Neurobiology, Pharmacology, and Medicinal Chemistry of Neuropeptide S and Its Receptor

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Abstract: Neuropeptide S (NPS) is the last neuropeptide identified via reverse pharmacology techniques. NPS selectively binds and activates a previous orphan GPCR, now named NPSR, producing intracellular calcium mobilization and increases in cAMP levels. Biological functions modulated by the NPS/NPSR system include anxiety, arousal, locomotion, food intake, memory, and drug addiction. The primary sequence of NPS (in humans SFRNGVGTGMKKTSFQRAKS) is highly conserved among vertebrates especially at the *N*-terminus. Ala- and D-scan studies demonstrated that this part of the molecule is crucial for biological activity. Focused structure–activity studies performed on Phe^2, Arg^3, and Asn^4 confirmed this indication and revealed the chemical requirements of these positions for NPSR binding and activation. The sequence Gly^5-Val^6-Gly^7 seems to be important for shaping the bioactive conformation of the peptide. Structure–activity studies on Gly^5 enabled identification of the first generation of peptidergic NPSR pure antagonists including [D-Cys(tBu)^5]NPS and [D-Val^5]NPS whose antagonist properties were confirmed in vivo. Finally, the pharmacological features of substituted bicyclic piperazine molecules (e.g. SHA 68 (3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide) were recently published making available the first generation of nonpeptide NPSR antagonists. The use in future studies of NPSR antagonists will be of paramount importance for understanding which biological functions are controlled by the NPS/NPSR system and for defining the therapeutic potential of selective NPSR ligands. Med Res Rev, 30, No. 5, 751–777, 2010

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1. INTRODUCTION

The reverse pharmacology concept and strategy[1] was validated in 1995 with the identification of the peptide nociceptin/orphanin FQ[2,3] as the endogenous ligand of a previous orphan G-protein coupled receptor now referred to as NOP. In the subsequent years more than 40 G-protein coupled receptors were deorphanized and novel peptide–receptor systems were identified including orexins, prolactin releasing peptide, apelin, ghrelin, metastatin, neuropeptides B and W and prokineticins 1 and 2. The novel information emerging from these studies 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.

Neuropeptide S (NPS) was the last neuropeptide identified via the reverse pharmacology approach. The identification of NPS as the endogenous ligand of the previous orphan G protein coupled receptor GPR154 was first reported in the patent literature.[4] Then an elegant study by Xu et al.[5] demonstrated that NPS bound with high affinity to the GPR154 receptor causing intracellular calcium mobilization. The receptor is widely distributed in the brain while the expression of the peptide precursor is limited to few discrete brain areas. In vivo studies in rodents showed that the supraspinal administration of NPS in the nmol range evokes robust anxiolytic-like effects that are associated with the stimulation of locomotor activity and clear arousal promoting effects.[5] Thus, NPS seems to act in the brain as a rather unique transmitter: an activating anxiolytic.[6]

Following the description by Xu et al.[5] in the present review the peptide will be indicated as NPS and its receptor as NPSR. However, as previously pointed out,[7] 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[8]).

In this review we will briefly summarize the available information regarding NPS and its receptor and the biological actions modulated by the NPS–NPSR system. Next we describe in detail structure and conformation activity studies on NPS and the focused studies performed on the most important amino acid residues of this peptide, which led to the identification of the first generation of peptide NPSR antagonists. Finally, the limited information regarding substituted bicyclic piperazine molecules as nonpeptide NPSR ligands will be analyzed.

2. THE NPS/NPSR SYSTEM

NPS: 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.[5] The NPS sequence is highly conserved among animal species with few variations located in the center and *C*-terminus of the peptide.[9] 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.[9]

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.[10] ppNPS mRNA expression in the brain displayed a very limited distribution. Most of the NPS expressing neurons were found in the following brain stem regions: the principle sensory trigeminal nucleus, the lateral parabrachial nucleus, and the locus coeruleus area.[11] Double label in situ hybridization studies

demonstrated that most of the NPS expressing cells in the locus coeruleus area are glutamatergic neurons, few are cholinergic, while none produce GABA. In the principle sensory trigeminal nucleus many of the NPS expressing neurons use glutamate as a neurotransmitter. Finally, in the lateral parabrachial nucleus NPS positive cells co-express CRF.[11] 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.[11]

The NPS receptor: The NPSR (also known as vasopressin receptor-related receptor VRR1[12] or G protein coupled receptor for asthma susceptibility (GPRA)[13]) is a typical G protein coupled receptor, which shows only moderate homology with other peptide receptors. In cells expressing the recombinant NPSR receptor, NPS both stimulates intracellular calcium levels and cAMP accumulation (Table I). This indicates that the NPSR can signal via both Gq and Gs to increase cellular excitability. Several splice variants and multiple single nucleotide polymorphisms have been reported for the human NPSR. The most intensely investigated NPSR isoforms are hNPSR Asn107 and hNPSR Ile107. This receptor polymorphism seems to have functional implications since the hNPSR Ile107 receptor displayed similar binding affinity but higher NPS potency (by approx. 10-fold) than hNPSR Asn107.[14] 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) (see Table I for data and quotations). It is worthy of mention that the rat and mouse NPSR contain Ile at position 107. However, the pathophysiological implications of NPSR polymorphisms are far from being fully understood. For detailed analysis of genetic linkage studies for the NPSR gene locus with asthma and psychiatric diseases the reader is referred to the review by Reinscheid.[15]

In contrast to ppNPS, the NPSR is widely distributed in the brain. In fact, high levels of NPSR mRNA expression were found in the cortex, olfactory nuclei, thalamus, hypothalamus, amygdala, and subiculum.[5,11] This profile of receptor expression suggests the involvement of the NPS–NPSR system in the regulation of multiple central functions.

3. BIOLOGICAL ACTIONS

A. In Vitro Studies

Table I summarizes the in vitro effects of NPS. It is evident that most of the studies were performed measuring intracellular calcium mobilization in cells expressing the recombinant NPSR. In these studies NPS displayed similar high potency at the rat, mouse, and human NPSR and, as mentioned above, 10-fold higher potency at the hNPSR Ile107 isoform than hNPSR Asn107.

Limited information is available on the in vitro effects of NPS in cells/tissues expressing 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 type reported to be sensitive to NPS is the macrophage, which responds to NPS with reduced adhesion and increased phagocytosis and chemotaxis.[16] 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[13] and gastrointestinal[17] systems, indicate that the NPS–NPSR system may have a role in modulating innate immunity and chronic inflammatory diseases of epithelial barrier organs.

