed by the Bonferroni test for multiple comparisons.

Subsequently the phenotype of NPSR(+/+) and NPSR(-/-) mice backcrossed on the CD-1 strain was investigated in the same assay. CD-1 mice were less sensitive to the hypnotic effects of diazepam compared to 129S6/SvEv mice. In fact after diazepam administration at the dose of 15 mg/kg, only the 85% of CD-1 mice lost the RR and the sleep time of mice responding to benzodiazepine was ~ 80 min. As shown in figure 42, no differences between NPSR(+/+) and NPSR(-/-) mice were recorded both in terms of percentage of animals responding to diazepam and in terms of sleep time (figure 42).

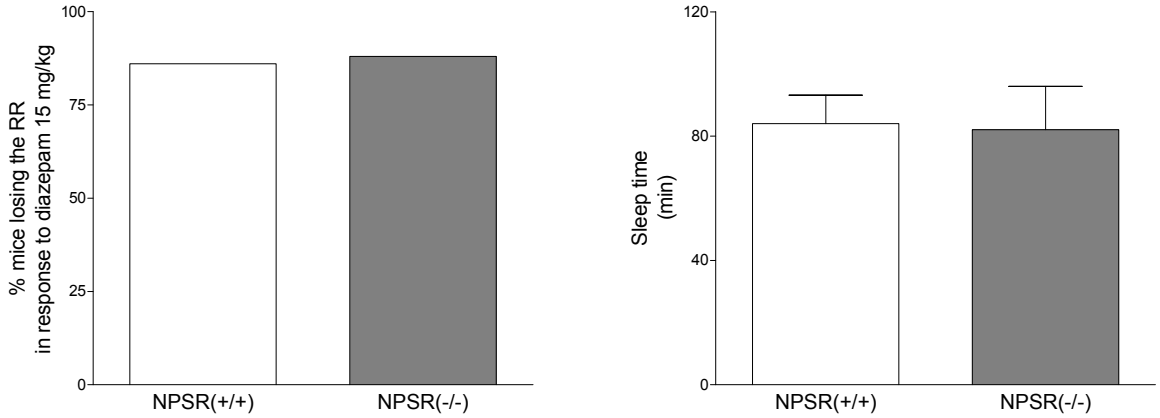

Figure 42 Recovery of righting reflex in NPSR(+/+) and NPSR(-/-) mice. The percent of animals losing the righting reflex in response to diazepam 15 mg/kg is displayed on the left panels, their sleep time is displayed on right panels. Sleep time is defined as the amount of time between the loss and regaining of the righting reflex. Data are mean ± s.e.m. of 10 mice per group.

Elevated plus maze

The behaviour of NPSR(+/+) and NPSR(-/-) mice was investigated in the EPM test. In this test, the average time spent in and the number of entries into open arms by NPSR(+/+) mice were 34.1 ± 6.4 s (corresponding to 21.4 ±3.6 % of the total time spent in open and closed arms) and 3.6 ± 0.7 (corresponding to 22.5 ± 3.9 % of the total entries into open and closed arms), respectively. Mice lacking the NPSR receptor did not exhibit any difference compared to NPSR(+/+) mice (figure 43). All the behavioural parameters measured in the EPM in NPSR(+/+) and NPSR(-/-) animals are summarised in table 13. Interestingly enough, NPSR(-/-) mice displayed a significant decreased number of rearings compared to NPSR(+/+) mice. No differences in the frequency of stretch attend postures, grooming, and head dipping were observed.

NPSR(+/+) and NPSR(-/-) mice were injected with saline or NPS 1 nmol and their behaviour was investigated in the EPM test. No genotype related differences were observed in mice treated with saline (figure 44). NPS 1 nmol had a significant anxiolytic action in NPSR(+/+) mice increasing both the number of entries in and the time spent in the open arms. On the contrary the peptide was inactive when given to NPSR(-/-) animals. All the measured EPM parameters are summarised in table 14.

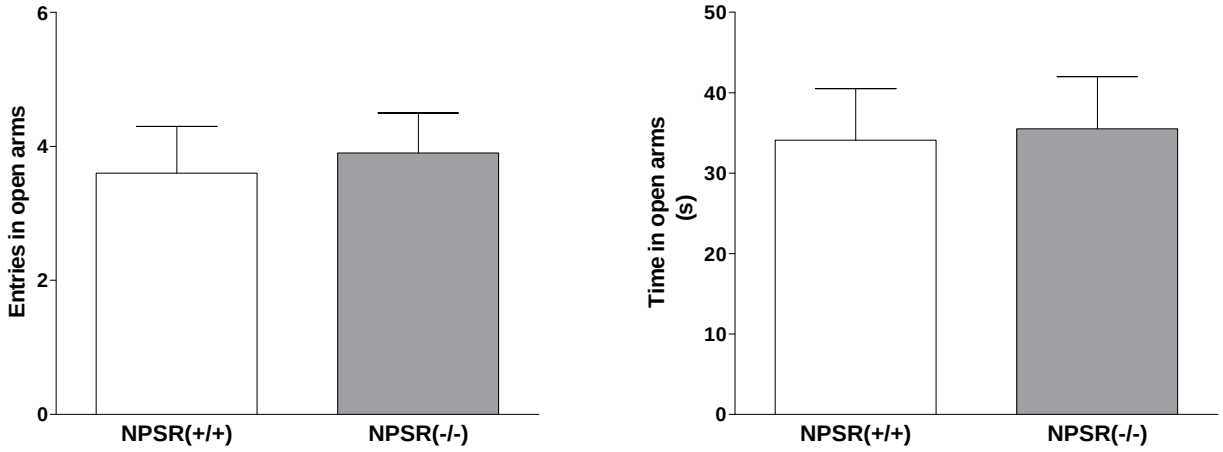

Figure 43 Elevated plus maze test performed with NPSR(+/+) and NPSR(-/-) mice. Effects of genotype on the time spent in (left panel) and on the number of entries into the open arms (right panel). Data are mean ± s.e.m. of 22 mice per group.

Table 13 Effects of genotype on various behavioural parameters displayed by NPSR(+/+) and NPSR(-/-) mice subjected to the EPM

Genotype	*Time in open arms (s)*	*Entries in open arms*	*Time in closed arms (s)*	*Entries in closed arms*	*Stretch attend posture*	*Head-dipping*	*Rearing*
NPSR(+/+)	34.1±6.4	3.6±0.7	110.9±5.9	10.2±0.8	21.5±1.6	9.7±1.5	20.7±1.8
NPSR(-/-)	35.5±6.5	3.9±0.6	107.8±4.4	11.8±1.1	21.5±1.5	7.4±1.0	14.1±1.5*

All values are expressed as mean ± s.e.m. of 22 mice per group.
*P<0.05 vs NPSR(+/+), Student's t-test for unpaired data.

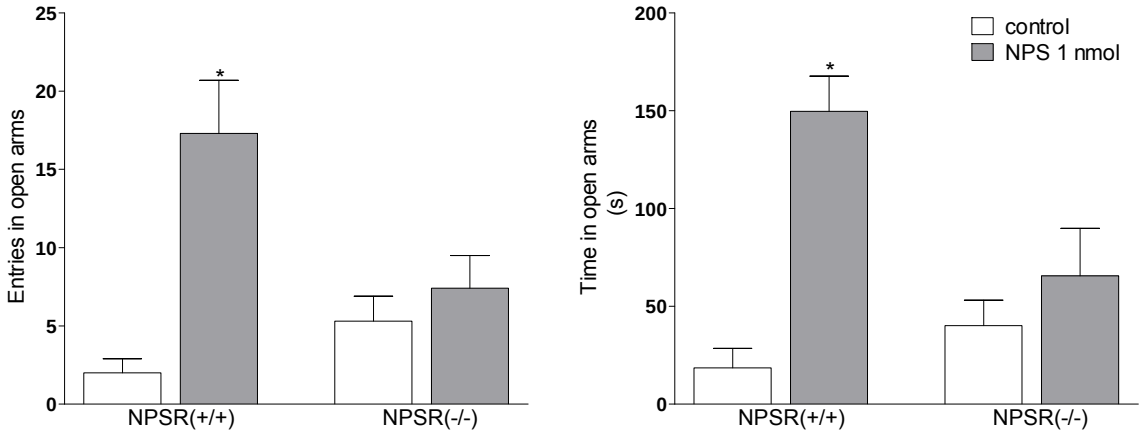

Figure 44 Elevated plus maze test performed with NPSR(+/+) and NPSR(-/-) mice. Effects of genotype and NPS (1 nmol, i.c.v.) on the time spent in (left panel) and on the number of entries into the open arms (right panel). Data are mean ± s.e.m. of 9 mice per group. *p<0.05 vs control, according to two-way (treatment and genotype) ANOVA followed by the Bonferroni test for multiple comparisons.

Table 14 Effects of genotype and treatment on various behavioural parameters displayed by NPSR(+/+) and NPSR(-/-) mice subjected to the EPM

Genotype	*Treatment*	*Time in open arms (s)*	*Entries in open arms*	*Time in closed arms (s)*	*Entries in closed arms*	*Stretch attend posture*	*Head-dipping*	*Rearing*
NPSR(+/+)	control	18.5±10.0	2.0±0.9	106.1±20.1	7.3±1.7	25.8±3.2	9.3±1.3	10.8±3.6
NPSR(+/+)	NPS 1 nmol	149.7±18.0*	17.3±3*	52.3±6.8*	9.7±1.2	5.0±1.6*	33.3±5.2*	16.4±2.7
NPSR(-/-)	control	40.2±12.9	5.3±1.6	92.7±14.1	9.9±1.3	21.4±3.2	13.2±2.3	10.0±3.1
NPSR(-/-)	NPS 1 nmol	65.6±24.2	7.4±2.1	76.0±16.4	9.9±1.4	11.0±3.1	19.1±4.7	11.9±0.6

All values are expressed as mean ± s.e.m. of 9 mice per group.
*p<0.05 vs control, according to two-way (treatment and genotype) ANOVA followed by the Bonferroni test for multiple comparisons.

Open field

When evaluated in the OF test no differences were observed between NPSR(+/+) and NPSR(-/-) mice in the total distance travelled, number of entries in and time spent in the central zone of the field (figure 45). However the frequency of rearing was significantly reduced in NPSR(-/-) mice compared to NPSR(+/+) mice (figure 45).

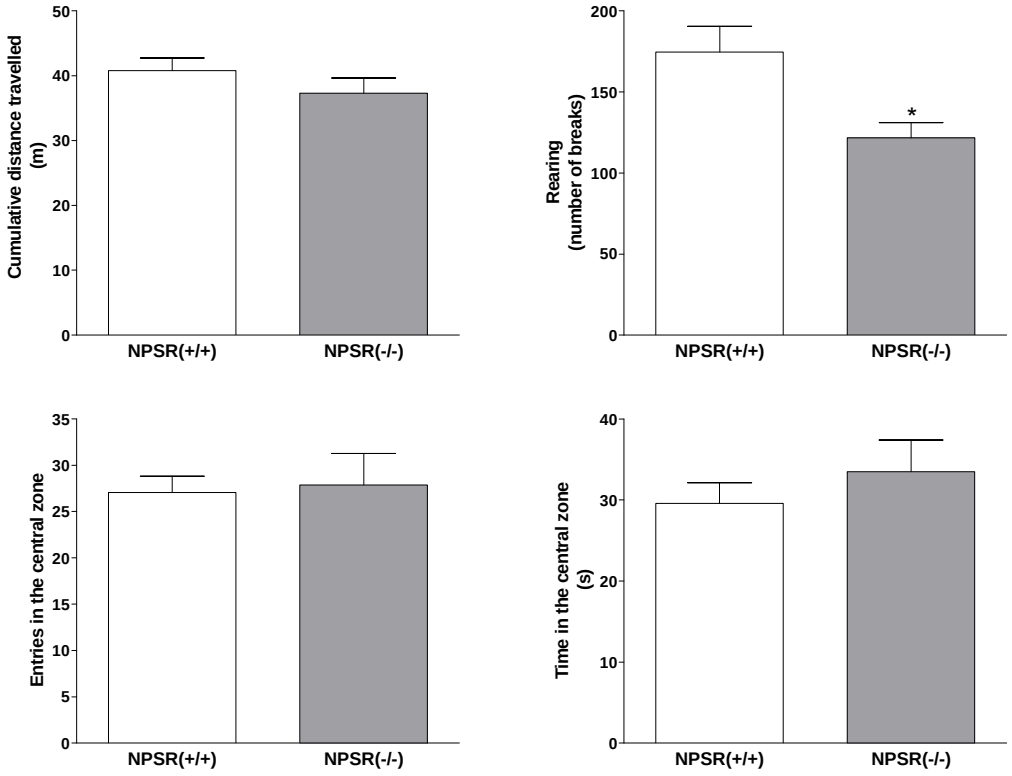

Figure 45 Open field assay in NPSR(+/+) and NPSR(-/-) mice. Effects of genotype on the total distance travelled (top left panel), number of rearings (top right panel), number of entries in the central zone (bottom left panel) and the time spent in this are of the field (bottom right panel). Data area mean ± s.e.m. of 20 mice per group. *P<0.05 vs NPSR(+/+), Student's t-test for unpaired data.

Next we evaluated NPS 1 nmol effects in the OF assay in NPSR(+/+) and NPSR(-/-) mice. NPSR(-/-) mice injected with saline displayed a significant decrease in the number of entries in the central zone of the field compared to NPSR(+/+) mice. No significant genotype effect were recorded in the other parameters detected. However there was a trend for of NPSR(-/-) animals towards reduced rearing behaviour, number of entries and time spent in the central zone of the field. NPS 1 nmol had an anxiolytic-like action in NPSR(+/+) mice and was able to increase their vertical activity and their frequency and the length of the visits in the central area of the OF. No significant effects were recorded in NPSR(-/-) mice in response to NPS injection (figure 46).