Table I. In Vitro Effects of NPS

Preparation	NPS action	pEC_{50}	Reference
HEK293-hNPSR	hNPS: Stimulation of $[Ca^{2+}]$i mobilization	8.03	5
HEK293-hNPSR	rNPS: Stimulation of $[Ca^{2+}]$i mobilization	8.49	5
HEK293-hNPSR	mNPS: Stimulation of $[Ca^{2+}]$i mobilization	8.52	5
HEK293-hNPSR Asn107	Stimulation of $[Ca^{2+}]$i mobilization	8.07	14
HEK293-hNPSR Ile107	Stimulation of $[Ca^{2+}]$i mobilization	8.75	14
HEK293-hNPSR Asn107	Stimulation of cAMP levels	7.50	14
HEK293-hNPSR Ile107	Stimulation of cAMP levels	8.46	14
Mouse macrophage RAW 264.7	Stimulation of phagocytosis	ND	16
Mouse macrophage RAW 264.7	Inhibition of macrophage adhesion onto fibronectin	ND	16
Mouse macrophage RAW 264.7	Stimulation of chemotaxis	ND	16
HEK293-hNPSR	Stimulation of $[Ca^{2+}]$i mobilization	8.39	7
CHO-hNPSR Asn107	Stimulation of $[Ca^{2+}]$i mobilization	8.08	57
CHO-hNPSR Ile107	Stimulation of $[Ca^{2+}]$i mobilization	8.85	57
HEK293-hNPSR Asn107	Stimulation of $[Ca^{2+}]$i mobilization	7.58	57
HEK293-hNPSR Ile107	Stimulation of $[Ca^{2+}]$i mobilization	8.67	57
HEK293-mNPSR	Stimulation of $[Ca^{2+}]$i mobilization	8.04	64
HEK293-mNPSR	Stimulation of $[Ca^{2+}]$i mobilization	8.96	65
HEK293-mNPSR	Stimulation of $[Ca^{2+}]$i mobilization	8.07	22
HEK293-hNPSR Asn107	Stimulation of $[Ca^{2+}]$i mobilization	$\approx$7.5	24
HEK293-hNPSR Ile107	Stimulation of $[Ca^{2+}]$i mobilization	$\approx$8.5	24
HEK293-mNPSR	Stimulation of $[Ca^{2+}]$i mobilization	7.99	66
Mouse frontal cortex synaptosomes	Inhibition of K^+ stimulated $[^3H]$5-HT release	10.5	20
Mouse frontal cortex synaptosomes	Inhibition of K^+ stimulated $[^3H]$NA release	Biphasic CRC	20
HEK293-hNPSR	Stimulation of the expression of actavin A, interleukin 8 matrix metalloprotease 10 and EPH receptor A2	7–6	71
Mouse coronal brain slices	Stimulation of BLA glutamatergic synaptic activity	ND	18
Mouse coronal brain slices	Stimulation of glutamatergic transmission to GABAergic neurons in the amygdala	ND	19

Unless otherwise indicated, human NPS was used in the different studies. ND, not determined; CRC, concentration response curve; BLA, basolateral amygdala.

As far as central nervous system preparations are concerned, NPS has been reported to modulate synaptic activity in mouse brain slices[18,19] and neurotransmitter release in mouse synaptosomes.[20] In particular, two complimentary studies performed in mouse coronal brain slices reported distinct actions of NPS in the amygdala. Meis et al.[18] 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.[18] Using the same preparation, Jungling et al.[19] 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[19] (see above). The electrophysiological actions of NPS modulating the activity of the circuit endopiriform cortex, lateral, basolateral, and central amygdala has been recently and nicely summarized by Pape et al.[21]

Finally, NPS has been recently 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.[20] 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.[20]). There is convincing evidence in the literature that elevation of 5-HT and NA levels is associated with anxiety like behaviors; 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.

B. In Vivo Studies

Available information regarding the in vivo actions of NPS is summarized in Table II.

Locomotor activity: In the pivotal study by Xu et al.[5] it has been reported that the supraspinal administration of NPS in mice stimulates locomotor activity. 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[7,22–26] and rats,[27] suggesting that the hyperlocomotor action of NPS is a robust phenomenon among experimental conditions and animal species. Interestingly enough, Castro et al.[25] demonstrated that NPS, at doses able to increase locomotor activity, attenuates oxidative stress and brain injury in mice. Further studies are needed to investigate the mechanisms underlining this action of NPS. Recently, the involvement of NPSR in the locomotor stimulatory effect of NPS has been demonstrated with the use of two chemically unrelated NPSR antagonists, the nonpeptide SHA 68[24] and the peptide [D-Val^5]NPS.[28] Both antagonists were able to counteract the stimulatory effect of NPS on locomotion being per se inactive. These findings demonstrate that this peptide action is due to NPSR activation and suggest that the endogenous system does not exert a tonic control on animal locomotor behavior. The brain areas and neurochemical mechanisms responsible for this NPS action are not known. Microinjection of NPS into the paraventricular nucleus[27] or amygdala[19] 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. In contrast, corticotrophin releasing factor signaling 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 no longer evident in $CRF_1(-/-)$ mice.[26]

Arousal and sleep: Electroencephalographic studies in rats demonstrated that i.c.v. injection of NPS reduced all stages of sleep promoting wakefulness.[5] Similar to the locomotor stimulant action in mice, this effect of NPS lasted for about 1 hr and can be evoked using low doses of peptide (0.1–1 nmol). These findings were later confirmed in a separate study.[29] The arousal promoting action of NPS has also been demonstrated using the righting

Table II. In Vivo Actions of NPS

Assay (route of administration)	NPS action	Effective dose (nmol)	Reference
Mouse locomotor activity (i.c.v.)	Stimulation of locomotor activity	0.1	[5]
Rat locomotor activity (i.c.v.)	Stimulation of locomotor activity	10	[27]
Rat plasma ACTH and corticosterone (i.c.v.)	Increase in plasma ACTH and corticosterone	0.1	[27]
Rat locomotor activity (iPVN)	Stimulation of rearing activity	0.1	[27]
Rat plasma ACTH and corticosterone (iPVN)	Increase in plasma ACTH and corticosterone	0.3	[27]
Mouse locomotor activity (i.c.v.)	Stimulation of locomotor activity	0.1	[7]
Mouse locomotor activity (i.c.v.)	Stimulation of locomotor activity	0.07	[22]
Mouse locomotor activity (i.c.v.)	Stimulation of locomotor activity	1	[24]
Mouse locomotor activity (i.c.v.)	Stimulation of locomotor activity	0.1	[23]
Mouse locomotor activity (i.c.v.)	Stimulation of locomotor activity	0.1	[25]
Mouse locomotor activity (i.c.v.)	Stimulation of locomotor activity	0.45	[26]
Mouse locomotor activity (iA)	Inactive	0.5	[19]
Rat EEG recording (i.c.v.)	Increase in wakefulness	1	[5]
Mouse righting reflex (i.c.v.)	Reduction of sleeping time	0.1	[23]
NPSR(+/+) mouse righting reflex (i.c.v.)	Reduction of sleeping time	1	[31]
NPSR(−/−) mouse righting reflex (i.c.v.)	Inactive	1	[31]
Rat righting reflex (i.c.v.)	Reduction of sleeping time	3	[30]
Mouse open field (i.c.v.)	Increase in center entries	0.1	[5]
Mouse light dark box (i.c.v.)	Increase in time in the light area	0.03	[5]
Mouse elevated plus maze (i.c.v.)	Increase in time on open arms	0.1	[5]
Mouse murble burying (i.c.v.)	Reduction of marbles buried	0.01	[5]
Mouse four plate (i.c.v.)	Increase in punished crossings	0.07	[22]
Mouse elevated zero maze (i.c.v.)	Increase in time on open zone	0.03	[22]
Mouse stress induced hyperthermia (i.c.v.)	Reduction of T_2–T_1	0.07	[22]
Mouse elevated plus maze (i.c.v.)	Increase in time on open arms	0.01	[23]
Mouse stress induced hyperthermia (i.c.v.)	Reduction of T_2–T_1	0.1	[23]
Mouse open field (iA)	Increase in center entries	0.5	[19]
Mouse elevated plus maze (iA)	Increase in time on open arms	0.5	[19]
Rat defensive burying (i.c.v.)	Reduction of burying behavior	1	[38]
Mouse light / dark box (i.c.v.)	Increase in time in the light area	0.45	[26]
Mouse murble burying (i.c.v.)	Reduction of marbles buried	0.45	[26]
Mouse tail suspension (i.c.v.)	Inactive	0.07	[22]
Mouse forced swimming (i.c.v.)	Inactive	1	Rizzi et al., unpublished
P and NP rat alcohol intake (i.c.v.)	Inhibition of alcohol intake in P but not NP rats	0.07	[45]
Mouse conditioned place preference (i.c.v.)	Inhibition of morphine conditioned place preference	1	[44]
Rat ethanol seeking (i.c.v.)	Increase of ethanol seeking	2	[46]