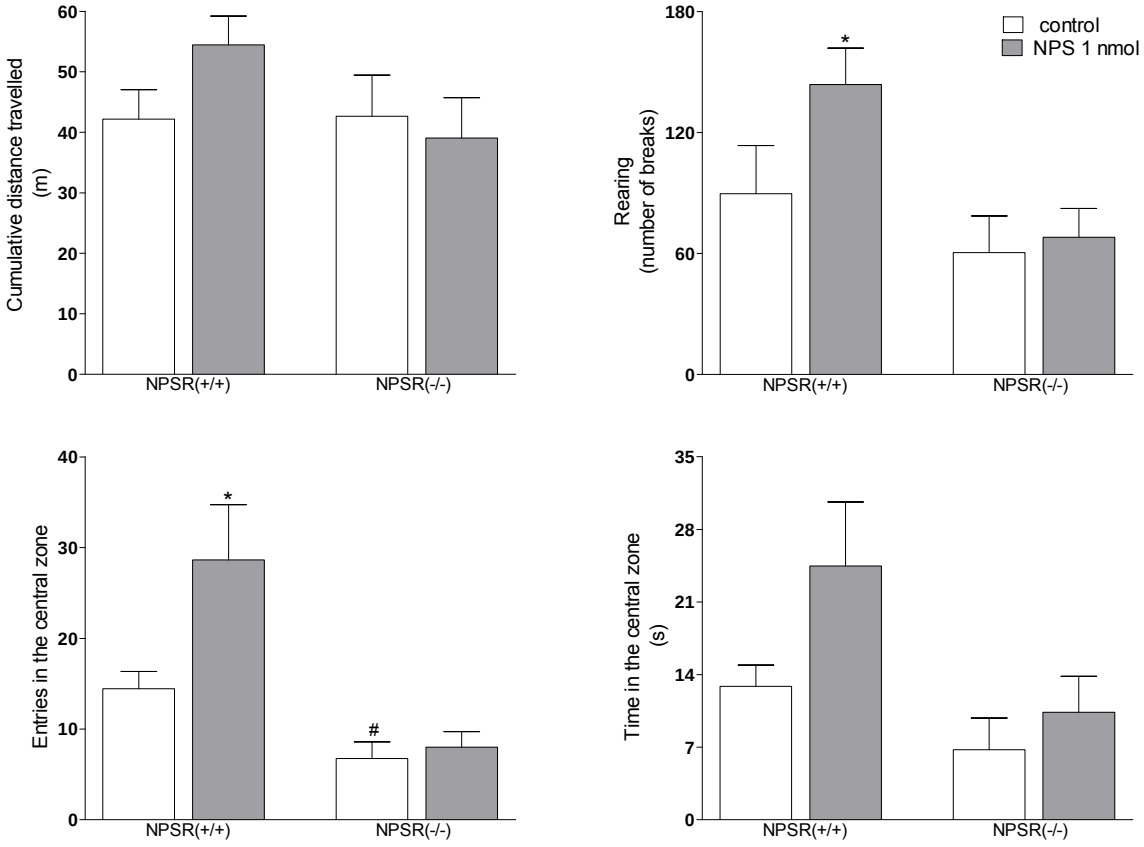

Figure 46 Open field assay in NPSR(+/+) and NPSR(-/-) mice. Effects of genotype and NPS (1 nmol, i.c.v.) on the total distance travelled (top left panel), number of rearings (top right panel), number of entries in the central zone (bottom left panel) and the time spent in this area of the field (bottom right panel). Data are mean ± s.e.m. of 9 mice per group. *p<0.05 vs control, # p<0.05 vs NPSR(+/+) according to two-way (treatment and genotype) ANOVA followed by the Bonferroni test for multiple comparisons.

Stress-induced hyperthermia test

Stress induced by the measurement of body temperature evoked an increase in T_2 compared with T_1 temperature in NPSR(+/+) and NPSR(-/-) mice. No differences were recorded in baseline temperature and in the SIH between NPSR(+/+) and NPSR(-/-) mice (figure 47).

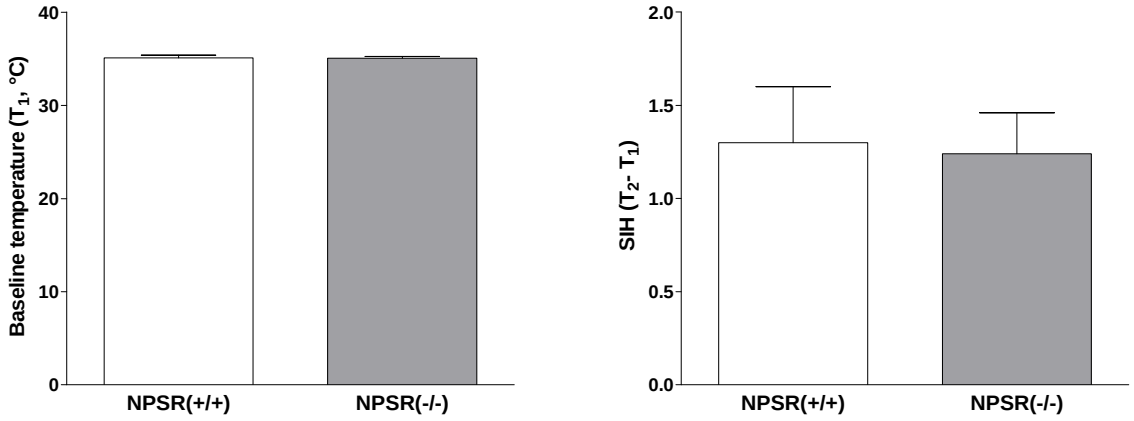

Figure 47 Sress-induced hyperthermia test in NPSR(+/+) and NPSR(-/-) mice. Effects of genotype on baseline temperature (T_1, left panels) and SIH (T_2–T_1, right panels). Data are mean ± s.e.m. of 7-10 mice per group.

Novel object recognition

The NPSR(+/+) and NPSR(-/-) performances were evaluated in the NOR test. In the sample trial no significant differences were observed in the time spent by mice exploring the two objects, both in NPSR(+/+)and NPSR(-/-) mice. Moreover the total time spent by NPSR(+/+) and NPSR(-/-) mice exploring the objects was superimposable (data not shown). After a 3 h delay, both NPSR(+/+) and NPSR(-/-) mice spent significantly more time exploring the new object. No alterations were observed in the NOR in NPSR(-/-) compared to NPSR(+/+) mice (figure 48).

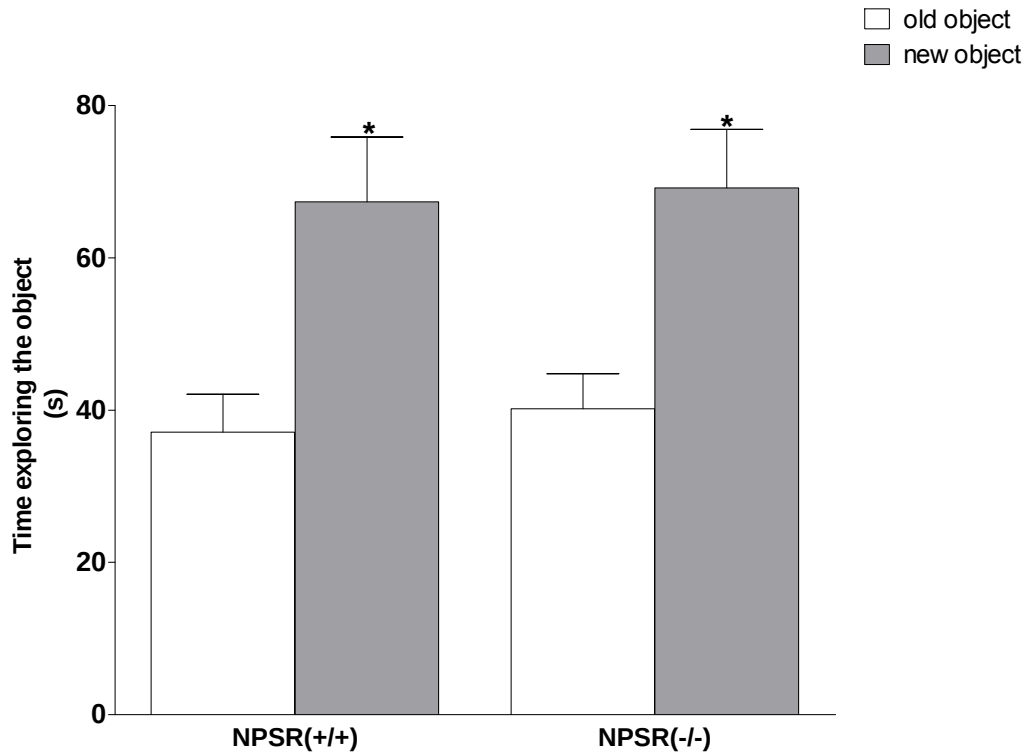

Figure 48 Novel object recognition test in NPSR(+/+) and NPSR(-/-) mice. Effects of genotype on time spent exploring the old and the new object during the test session. *p<0.05 vs old object, according to two-way (genotype and object) ANOVA followed by the Bonferroni test for multiple comparisons.

Forced swimming test

No differences were measured between the behaviour of NPSR(+/+) and NPSR(-/-) mice subjected to the FST (figure 49).