Table II. *Continued*

Assay (route of administration)	NPS action	Effective dose (nmol)	Reference
Rat ethanol seeking (iLH)	Increase of ethanol seeking	0.5	46
Mouse cocaine seeking (i.c.v.)	Increase of cocaine seeking	0.45	26
Rat food intake (i.c.v.)	Inhibition of food intake	0.3	47
Rat food intake (i.c.v.)	Slight inhibition of food intake	10	27
Rat food intake (iPVN)	Inhibition of food intake	0.1	27
Rat food intake (i.c.v.)	Inhibition of palatable food intake	0.1	48
Rat food intake (i.c.v.)	Stimulation of food intake	1	51
Chick food intake (i.c.v.)	Inhibition of food intake	0.4	49
Chick food intake (iLH or iPVN)	Inhibition of food intake	0.2	49
LWS and HWS chick food intake (i.c.v.)	Inhibition of food intake greater in LWS than HWS	0.56	50
Mouse conditioned fear behavior (iEPN)	Reduction of contextual freezing	0.1	18
Mouse conditioned fear behavior (iA)	Reduction of freezing; facilitation of fear extinction	0.5	19
Mouse Morris water maze (i.c.v.)	Increase in spatial memory	1	54
Mouse antinociceptive action (i.c.v.)	Antinociceptive effect in the TW and HP tests	0.03	55
Mouse colonic transit (i.c.v.)	Inhibition of bead expulsion time	0.01	56
Mouse colonic transit (i.p.)	inactive	10	56

EEG, electroencephalogram; iPVN, intra paraventricular nucleus; iLH, intra lateral hypothalamus; LWS, low body weight; HWS, high body weight; P, alcohol preferring; NP, alcohol nonpreferring; iEPN, intra endopiriform nucleus; iA, intra amygdala; $NPSR^{+/+}$, wild type mice; $NPSR^{-/-}$, mice knockout for the NPSR gene; TW, tail withdrawal; HP, hot plate.

reflex assay. In fact, in mice treated with a hypnotic dose of diazepam (15 mg/kg) NPS dose-dependently (0.01–1 nmol) reduced the proportion of animals losing the righting reflex in response to the benzodiazepine and their sleep time.[23] Similar results were also found in rats where NPS injected i.c.v. dose-dependently reduced ketamine-induced anesthesia time.[30] The following evidence demonstrated that the arousal promoting action of NPS is due to selective NPSR activation: first, the NPSR peptide antagonist [D-Cys(tBu)^5]NPS dose-dependently (1–10 nmol) counteracted the action of NPS in the mouse righting reflex assay;[31] second, in the same assay, NPS reduced sleep time induced by diazepam in NPSR(+/+) but not in NPSR(−/−) mice.[31] Interestingly, [D-Cys(tBu)^5]NPS did not modify per se the hypnotic effect of diazepam and NPSR(+/+) and NPSR(−/−) animals did not show any phenotype difference in the righting reflex assay.[31] These findings indicate that the endogenous NPS–NPSR system is not tonically activated to maintain wakefulness (at least under these experimental conditions); this is at variance to the peptidergic arousal promoting orexin system.[32] In fact, orexin receptor antagonists prolonged barbiturate sleep time in rats[33] and emergence from general anesthesia,[34] and are also able per se to promote sleep in rats, dogs, and humans.[35] Clearly further studies are needed to investigate the role of the endogenous 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.[11] In particular, the thalamic midline nuclei, which integrate the arousal circuit reticular formation-thalamus-cortex, express high levels of NPSR mRNA. Micro-injection and electrophysiological studies are now needed to establish the role of the above mentioned brain structures in the arousal promoting actions of NPS. Interestingly, a recent genetic epidemiology study performed on 749 subjects found a clear association between the Asn/Ile 107 NPSR polymorphism and mean bedtime delay.[36] 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: Supraspinal administration of NPS in the 0.01–1 nmol range evoked clear anxiolytic-like effects in mice subjected to a battery of validated assays including the elevated plus maze, the light-dark box, and the open field.[5] These initial observations were later confirmed (elevated plus maze,[23] light-dark box[26]) and extended to other assays such as the four-plate test and elevated zero maze.[22] 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 behaviors. However, the following evidence converge in suggesting that the anxiolytic-like action of NPS is indeed a genuine effect: (i) NPS reduces in a dose dependent manner the number of marbles buried in the marble burying test,[5,26] a model which is not biased by locomotion since anxiety levels are measured as an active behavior; (ii) two separate studies[22,23] demonstrated that similar anxiolytic-like effects can be measured in response to NPS in mice in the stress-induced hyperthermia test. This assay measures a physiological parameter poorly sensitive to locomotion;[37] (iii) recently, the anxiolytic-like effects of NPS were confirmed and extended to a different species, the rat, and a different assay, defensive burying.[38] It was demonstrated that NPS (0.1–10 nmol) reduces cumulative burying behavior in a dose-dependent manner without modifying other parameters including latency to contact the probe, burying behavior latency, number of shocks received, or immobility/freezing duration.[38] Again, since the main parameter predictive of anxiolysis in this assay is the inhibition of an active defensive behavior, motor activity can be considered a minor bias in the outcome of the defensive burying test.[39]

Interestingly, the effect of NPS on emotional behavior seems to be restricted to anxiety since the peptide was found inactive in tests such as the tail suspension[22] or the forced swimming (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 behaviors in a rat genetic model of depression. In fact, in flinders sensitive rats, NPS decreased depression-like behavior in the forced swimming test and anxiety-related behavior on the elevated plus-maze in a dose-dependent manner (0.05–1 nmol, i.c.v.). In contrast, NPS did not alter the behavior of flinders resistant animals.[40] Clearly, further studies are needed to define the possible role of the NPS/NPSR system in mood regulation.