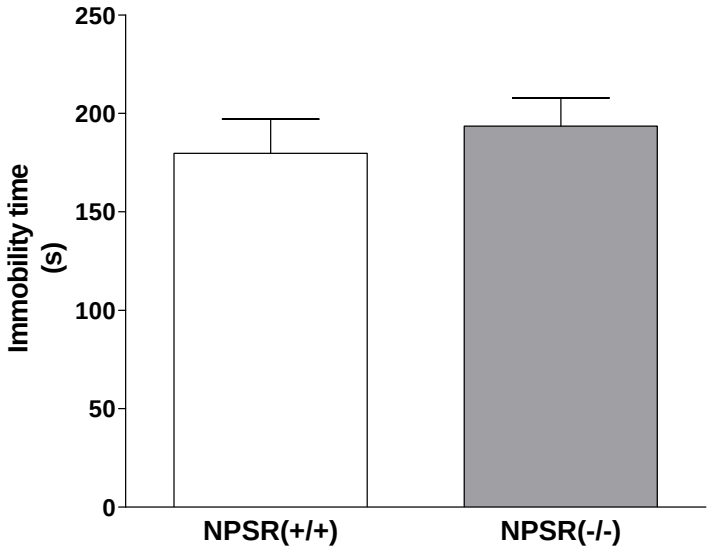

Figure 49 Forced swimming test in NPSR(+/+) and NPSR(-/-) mice. Effects of genotype on immobility time. Data are mean ± s.e.m. of 8-10 mice per group.

Formalin test

The intraplantar injection of 30 µl of 1.5% formalin solution into the dorsal surface of the right hind paw produced a biphasic nociceptive response both in NPSR(+/+) and NPSR(-/-) mice. As shown in figure 51 the first phase started immediately after formalin injection and lasted for 10 min, while the second phase was prolonged, starting approximately 15 min after the injection and lasting for about 30 min. No differences were observed between the nociceptive behaviours of NPSR(+/+) and NPSR(-/-) mice, both in the first and second phase (figure 50).

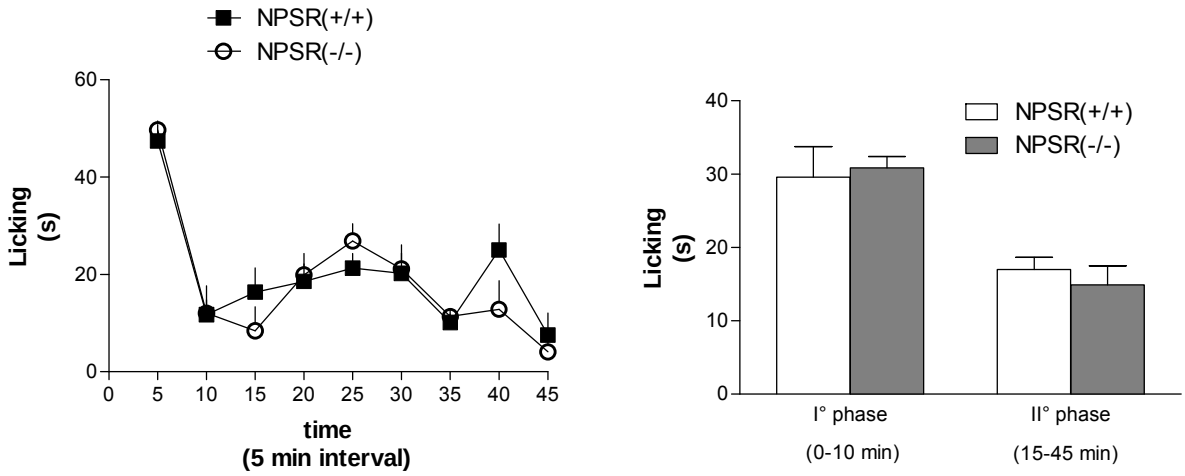

Figure 50 Formalin test in NPSR(+/+) and NPSR(-/-) mice. Effect of genotype on the time course of formalin-induced pain behaviour (left panel) and on the cumulative formalin-induced pain behaviour during the I° and II° phase (right panel). Data are mean ± s.e.m. of 8-10 mice per group.

The present study reports the behavioural phenotype of mice lacking the NPSR receptor gene. Specifically we assessed the effects of the NPSR absence in locomotion, benzodiazepine induced sleep, anxiety, depression, memory and pain. Subsequently we evaluated the involvement of NPSR in NPS induced iperlocomotion, arousal and anxiolytic-like effects.

What is unique in the present work is the use of NPSR(-/-) mice backcrossed to CD-1 strain. In fact the previously published phenotype studies were performed with 129S6/SvEv [36] and C57BL/6 [37-38] NPSR(-/-) mice. It is well know that the strain of mouse can greatly affect the behavioural phenotype and this fact must be taken into account before the behavioural screening starts. In particular, we decided to avoid the use of 129S6/SvEv mice obtained from Taconic because of their low locomtor activity and their high anxious profile. After 8 cycles of backcross to CD-1 strain we obtained mice more active and less anxious in the OF test. The behaviour of these mice is similar to that of the CD-1 mice we used to set up our experimental conditions and to evaluate the effects of selective NPSR ligands (see section 3.3).

NPSR(+/+) and NPSR(-/-) mice were tested in a battery of tests to characterize their phenotype. To minimize the influence of experience of one behavioural test to another, we divided mice in two group (see section 2.5.1) and we arranged the tests in order from the least to the most stressful. The behaviour of NPSR(+/+) mice in each test was very similar to that of CD-1 naïve mice, suggesting the absence of significant carry over effects of one test to another.

No differences were observed between NPSR(+/+) and NPSR(-/-) mice in terms of locomotor activity, both in naïve and in habituated mice. These data suggest that the NPS/NPSR system does not tonically control locomotion. The present results are in line with what reported by other research groups [36-38] and they match the data obtained with the NPSR antagonists [D-Val^5]NPS, [^{t}Bu-D-Gly^5]NPS and SHA 68 (see sections 3.4 and 3.5). In fact the absence of differences between NPSR(+/+) and NPSR(-/-) mice parallels the lack of effects of the NPSR antagonists *per se* in LA test and corroborate the hypothesis that NPS signalling is not activated under the present experimental conditions. When NPS 1 nmol is given i.c.v., it produced an increase in mice locomotion (horizontal and vertical) in NPSR(+/+) mice but not in NPSR(-/-) mice. Thus, the present study give evidence that the mechanism by which NPS promotes stimulation of LA is the selective activation of the NPSR protein. This results are consistent with the results obtained in several laboratories with the NPSR(-/-) mice [36-38] and with the NPSR antagonists [D-Val^5]NPS, [^{t}Bu-D-Gly^5]NPS and SHA 68, that inhibited the stimulatory effect of NPS on LA [33] (see sections 3.4 and 3.5).

Similarly, in the RR test, NPSR(+/+) and NPSR(-/-) shown the same sensitivity to diazepam, both in terms of percentage of mice losing RR and in terms of sleep time. These results were in a first series of experiments obtained with 129S6/SvEv mice and then confirmed with CD-1 mice. 129S6/SvEv mice were more sensitive than CD-1 mice to the hypnotic effect of benzodiazepine as suggested by the difference in percentage of mice losing the RR (~ 100% *vs* ~ 85%, respectively) and particularly in sleep time (~ 200 *vs* ~ 80 min, respectively). This diverse diazepam sensitivity can be due by the difference in genetic background (CD-1 *vs* 129S6/SvEv) and/or age (3 *vs* 6 months). However the results obtained with the two different mice strains in terms of lack of phenotype were perfectly superimposable. Furthermore, NPS was able to reduce the sleep time only in NPSR(+/+) and not in NPSR(-/-), demonstrating that the NPSR receptor is required for the NPS arousal promoting effect. Also in this case the data from knockout studies exactly match the results obtained with the NPSR antagonists [D-Cys(^{t}Bu)^5]NPS, [^{t}Bu-D-Gly^5]NPS and SHA 68 (see section 3.4 and 3.5). All these NPSR antagonists were in fact inactive *per se* in RR test, suggesting that the NPSR receptor is not tonically activated under the present experimental conditions, but, they were able to block the NPS wake promoting action, corroborating the proposal

that the mechanism by which NPS induces arousal is the selective NPSR activation. Obviously our data are limited to the mice sensitivity to diazepam and do not exclude the possibility that the endogenous NPSergic pathways can be activate to promote wakefulness and control circadian sleep/wakefulness cycles. In fact it has been recently reported that thiopental and ketamine anaesthesia time is reduced by NPS while enhanced by the NPSR antagonist [D-Cys(^{t}Bu)^{5}]NPS. Moreover NPSR(-/-) mice displayed reduced late peak wheel running (an index of activity of the internal clock) [36] and reduced LA during the dark phase [37] compared to NPSR(+/+) mice. Furthermore the functional hNPSR polymorphism Ile^{107} has been associated with delayed average bedtime in humans [20]. Thus it seems that the endogenous NPS system can be activated under defined situations/conditions to promote wakefulness.

Since NPS produces a clear anxiolytic-like action when administered supraspinally in rodent [9,43-44] (see section 3.3), we evaluated the behavior of NPSR(+/+) and NPSR(-/-) mice in a battery of paradigms predictive of anxiety, including EPM, OF and SIH assays. The behaviour of NPSR(+/+) and NPSR(-/-) mice in the EPM, OF and SIH tests was superimposable, indicating that the absence of NPSR receptor does not alter anxiety levels under basal conditions. The results of our studies are in line with that reported by Zhu *et al*. in the OF and elevated zero maze tests [38] and Fendt *et al*. [37] in the OF and EPM assays. However they are in contrast to the data published by Duangdao [36], who reported an anxiogenic-like phenotype in NPSR(-/-) mice in the EPM, OF and light-dark box assays. This discrepancy can be explain by the fact that the present study was performed with mice backcrossed on CD-1 strain, instead Duangdao and colleagues used mice on a 129S6/SvEv genetic background. This difference is of particular importance because of 129S6/SvEv mice display high anxiety-levels [105], as demonstrated by the few time spent in the open arms of the EPM (< 6 s) and in the central zone of the OF (< 5 s) by Duangdao 129S6/SvEv mice [36] compared to our CD-1 mice (~ 35 s; ~ 30 s, respectively). It may be that the endogenous NPS/NPSR system modulates anxiety levels only in case of high anxiety basal conditions (129S6/SvEv mice) but not in case of normal anxiety basal conditions (CD-1 mice). This hypothesis is supported by the results obtained by Zhu *et al*. and Fendt *et al*. [37-38] using C57BL/6, who reported no differences between NPSR(-/-) and NPSR(+/+) mice in different models of anxiety. Furthermore, when we evaluated the behaviour of NPSR(-/-) and NPSR(+/+) mice treated with an i.c.v. injection of saline or NPS in the OF test, an effect of genotype was recorded. In fact control mice lacking the NPSR receptor displayed a tendency to a more anxious behaviour compared to wild type animals. This discrepancy between the data obtained with naïve and i.c.v. treated mice can be possibly explained considering that the injection evokes a significant stress for mice. This may increase mice anxiety levels, in fact naïve mice spent ~ 30 s in the central zone of the OF, instead i.c.v. injected mice spent less then 14 s in

this zone (figures 46 and 47). Thus it might be possible that the endogenous NPS system is activated in high anxious mice (i.e. 129S6/SvEv mice and i.c.v. treated mice) but not in middle anxious mice (i.e. C57BL/6 mice and CD-1 naïve mice). Further studies are needed to firmly identify the conditions under which the endogenous NPS signalling is activated.

NPS 1 nmol failed to elicit anxiolytic-like effects in NPSR(-/-) mice, both in the EPM and in the OF test. Thus, with the present study we demonstrated that the NPSR protein is required for the NPS anxiolytic-like action. Our data are consistent with the results reported by Zhu and colleagues [38] in the OF and elevated zero maze assays. Up to now no data with NPSR antagonists in anxiety paradigms are available in mice. However SHA 68 and [^{t}Bu-D-Gly^{5}]NPS are reported to be able to completely inhibit the NPS effects in the EPM in rats [47], corroborating the hypothesis that the mechanism by which NPS promotes anxiolysis is the selective activation of the NPSR receptor.

Interestingly, NPSR(-/-) mice displayed a significantly reduced rearing behaviour, both in the EPM and in the OF test. Rearing is an exploratory act displayed by mice when placed in a novel situation such as the EPM and the OF tests. Actually, there is not an unique interpretation of this kind of spatial exploration, however it seems to represent a response to novelty [108]. Thus, we can suppose a tonic control of the NPS/NPSR system on this particular animal behaviour. Our hypothesis is consistent with pharmacological studies with the NPSR antagonists, in fact there was a clear tendency of animals treated with [D-Val^{5}]NPS 10 nmol to reduce their rearing behaviour (see section 3.4), furthermore, SHA 68 at doses able to counteract the stimulatory effect of exogenously applied NPS produced *per se* a reduction of mouse vertical activity [33].

NPS facilitates spatial memory acquisition [67] and enhances aversive and non aversive memory consolidation [69]. Thus, we investigated the NPSR(-/-) and NPSR(+/+) mice performance in the NOR paradigm. In the sample trial animals explored the two equal objects for the same time and no differences were recorded between NPSR(-/-) and NPSR(+/+) mice in this phase. After 3 hours, when tested for memory retention, both NPSR(-/-) and NPSR(+/+) mice spent more time with the novel object, indicating that they have memorized the familiar object. Also in this trial, the performance of NPSR(-/-) mice was superimposable to that of NPSR(+/+) mice, suggesting that the endogenous NPS system does not modulate learning and memory in the NOR paradigm, at least under the present experimental conditions. Our data are in contrast to those reported by Okamura *et al.* [69], who observed a deficit in NPSR(-/-) mice memory, not only in the NOR test, but also in the inhibitory avoidance paradigm, demonstrating that the NPS/NPSR system is required for memory consolidation. Of note, the experimental protocol adopted by Okamura *et al.* is different from the our protocol. In fact Okamura *et al.* performed the second trial after 24 hours from the first trial [69], instead we performed the second trial only 3 hours from the first trial. Thus it is possible that

NPSergic pathways have a role in modulating long term (24 hours delay) but not short term (3 hours delay) memory consolidation. The discrepancy between our and Okamura *et al.* data can also be due to the different mice strain used (129S6/SvEv *vs* CD-1). On note, our results are in line with that reported by Zhu and colleagues [38], which recorded no differences between NPSR(+/+) and NPSR(-/-) mice in spatial learning and memory evaluated in the Morris water maze task. Obviously, further studies are needed to firmly establish the role of played by the endogenous NPS in regulating memory acquisition, consolidation and retrieval.

The FST was used to assess depressive-like behaviours. During this test NPSR(+/+) and NPSR(-/-) mice spent the same amount of time immobile, indicating that the lack of the NPSR receptor does not influence this kind of behaviour in mice. This results is consistent to that reported by Okamura *et al.* [69] but it is in contrast with the results reported by Zhu *et al.* [38], who observed that NPSR(-/-) mice display an enhanced depressive behaviour compared to NPSR(+/+) mice. It is worthy of note that this different phenotype was detected only in male but not in female mice and only in the FST but not in the tail suspension test [38].

NPS was reported to be able to reduce pain transmission in different mice models, including the tail withdrawal test, the hot-plate test [71] and the formalin test [72]. Thus, we decided to investigate the sensitivity of NPSR(+/+) and NPSR(-/-) mice in the formalin test. No differences were recorded between wild type and knock out animas, demonstrating that the endogenous NPS signalling is no activated during the formalin test. This results parallel the results obtained with the peptide NPSR antagonists [D-Cys(^{t}Bu)^5]NPS and [D-Val^5]NPS. In fact [D-Cys(^{t}Bu)^5]NPS did not induce hyperalgesia or antinociception *per se* in the tail withdrawal test and in the hot-plate test [71]; similarly [D-Val^5]NPS was found inactive *per se* in the formalin test [72]. These data obtained with the NPSR antagonists corroborate the hypothesis that NPSergic pathways do not tonically modulate pain transmission.