As far as brain areas implicated in the action of NPS on stress and anxiety are concerned, the study by Xu et al.[11] demonstrated that NPSR mRNA is expressed in various stress-related regions, including the amygdala, bed nucleus of the stria terminalis, hypothalamus, raphe nucleus, and ventral tegmental area. The presynaptic inhibitory effects exerted by NPS on the release of 5-HT and NA from the cortex[20] could be involved in the anxiolytic-like effects of this peptide. The recent findings by Jungling et al.[19] indicate, however, that the amygdala could be the brain area crucial for NPS-mediated anxiolytic-like actions. In fact, intra amygdala injection of NPS promoted clear anxiolytic-like effects in the mouse elevated plus maze and open field assays, mimicking the effects of the peptide given i.c.v.[5]

In contrast to locomotor activity and arousal, the endogenous NPS/NPSR system might tonically control anxiety levels since anxiogenic effects were observed in mice subjected to the open field test in response to intra amygdala injection of the NPSR antagonist SHA 68.[19] Furthermore, preliminary findings indicate that NPSR(−/−) mice display increased levels of defensive behaviors compared to their NPSR(+/+) littermates in the light-dark box, elevated plus maze and open field assays.[41] These initial indications however need 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(−/−)[42] and possibly ppNPS(−/−) (which are not yet described in the literature) mice in different assays predictive of anxiety.

Thus, the initial findings by Xu et al.[5] were replicated and extended in different laboratories confirming that over the same dose range NPS promotes stimulation of locomotor activity and arousal associated to a genuine anxiolytic-like action. Thus, the proposal of NPS as an activating anxiolytic,[5,6] is confirmed after five 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 that shares this behavioral profile with NPS is nicotine, which increases arousal and wakefulness and produces, at least in smokers, anxiolysis and anti-stress effects.[6] Interestingly chronic nicotine treatment in rats increases both NPS and NPSR expression in the brainstem and NPSR in the hypothalamus.[43] 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 rewarding 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 the rewarding activities of morphine.[44]

The effects of NPS were also evaluated on ethanol drinking in alcohol-preferring and nonpreferring rats.[45] NPS given i.c.v. reduced ethanol intake in alcohol-preferring, but not in nonpreferring rats. The peptide neither altered anxiety-like behavior in the elevated plus maze test nor modified general motor activity. However, there was an increase in the amount of time spent in the center of the activity monitors following infusions of 0.6 nmol of NPS in preferring, but not in nonpreferring 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.[45]

Very recently two complimentary studies investigated the role of NPS in drug seeking behavior.[26,46] Cocaine-seeking behavior was evaluated in mice by Paneda et al.[26] It was demonstrated that i.c.v. NPS reinstated 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.[26] Ethanol seeking behavior was investigated in rats by Cannella et al.[46] 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.

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 pretreatment 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.[46] Collectively, these pivotal studies suggest that the NPS/NPSR system may be an important target for drug abuse research.

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.[47] 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.[47] This latter finding was independently replicated in a different laboratory.[48] Smith et al.[27] later demonstrated that the injection of low NPS doses in the paraventricular nucleus produces robust anorexigenic effects. The observations in rats were confirmed in chicks where NPS inhibited food intake when both injected i.c.v. and in the paraventricular nucleus or lateral hypothalamus.[49] In a follow-up study[50] the same authors demonstrated that chicken 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. While the above mentioned studies converge indicating that NPS reduces food intake, the report by Niimi[51] produced the 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 in the actions of NPS on food intake.

The mechanism by which NPS regulates food intake is at present unknown. The only available data suggesting that the NPSR is involved in the anorectic action of NPS are those described by Ciccocioppo et al.[48] who demonstrated that the NPSR partial agonist [Ala^3]NPS7 counteracts the inhibitory effect of NPS on palatable food intake in rats. Receptor antagonist and knockout studies are needed to confirm this evidence and more importantly to shed light on the role of the endogenous NPS/NPSR system in the regulation of feeding behavior.

Interestingly caffeine shares with NPS the anorectic, arousal promoting, and locomotor stimulant effects (but produces the opposite effects on anxiety; see[23,38] for parallel studies on the behavioral actions of caffeine and NPS). It has been reported that acute and chronic administration of caffeine can regulate the expression of NPS and NPSR in the rat hypothalamus and brainstem.[52]

Learning and memory: Recent findings implicate the NPS/NPSR system in the regulation of memory processes. In fact, local injection of NPS to the endopiriform nucleus interferes with the expression of contextual, but not auditory cued fear memory in mice.[18] On the other hand, NPS facilitates extinction of conditioned fear responses when administered into the amygdala in mice.[19] Moreover, the injection of the NPSR antagonist SHA 68, under the same experimental conditions, produced opposite effects increasing the freezing response of mice to the conditioned stimulus. These results demonstrated that the endogenous NPS/NPSR system in the amygdala plays a major role in facilitating extinction of aversive memories.[19] In addition, preliminary data from Reinscheid group demonstrated that post-training, but not pretraining, i.c.v. injection of NPS enhanced memory formation in a time- and dose-dependent manner in mice subjected to the step-through inhibitory avoidance test. Furthermore, NPSR(−/−) mice displayed significant deficits in this assay. In addition, NPS

also improved cognitive performance in the novel object recognition task.[41,53] Finally, very recent findings[54] demonstrated that supraspinally injected NPS facilitated spatial memory in mice subjected to the Morris water maze test. Moreover, NPS was able to partially counteract the memory impairment induced by the selective *N*-methyl-D-aspartate receptor antagonist dizocilpine.

Very recently, the group of Prof. R. Wang investigated novel actions elicited by NPS after supraspinal administration in mice. Using the tail withdrawal and hot-plate test they demonstrated that NPS (0.01–1 nmol, i.c.v.) caused a dose-dependent antinociceptive effect.[55] In both the assays, the action of NPS (0.1 nmol) was not affected by naloxone while sensitive to the peptide antagonist [D-Cys(tBu)^5]NPS (3 and 10 nmol). The NPSR antagonist given alone did not modify nociceptive transmission.[55] These results revealed that NPS could produce antinociceptive effects through NPSR and that opioid systems are not involved in this action.

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.[56] 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.[56]

In conclusion there is convincing evidence in the literature that the NPS/NPSR system regulates multiple biological functions including locomotor activity, arousal and wakefulness, stress and anxiety, food intake and gastrointestinal functions, drug addiction, memory processes, and possibly pain transmission. Our knowledge in this field was however limited in the past by the lack of selective antagonists for the NPSR. The next two sections will describe in detail the studies that led to the identification of the first generation of peptide and nonpeptide NPSR antagonists. Future studies performed with these molecules will be of paramount importance for increasing our knowledge on this peptidergic system and for firmly identifying the therapeutic potential of selective NPSR ligands.