In conclusion the present study indicate that the endogenous NPSergic pathway does not modulate locomotion, sensitivity to benzodiazepine, anxiety, memory, depressive-like behaviours and pain transmission in mice. However we demonstrated that the NPS/NPSR system has role in controlling mice rearing behaviour in a novel environment. Furthermore our results clearly show that the NPSR protein is not a redundant receptor for NPS in the brain; on the contrary, it represents the mandatory protein for all the biological effects of NPS so far described.

4. GENERAL CONCLUSIONS

The present thesis summarizes the work we performed in the last three years in the field of NPS and its receptor. Following the identification in 2004 of NPS as the endogenous ligand of the NPSR receptor an extensive research has started, both in academic and industrial laboratories, to assess the biological functions regulated by this new peptidergic system and to foresee the possible therapeutic indications of drugs interacting selectively with the NPSR receptor. To this aim, different group focused their attention to the identification of selective NPSR ligands, particularly antagonists, since they represent a tool of paramount importance to elucidate which biological functions and how they are controlled by the NPS/NPSR system and the neurobiological mechanisms of NPS actions. Another important tool instrumental for investigating this new peptidergic system is represented by NPSR knockout animals. In fact the study of their phenotype is useful to elucidate the biological functions tonically regulated by endogenous NPS, furthermore they can be used to demonstrate the *in vivo* selectivity of action of NPS and synthetic NPSR agonists.

The aim of our research was to contribute to the study of the pharmacology and the neurobiology of this new petidergic system. We performed parallel studies aimed at the identification of new and interesting peptide NPSR ligands, in particular antagonists, and at the determination of the biological actions elicited by NPS in mice. New identified NPSR antagonists as well as NPSR(-/-) mice were used to deeply investigate the neurobiology of the NPS/NPSR system.

Our major achievements in this field were:

1. systematic structure and conformation activity studies that allowed the identification of the first generation of peptide NPSR antagonists
2. detailed investigation of NPS actions in mice
3. first *in vitro* and *in vivo* characterization of NPSR antagonists
4. generation of a CD-1 congenic NPSR(-/-) mouse colony and first investigation of their phenotype and NPS sensitivity

The studies focused on NPS position 2, 3 and 4, that represent the peptide message domain, revealed the chemical characteristics needed for high potency binding to NPSR. However no useful and interesting NPSR ligands were identified in the context of such studies. The SAR studies

focused on NPS position 5 demonstrated that peptide efficacy was not modified by L amino acid residues while being strongly reduced or even abolished by D residues. This clearly suggests that modifications of the relative spatial disposition of the N- (message) and C-terminal domains of NPS induced by chirality changes in position 5 have little effect on peptide potency while having a profound impact on peptide efficacy, with L amino acid favouring agonist and D amino acids inducing antagonist bioactive conformations. Thus these studies led to the identification of the first generation of peptide NPSR antagonists, that derived from the substitution of Gly^5 with D amino acids with a short lipophilic-branched side chain. The most important chemical modifications applied in NPS position 5 that were instrumental for the identification of NPSR peptide antagonists are displayed in figure 52. The most potent NPSR pure antagonists identified were the compounds [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS.

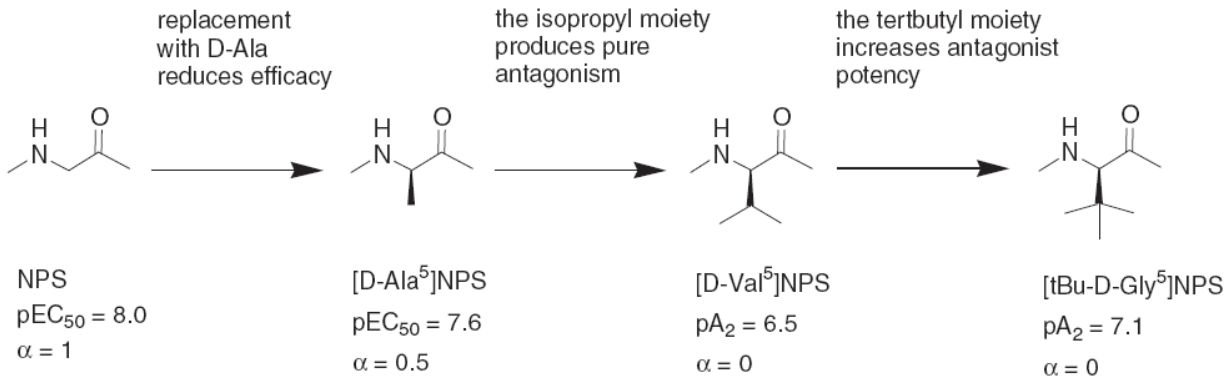

Figure 51 Chemical modifications of NPS Gly^5: critical steps toward the identification of NPSR peptide antagonists.

Knowledge derived from the SAR studies focused on NPS sequence are summarized in figure 53.