4. 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.[7,14,57] 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 Ca^{2+} mobilization assay using HEK293 cells expressing the hNPSR receptor by Roth et al.[7] 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.[57] 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.[7] 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 potent 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.[7]

N- and *C*-terminal truncation studies[7,14,57] 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^1 produced a moderate decrease in peptide potency while further deletion of Phe^2 generated an inactive analog.[7,57] 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.[7,14,57] Further, *C*-terminal shortening of the NPS sequence produced different results with Roth et al.[7] reporting loss of activity while Bernier et al.[57] 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.[7] 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.

In the context of these studies [Ala^3]NPS was identified as the first NPSR ligand with reduced efficacy.[7] As mentioned before, [Ala^3]NPS was tested in vivo in rats in palatable food intake experiments. In this assay NPS given i.c.v. at 0.1, 0.3, and 1 nmol elicited a dose-dependent inhibition of palatable food intake. [Ala^3]NPS did not modify palatable food intake at 30 and 60 nmol i.c.v.; however, at the highest dose it fully prevented the inhibitory effect of 1 nmol NPS. Thus, in this assay [Ala^3]NPS behaves as a pure NPSR antagonist.[48] [Ala^3]NPS was further investigated in vivo in mice in locomotor activity and righting reflex experiments performed under the experimental conditions and using the protocols previously described in detail.[23] In mice habituated to the test chamber for 1 hr prior to the i.c.v. injection, NPS produced a dose-dependent (0.01–1 nmol) stimulatory effect on locomotor activity, the first dose producing a statistically significant effect was 0.1 nmol.[23] This NPS dose was selected to be challenged with [Ala^3]NPS at 1 and 10 nmol. As shown in Figure 1, in habituated mice the i.c.v. injection of 1 (top panels) or 10 (bottom panels) nmol [Ala^3]NPS produced a stimulatory effect on locomotor activity equal to approximately half of that evoked by 0.1 nmol of NPS. However, in both cases, this effect did not reach the level of statistical significance. When [Ala^3]NPS was coinjected with NPS, the resultant effect was similar to that evoked by [Ala^3]NPS alone (Fig. 1).

Similar experiments were performed in mice sedated with diazepam. Pretreating mice with 5 mg/kg diazepam elicited a virtual suppression of locomotor activity (Fig. 2), although these animals did not completely lose the righting reflex. Under these experimental conditions, NPS produced a dose-dependent stimulatory effect.[23] The i.c.v. injection of 1 (top panels) or 10 (bottom panels) nmol [Ala^3]NPS did not produce statistically significant effects. However, the peptide at 10 nmol completely prevented the stimulatory effect evoked by 0.1 nmol NPS (Fig. 2, bottom panels). Thus, in locomotor activity experiments [Ala^3]NPS displayed some effects per se associated with the ability to counteract the hyperlocomotor action of NPS; in other words [Ala^3]NPS behaves as a partial agonist in this assay.

Finally, [Ala^3]NPS was assessed in the righting reflex test. In this assay NPS dose-dependently reduces the number of animals losing the righting reflex in response to the

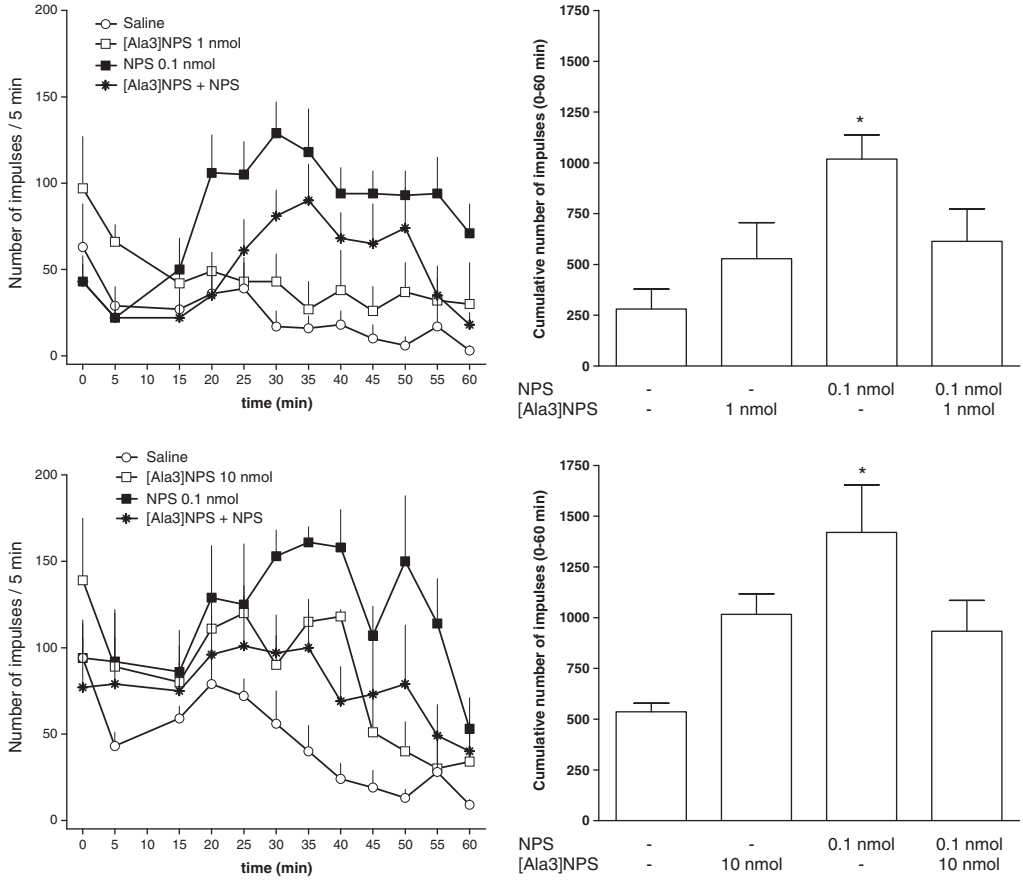

Figure 1. Effects of NPS and [Ala^3]NPS given i.c.v. on locomotor activity in mice habituated to the test chamber. Locomotor activity of mice is displayed over the time course of the experiment in the left panels and as cumulative impulses over the 60 min observation period in the right panels. Data are mean ± SEM of 16 mice per group. $^*P < 0.05$ vs. control, according to one way analysis of variance followed by the Dunnett's test for multiple comparison.

hypnotic dose of 15 mg/kg of diazepam and their sleep time.[23] As shown in Figure 3, [Ala^3]NPS given i.c.v. in the dose range 0.1–10 nmol mimicked the arousal promoting action of NPS, reducing at the dose of 10 nmol the percent of animals losing the righting reflex in response to diazepam and their sleep time in a statistically significant manner.

In a separate series of experiments [Ala^3]NPS 1 nmol was tested against NPS 0.1 nmol. The peptide did not modify the arousal promoting action of NPS (data not shown). Thus, in the righting reflex assay only NPSR agonist properties were evident using [Ala^3]NPS. Collectively these studies demonstrated that in vivo in various assays [Ala^3]NPS displayed different pharmacological activities ranging from pure antagonism (rat food intake) to partial (mouse locomotor activity) or full (mouse righting reflex) agonism. Different pharmacological behaviors of low efficacy agonists dependent on the different assay or biological function under study are a relatively common finding.[58] In our experience we may mention in this regard the findings obtained with the nociceptin/orphanin FQ receptor partial agonist [Phe$^1\psi$(CH$_2$-NH)Gly^2]N/OFQ(1-13)-NH$_2$[59]or with the urotensin-II receptor ligand [Orn^8]U-II.[60] Both peptides behaved as full agonists in recombinant systems while they showed partial agonist or even pure antagonist activities in isolated tissues and in in vivo assays.[60–63] Independently from this variable efficacy, [Ala^3]NPS displayed in vivo values of

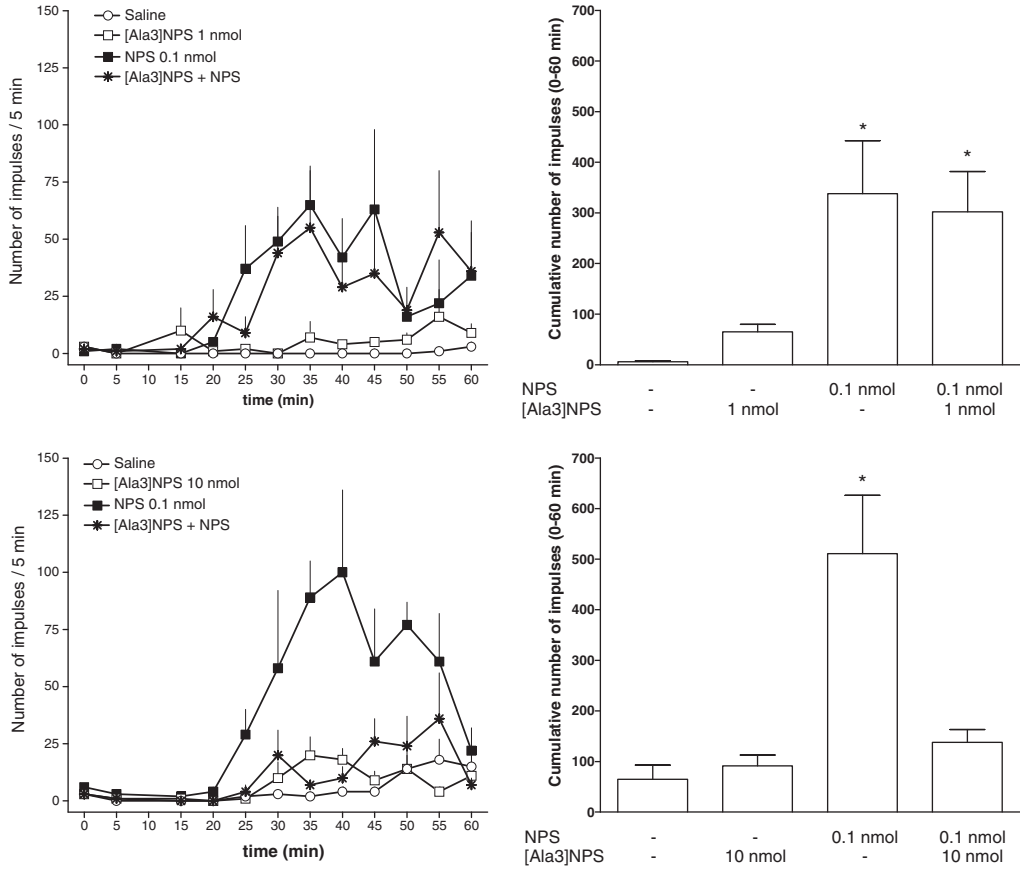

Figure 2. Effects of NPS and [Ala^3]NPS given i.c.v. on locomotor activity in mice treated with diazepam 5 mg/kg. Locomotor activity of mice is displayed over the time course of the experiment in the left panels and as cumulative impulses over the 60 min observation period in the right panels. Data are mean ± SEM of 16 mice per group. $^*P < 0.05$ vs. control, according to one way analysis of variance followed by the Dunnett's test for multiple comparison.

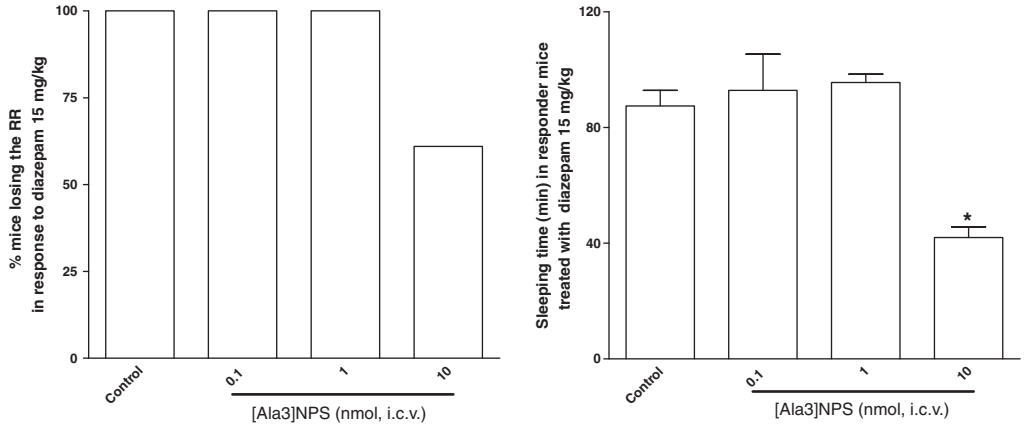

Figure 3. Dose–response curve to [Ala^3]NPS in the righting reflex assay in mice. The left panel displays percentage of animals losing of the righting reflex in response to diazepam 15 mg/kg i.p. while the right panel displays their sleeping time. Sleeping time is defined as the amount of time between the loss and regaining of the righting reflex. Data are mean ± SEM of 16 mice per group. $^*P < 0.05$ vs. control, according to one way analysis of variance followed by the Dunnett's test for multiple comparison.

potency that match in vitro results. In fact, the concentration ratio between [Ala^3]NPS and NPS in vitro was approximately 10 and consistent in vivo effects with this peptide (either as NPSR agonist or antagonist) were obtained in the dose ratio 60–100. This suggests that the in vivo actions of both NPS and [Ala^3]NPS likely derive from their ability to interact with the NPSR protein. The in vitro and in vivo results obtained with [Ala^3]NPS are summarized in Table III.