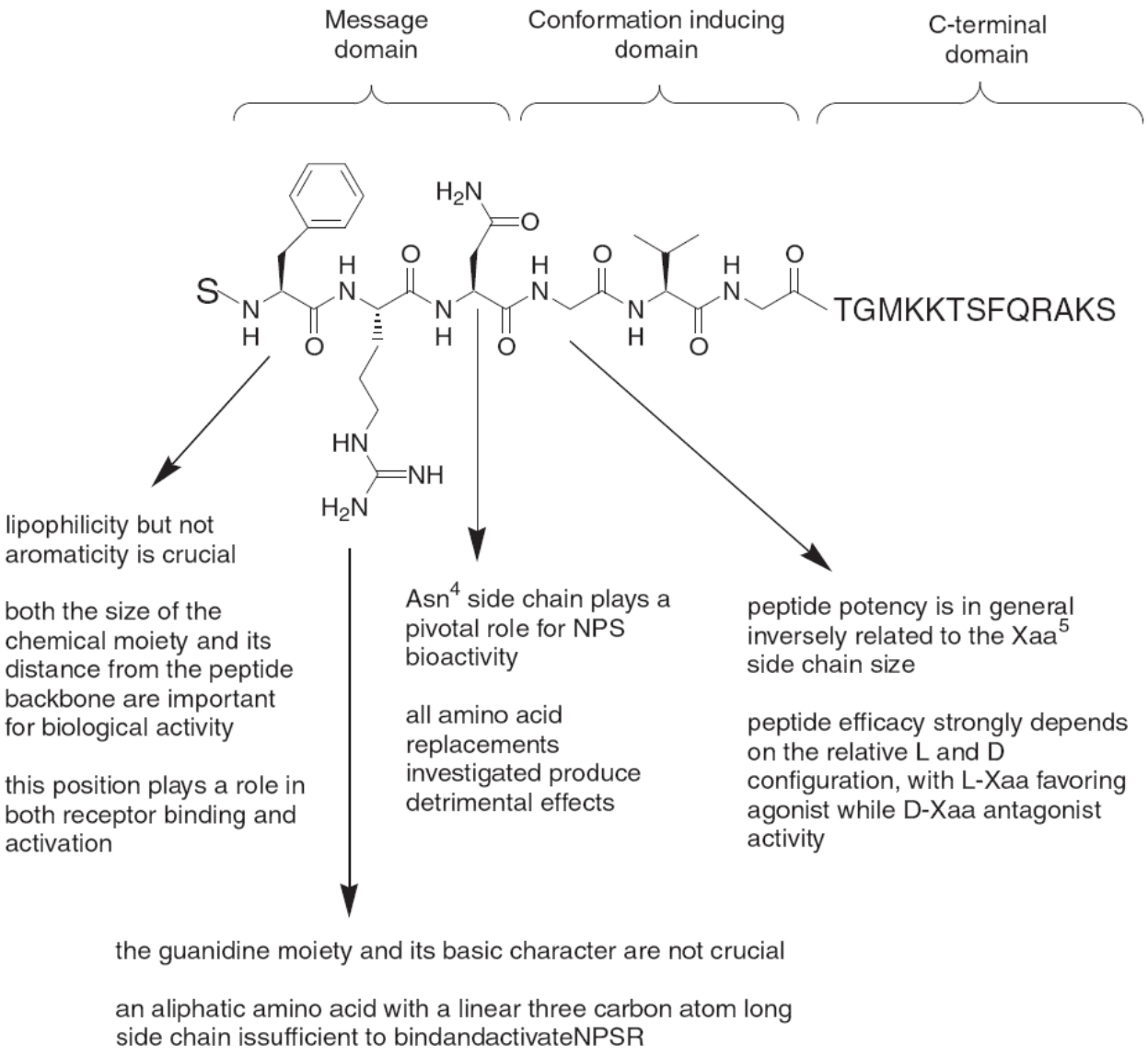

Figure 52. Summary of SAR studies on NPS. The message domain is crucial for NPSR binding and activation. The conformation inducing domain plays a critical role in shaping the bioactive conformations of NPS. The C-terminal domain is required for *in vivo* biological activity.

We successfully set up the experimental conditions to study NPS actions *in vivo* and we provided further evidence that NPS, given supraspinally in mice, elicits an unique pattern of biological effects: stimulation associated with anxiolysis. We demonstrated that NPS increases mice locomotion and reduces benzodiazepine induced sleep. Moreover NPS has a clear anxiolytic effect in different mice models, such as EPM, OF and SIH tests. In LA and RR assays, NPS mimics the stimulatory effect of caffeine, instead in EPM, OF and SIH tests NPS mimics the anxiolytic effect of diazepam. Our data corroborate and extend the findings obtained in the pivotal study by Xu and colleagues [9], that reported that NPS is able to increase mice locomotion and to stimulate

wakefulness in rat and at the same time it shows clear anxiolytic-like actions in EPM, OF light-dark box and marble burying tests. The locomotor stimulant effect of NPS seems to be highly consistent among experimental conditions and animal species, in fact it was replicated in different laboratories [9,32-34], not only in mice but also in rats [31,35]. We also demonstrated that NPS has arousal promoting proprieties in the RR assay, since it is able to reduce the number of animals losing RR in response to an hypnotic dose of diazepam and markedly decrease the sleep time in those animals responding to the benzodiazepine. Our findings were recently confirmed by Kushikata *et al.* [40] who reported similar NPS effects in rats anesthetized with ketamine or thiopental. Finally, we demonstrated that NPS acts as an anxiolytic drug in the EPM, OF and SIH assays, confirming the data previously reported by Xu *et al.* [9]. During the last few years the NPS anxiolytic effects were replicated in different laboratories [25,34,38,43] and extended to rats [44,47] Thus, similar to the stimulant effects of NPS, its anxiolytic like action also seems to be a robust effect easily reproduced in different laboratories, species and assays. Collectively the present work confirms and extends the hypothesis of NPS as a unique neuropeptidergic signal: an activating anxiolytic. Furthermore we successfully set up the experimental conditions to evaluate the *in vivo* actions of new synthesized NPSR ligands in a battery of behavioural assays. The results of this study suggest that the NPS/NPSR system can represent an innovative and unique pharmacological target for the treatment of sleep and anxiety disorders.

Our major goal was, in the frame of the SAR studies focused on NPS position 5, the discovery of the first generation of peptide NPSR antagonists, including the compounds [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS. We performed an extensive *in vitro* and *in vivo* pharmacological characterization of the actions of these new NPSR antagonists. In calcium mobilization assay [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS acted as pure and selective NPSR antagonists, with the following rank order of potency [^{t}Bu-D-Gly^5]NPS > [D-Val^5]NPS > D-Cys(^{t}Bu)^5]NPS. The NPSR antagonistic action of these molecules was also confirmed *in vivo* in the LA ([D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS) and RR ([D-Cys(^{t}Bu)^5]NPS and [^{t}Bu-D-Gly^5]NPS) tests. From these studies [^{t}Bu-D-Gly^5]NPS resulted the most potent peptide NPSR antagonist available up to now. Both [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS were able to completely prevent the stimulant NPS effects in LA test, with [^{t}Bu-D-Gly^5]NPS 3 fold more potent than [D-Val^5]NPS, but the peptide injected alone did not change mice locomotion. Similar results were obtained in the RR test, where [D-Cys(^{t}Bu)^5]NPS and [^{t}Bu-D-Gly^5]NPS counteracted NPS arousal promoting action, with [^{t}Bu-D-Gly^5]NPS being 10 fold more potent than [D-Cys(^{t}Bu)^5]NPS. Thus we provided first evidence that the NPS stimulant and arousal promoting

actions are selectively due to the NPSR receptor activation and that the endogenous NPSergic pathway is not involved in the control of mice locomotion and sensitivity to benzodiazepine under the present experimental conditions. [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS represent pharmacological tools, available to the scientific community, of paramount importance to investigate which biological functions are controlled by the NPS/NPSR system and to elucidate the role plays by NPSR in the actions evoked by NPS. In fact, as summarized in table 15, the NPSR antagonists we identified were largely used by different research groups in the world. Specifically, using [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS and [^{t}Bu-D-Gly^5]NPS different laboratories demonstrated that the NPS anorectic effects are selectively due to the NPSR activation and that the endogenous NPS does not modulate feeding behaviours both in mice and in rat [63-65]. [D-Cys(^{t}Bu)^5]NPS and [D-Val^5]NPS were instrumental to shown that the NPS induced antinociception is selectively mediated by the NPSR protein and that the NPS/NPSR system does not control this biological function [71-72]. Moreover, [D-Val^5]NPS was useful to demonstrate that the inhibition of mouse colonic transit caused by NPS is due to the NPSR activation and that endogenous NPS does not control colonic transit [73]. Finally, Kushikata and colleagues [40]reported that [D-Cys(^{t}Bu)^5]NPS was able to counteract the NPS arousal promoting action in ketamine and thiopental induced anaesthesia, demonstrating that NPS actions in RR test are due to the NPSR activation. [D-Cys(^{t}Bu)^5]NPS prolonged *per se* anaesthesia duration, suggesting that the NPS/NPSR system tonically controls the anaesthesia state [40]. Of note the data by Kushikata *et al.* are opposite to our findings, in fact neither [D-Cys(^{t}Bu)^5]NPS nor [^{t}Bu-D-Gly^5]NPS increased diazepam induced sleep time under our experimental conditions. This discrepancy may be possible due the differences in anaesthetics used and in animal species (mouse *vs* rat). However, based on Kushikata findings, NPSR may represent a pharmacological target for developing novel anaesthetic.

SHA 68 has been identified as a novel non peptide selective NPSR antagonist [33]. We performed a complete pharmacological characterization of the actions of this interesting molecule. We confirmed and extended previous findings demonstrating that SHA 68 behaves as a selective NPSR antagonist *in vitro* and *in vivo*. In calcium mobilization assay SHA 68 resulted 10 fold more potent than the most potent peptide NPSR antagonist [^{t}Bu-D-Gly^5]NPS. Despite its high potency at the mNPSR *in vitro*, *in vivo* SHA 68 at high doses was able to completely prevent only the NPS arousal promoting effect in the RR test, instead in the LA test it only partially counteracted NPS actions. SHA 68 did not antagonize NPS anxiolytic-like effects in the OF assay. Those differences between *in vitro* and *in vivo* effectiveness are likely due to the poor pharmacokinetic features of SHA 68. Thus the usefulness of SHA 68 as pharmacological tool for the investigation of the

NPS/NPSR system is limited. During the last few years new non peptide NPSR antagonists were identified by industrial research groups and others will be available in the next future. The comparison of the biological effects of these molecules, characterized by unrelated chemical structures, will allow a specific determination of the therapeutic indications of this new drug class. However SHA 68 may represent an useful template for the identification of NPSR selective antagonists characterized by better pharmacokinetic features. As summarized in table 15 and similar to peptide NPSR antagonists, SHA 68 was used during these years by different research groups to investigate the mechanisms of NPS actions and the biological functions controlled by the NPS/NPSR system.