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.[64] However, several NH-NH cross peaks were observed by Bernier et al.[57] 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 favor the formation of a stable α helix structure.

On the other hand, disordered NPS conformations were observed in different NMR solvent mixtures that may favor helicity.[64] This NPS behavior 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 α 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.[64] 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.[64] 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 centered 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.[64] This suggests that an α helix conformation (favored 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.[64] 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 favor 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.

Based on the findings obtained in these pivotal conformation and structure–activity studies, focused studies were performed for investigating the detailed chemical features of NPS position 2, 3, 4, and 5 for biological activity.

Phe^2 was replaced with ten different natural amino acids.[65] Among them, Tyr, Trp, and Leu produced NPS derivatives with potency comparable with the reference peptide; [His^2]NPS behaved as a low potency NPSR ligand while other amino acids characterized by hydrophilic basic, acid, or neutral side chains produced peptides with strongly reduced

Table III. Summary of In Vitro and In Vivo NPSR Antagonist Studies

Preparation/ assay	NPS effect (pEC_{50} or dose)	Compound	Antagonist action (pEC_{50}/pA_2 or dose)	Reference
HEK293-hNPSR	Stimulation of $[Ca^{2+}]i$ mobilization (pEC_{50} 7.9)	$[Ala^3]$NPS	Partial agonist ($\alpha = 0.49$, pEC_{50} 6.9)	[7]
Mouse locomotor activity	Stimulation of locomotor activity (0.1 nmol, i.c.v.)	$[Ala^3]$NPS	Counteracts NPS stimulatory effects showing residual agonist activity (10 nmol, i.c.v.)	Present article
Mouse righting reflex	Reduction of sleeping time (0.1 nmol, i.c.v.)	$[Ala^3]$NPS	Mimicks NPS effects (10 nmol)	Present article
Rat food intake	Reduction of palatable food intake (1 nmol, i.c.v.)	$[Ala^3]$NPS	Antagonizes NPS effects with no residual agonist activity (60 nmol, i.c.v.)	[48]
HEK293-mNPSR	Stimulation of $[Ca^{2+}]i$ mobilization (pEC_{50} 8.9)	[D-Cys(tBu)^5]-NPS	Competitive antagonist (pA_2 6.4)	[31]
Mouse righting reflex	Reduction of sleeping time (0.1 nmol, i.c.v.)	[D-Cys(tBu)^5]-NPS	Antagonizes NPS effects, no effects per se (10 nmol, i.c.v.)	[31]
Mouse TW and HP tests	Antinociceptive action (0.1 nmol, i.c.v.)	[D-Cys(tBu)^5]-NPS	Antagonizes NPS effects, no effects per se (10 nmol, i.c.v.)	[55]
HEK293-mNPSR	Stimulation of $[Ca^{2+}]i$ mobilization (pEC_{50} 8.6)	[D-Val^5]NPS	Competitive antagonist (pA_2 7.0)	[28]
Mouse locomotor activity	Stimulation of locomotor activity (0.1 nmol, i.c.v.)	[D-Val^5]NPS	Antagonizes NPS effects, no effects per se but tendency to reduce rearings (10 nmol, i.c.v.)	[28]
Mouse colonic transit	Inhibition of bead expulsion time (0.01 nmol, i.c.v.)	[D-Val^5]NPS	Antagonizes NPS effects, no effects per se (1 nmol, i.c.v.)	[56]
HEK293-hNPSR Asn107	Stimulation of $[Ca^{2+}]i$ mobilization ($pEC_{50} \approx 7.6$)	SHA 68	Competitive antagonist (pA_2 7.8)	[24]
HEK293-hNPSR Ile107	Stimulation of $[Ca^{2+}]i$ mobilization ($pEC_{50} \approx 8.6$)	SHA 68	Competitive antagonist (pA_2 7.6)	[24]
HEK293-mNPSR	Stimulation of $[Ca^{2+}]i$ mobilization (pEC_{50} 8.3)	SHA 68	Competitive antagonist (pA_2 8.0)	Camarda et al., unpublished

Table III. *Continued*

Preparation/ assay	NPS effect (pEC_{50} or dose)	Compound	Antagonist action (pEC_{50}/pA_2 or dose)	Reference
Mouse locomotor activity	Stimulation of locomotor activity (1 nmol, i.c.v.)	SHA 68	Antagonizes NPS effects, no effects per se but tendency to reduce rearings (50 mg/kg, i.p.)	[24]
Mouse open field	Increase in center entries (0.5 nmol, iA)	SHA 68	Reduction of center entries (2 nmol, iA)	[19]
Mouse conditioned fear behavior	Reduction of freezing (0.5 nmol, iA)	SHA 68	Increase in freezing (2 nmol, iA)	[19]

iA, intra amygdala; TW, tail withdrawal; HP, hot plate.

potency. This first series of [Xaa^2]NPS analogues suggested lipophilicity in this position is a critical chemical requirement for NPSR interaction. Moreover, none of the amino acids investigated produced an increase of peptide potency; thus, the aromatic benzyl moiety of Phe^2 seems to be the most effective pharmacophore in promoting NPSR binding. To investigate in detail the chemical requirements of the Phe^2 benzyl moiety, a second round of [Xaa^2]NPS analogues was generated modifying the side chain electronic asset, length, size, and conformational freedom. The replacement of Phe with the parent nonaromatic amino acid cyclohexylalanine (Cha) is fully tolerated. This result, together with the data obtained for [Leu^2]NPS, confirmed that aromaticity in position 2 is not crucial for NPSR interaction. Introduction of halogen atoms or small chemical groups with electron donor or withdrawing properties in para position of the phenyl ring seems to be also well tolerated, producing NPS analogues with potency and efficacy superimposable to that of the natural sequence. Increase of Phe^2 side chain length by one carbon atom produced a fairly potent NPS analogue while its shortening or shift with respect to the peptide backbone was detrimental for biological activity. The insertion in position 2 of constrained Phe analogues such as Tic, Aic, and Atc produced peptides with 10- to 30-fold reduced potency. Biological effects induced by increasing Phe^2 side chain size were also investigated. The combination of two phenyl rings having two common carbon atoms as in 1Nal and 2Nal produced NPS derivatives about 3-fold less potent, while the insertion of a phenyl ring in para position of the benzyl moiety as in [Bip^2]NPS produced a 10-fold reduction of peptide potency associated with a statistically significant reduction in peptide efficacy. Finally, the addition of a further phenyl ring on the Cβ Phe^2 carbon atom generated a compound more than 1,000-fold less potent than the parent natural peptide.

Collectively, the results obtained substituting NPS position 2 suggest that the NPSR ligand-binding pocket harboring the Phe side chain does not show particularly stringent electronic and/or steric chemical requirements. Moreover, the statistically significant reduction in efficacy showed by [Bip^2]NPS suggests an important role for the correct position of the Phe^2 side chain to access the corresponding NPSR ligand binding pocket not only for receptor binding but also for its activation.