Interestingly, in a recent study Kallupi *et al.* [59] demonstrated that [D-Cys(^{t}Bu)^5]NP and SHA 68 injected alone are able to prevent cue-induced drug seeking. This evidence suggests that the NPS/NPSR system may have a role in the pathophysiology of drug relapse and that NPSR receptor may represent an important and unique target for the treatment of drug craving and the prevention of relapses in addicted patients.

Table 15. Summary of NPSR antagonist *in vivo* studies

Assay	NPS effects (dose)	Antagonist	Antagonist actions (dose)	Reference
Mouse RR (diazepam)	Arousal promoting action (0.1 nmol)	[D-Cys(^{t}Bu)^5]NPS	Antagonizes NPS effects, no effects *per se* (10 nmol)	present work
Rat cue-induced cocaine seeking	Increasing of drug-seeking (2 nmol)	[D-Cys(^{t}Bu)^5]NPS	Prevented *per se* drug seeking (30 nmol)	59
Rat palatable food intake	Anorectic action (1 nmol)	[D-Cys(^{t}Bu)^5]NPS	Antagonizes NPS effects, no effects *per se* (60 nmol)	63
Rat palatable food intake	Anorectic action (1 nmol)	[D-Cys(^{t}Bu)^5]NPS	Antagonizes NPS effects, no effects *per se* (60 nmol)	65
Mouse TW and HP tests	Antinociceptive action (0.1 nmol)	[D-Cys(^{t}Bu)^5]NPS	Antagonizes NPS effects, no effects *per se* (10 nmol)	71
Rat RR (ketamine and thiopentale)	Arousal promoting action (1 nmol)	[D-Cys(^{t}Bu)^5]NPS	Antagonizes NPS effects, prolonged *per se* anaesthesia duration (20 nmol)	40
Mouse LA	Stimulant action (0.1 nmol)	[D-Val^5]NPS	Antagonizes NPS effects, no effects *per se* (10 nmol)	present work
Mouse colonic transit	Inhibition of bead expulsion time (0.01 nmol)	[D-Val^5]NPS	Antagonizes NPS effects, no effects *per se* (1 nmol)	73
Mouse food intake	Anorectic action (0.1 nmol)	[D-Val^5]NPS	Antagonizes NPS effects, no effects *per se* (10 nmol)	64
Mouse formalin test	Antinociceptive action (0.01 nmol)	[D-Val^5]NPS	Antagonizes NPS effects, no effects *per se* (10 nmol)	64
Mouse LA	Stimulant action (0.1 nmol)	[^{t}Bu-D-Gly^5]NPS	Antagonizes NPS effects, no effects *per se* (3 nmol)	present work
Mouse RR (diazepam)	Arousal promoting action (0.1 nmol)	[^{t}Bu-D-Gly^5]NPS	Antagonizes NPS effects, no effects *per se* (1 nmol)	present work
Rat palatable food intake	Anorectic action (1 nmol)	[^{t}Bu-D-Gly^5]NPS	Antagonizes NPS effects, no effects *per se* (60 nmol)	65
Mouse LA	Stimulant action (0.1 nmol)	SHA 68	Antagonizes NPS effects, no effects *per se* (50 mg/kg)	33
Mouse OF	Anxiolytic-like activity (0.5 nmol)	SHA 68	Anxiogenic-like activity (2 nmol)	25
Mouse conditioned fear behaviour	Reduction of freezing (0.5 nmol)	SHA 68	Increase in freezing (2 nmol)	25
Rat EPM	Anxiolytic-like activity (1 nmol)	SHA 68	Antagonizes NPS effects, no effects *per se* (50 mg/kg)	47
Rat DB	Anxiolytic-like activity (1 nmol)	SHA 68	Antagonizes NPS effects, no effects *per se* (50 mg/kg)	47
Mouse OF	Anxiolytic-like activity (0.1 nmol)	SHA 68	Inactive (50 mg/kg)	present work
Mouse LA	Stimulant action (0.1 nmol)	SHA 68	Antagonizes NPS effects, no effects *per se* (50 mg/kg)	present work
Mouse RR	Arousal promoting action (0.1 nmol)	SHA 68	Antagonizes NPS effects, no effects *per se* (50 mg/kg)	present work
Rat cue-induced cocaine seeking	Increasing of drug-seeking (2 nmol)	SHA 68	Prevented *per se* drug seeking (30 mg/kg)	59
Inhibitory avoidance paradigm	Increasing aversive memory (1 nmol)	SHA 68	Antagonizes NPS effects, no effects *per se* (50 mg/kg)	69

NPS was given i.c.v. with the exception of [25] where it was given intra amygdala; peptide NPSR antagonists were given i.c.v. with the exception of [59] where they were given intra lateral hypothalamus; SHA 68 was given i.c.v., with the exception of [25] where it was given intra amygdala

NPSR(-/-) mice represent an useful genetic tool for the investigation of the neurobiology of the NPS/NPSR system. The first study using these mice was performed in 2006 by Allen *et al.* [83], next the behaviour of NPSR(-/-) mice and their sensitivity to NPS were evaluated in different laboratories [36-38,50]. We contributed to this field performing a deep investigation of the behavioural profile of NPSR(-/-) mice backcrossed on CD-1 strain. Our study revealed no locomotor differences between NPSR(+/+) and NPSR(-/-) mice, with the exception of rearing behaviour that was reduced in knockout animals. Furthermore, the behaviour of NPSR(+/+) and NPSR(-/-) mice in the EPM, OF and SIH tests was found to be superimposable. Similarly no differences were detected in the novel object recognition, forced swimming, RR and formalin assays. However, the stimulant actions of 1 nmol NPS in RR and in LA tests and the NPS anxiolytic-like effects in the EPM and OF assays were detected in NPSR(+/+) but not in NPSR(-/-) mice. Collectively our data suggest that endogenous NPS/NPSR system does not play a role in the control of locomotion, anxiety, depression and memory, at least under the present experimental conditions. Moreover these results demonstrate that the NPS stimulant and anxiolytic effects are selectively due to the NPSR activation. Thus the outcomes of this study corroborate the findings derived from the studies with the NPSR antagonists. Our CD-1 congenic NPSR(-/-) mice will be a powerful tool available to the scientific community to investigate the different biological function of the NPS system. Furthermore these mice will represent a tool of paramount importance to investigate the *in vivo* selectivity of action of novel NPSR ligands.

In conclusion, the research activity performed during my PhD program led to the development of two different tools useful for the study of the NPS and its receptor: NPSR antagonists and NPSR(-/-) mice. In particular we identified the first generation of NPSR peptide antagonists. The use of these research tools in parallel with knockout studies generated converging evidence on the biological effects induced by the selective activation of NPSR. For performing more detailed and systematic studies in this field other tools and knowledge need now to be identified and developed, such as more potent peptide and non peptide NPSR antagonists; selective non peptide NPSR agonists able to elicits their effects after peripheral administration; ppNPS(-/-) mice. The availability of these tools will allow in the future a more detailed and deep investigation of NPS/NPSR neurobiology and pharmacology.

From the limited information available nowadays it is possible to foreseen that NPSR agonists (and in some instances partial agonists) may be useful in the treatment of sleep, food intake, memory disorders, and anxiety states, while NPSR antagonists might possibly represent novel therapeutics for the control of drug addiction, in particular for inhibiting relapse.

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