To investigate the structure–activity relationships of NPS position 3 and 4, 38 NPS analogues were synthesized using natural and non-natural amino acids.[66] Replacement of Arg^3 with natural amino acids characterized by an aromatic or aliphatic side chain in both

cases produced a dramatic reduction in peptide potency. The best compound of this series, [His^3]NPS, was 100-fold less potent than the natural peptide. The substitution of position 3 with hydrophilic neutral, basic, or acidic residues produced low potency or inactive NPS analogues. Among these, only [Lys^3]NPS displayed a potency value similar to that of NPS. Interestingly, [Gln^3]NPS was 10-fold less potent than NPS while [Asn^3]NPS, which maintains an amide side chain but with one less carbon atom, was found to be inactive. A similar dramatic loss of potency was observed with [Glu^3]NPS and [Asp^3]NPS. This series of data, obtained substituting Arg^3 with natural amino acids, suggested that position 3 of NPS does not tolerate substitutions with aromatic and aliphatic branched residues. Moreover, an amino acid characterized by a linear 3 carbon atom side chain seems to be needed for binding to the respective NPSR pocket that recognizes the Arg^3 side chain. In addition, this same pocket does not tolerate an acidic side chain as demonstrated by the lack of activity of [Glu^3]NPS. Importantly, data obtained by replacement Arg/Gln suggested that basicity in position 3 is not crucial for bioactivity. Further investigations performed using non-natural amino acids with side chains chemically related to that of Arg indicated that for NPSR binding (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 i.e. hArg, Arg(NO_2) or Arg($Me)_2$ but not Arg(Tos).

Position 4 was investigated substituting Asn with a limited series of natural and non-natural amino acids. The introduction in this position of amino acids with acidic or aromatic side chains produced inactive derivatives.[64,66] A 10-fold reduction of potency has been obtained by replacing Asn with Thr while Gln produced a larger decrease in biological activity (55-fold), suggesting an important role of both the length and the amide moiety of the Asn side chain. To investigate in detail the contribution of the primary amide function of Asn^4 to biological activity, we substituted either alone or in combination the C=O and $-NH_2$ groups of the amide function with $-CH_2$ and $-CH_3$, respectively. A profound loss of biological activity (>1,000-fold) was observed with all these chemical modifications. Similar negative results were also obtained by mono- or dimethylation of the amide function. These results, together with indications coming from previous Ala- and D-amino acid scan studies,[7,57] highlight the importance of Asn^4 for NPS biological activity and suggested quite strict chemical requirements of the NPSR binding pocket allocating the side chain of this residue.

The reduction in efficacy obtained by replacement of Gly^5 with D-Ala^{64} suggests that this position could be instrumental for the identification of NPSR low efficacy ligands and possibly pure antagonists. In a first explorative study, Gly^5 was substituted with natural amino acids characterized by hydrophobic aromatic or aliphatic side chains (Phe, Trp, Leu, Val, Met, and Cys) and with their D enantiomers.[28] Hydrophobic aromatic side chains (Phe, Trp) produced a drastic decrease (>300-fold) in peptide potency. In contrast, natural amino acids with hydrophobic aliphatic side chains (Leu, Val, Met, and Cys) 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. To investigate the possible role of chirality in this position, the D enantiomers of the same amino acids were used. Substitution of Gly^5 with D amino acids with hydrophobic aromatic side chains produced a complete elimination of efficacy and, as in the case of their L enantiomers, an important reduction (approximately 100-fold) in potency. Replacement of Gly^5 with D amino acids with hydrophobic aliphatic side chains 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 potency. These results clearly indicate that the insertion of a Cα chiral carbon with relative D-configuration in NPS position

5 produces, depending on the chemical features of the side chain, an important reduction in efficacy or its total elimination. Independently from their efficacy, there was a good match between the rank order of potency of [L-Xaa^5]NPS and [D-Xaa^5]NPS derivatives. This suggests that the amino acid side chain size of Xaa^5 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. These peptides behaved as pure antagonists with [D-Cys(tBu)^5]NPS displaying the highest potency.

Thus this study allowed identification of the first generation of NPSR peptide antagonists. The [D-Cys(tBu)^5]NPS and [D-Val^5]NPS NPSR antagonist properties were confirmed in in vivo studies. In particular, [D-Cys(tBu)^5]NPS antagonized the arousal promoting[31] and antinociceptive effect[55] of supraspinal NPS and [D-Val^5]NPS antagonized its locomotor stimulant action[28] and inhibitory effect on colonic transit[56] (Table III).

In a follow-up study, to further investigate chemical requirements of the NPS D-Xaa^5 side chain that are critical in generating NPSR antagonism, 11 novel peptides were synthesized and characterized.[67] In this study [D-Val^5]NPS was used as a reference NPSR antagonist. Replacement of the isopropyl group with a sec-butyl group (D-Ile or D-allo-Ile) produced a moderate reduction of potency independently from the side chain chiral center. Similar results were obtained substituting a methyl of the isopropyl group of D-Val with an oxydril function (D-Thr or D-allo-Thr). A linear three carbon atom side chain (D-Nva) produced an analogue that behaved as a NPSR low efficacy partial agonist 10-fold less potent than [D-Val^5]NPS. Collectively, these findings indicate that the isopropyl moiety of the D-Val side chain is highly important for NPSR antagonist binding. The introduction in position 5 of cyclohexyl or methyl-cyclohexyl moieties (cyclohexyl-D-Gly and D-Cha) generated inactive derivatives while the introduction of a phenyl ring (D-Phg) produced only a 3-fold reduction in potency compared to [D-Val^5]NPS. Confirming previous findings[28] these data suggest that the increase in the side chain size has a detrimental effect on peptide antagonist potency. Next, the effects of insertion of a CH_3 (tBu-D-Gly) or SH (D-Pen) group in the D-Val^5 isopropyl moiety was evaluated. In both cases the chemical modification did not modify pharmacological activity of the peptides i.e. they behaved as pure antagonists, but produced a 3-fold increase in potency. Thus, [tBu-D-Gly^5]NPS and [D-Pen^5]NPS are the most potent NPSR peptide antagonists so far identified. Finally, the insertion of a carbon atom between the tBu moiety and the peptide backbone (tBu-D-Ala) caused an important reduction of peptide potency associated with a clear increase in efficacy. Similar results were obtained with [D-Val^5]NPS and [D-Leu^5]NPS.[28] Collectively, these findings indicate that a short side chain favors high potency and pure receptor antagonism. In addition, comparison of the effects of the side chain structures (tBu and isopropyl) clearly indicates that aliphatic branched moieties are better recognized by the NPSR. The best results are obtained with the tBu moiety in which substitution of a CH_3 with SH did not change biological activity.

The most important chemical modifications applied in NPS position 5 that were instrumental for the identification of NPSR peptide antagonists are displayed in Figure 4.

Collectively structure–activity studies on Gly^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 favoring agonist and D amino acids inducing antagonist bioactive conformations.

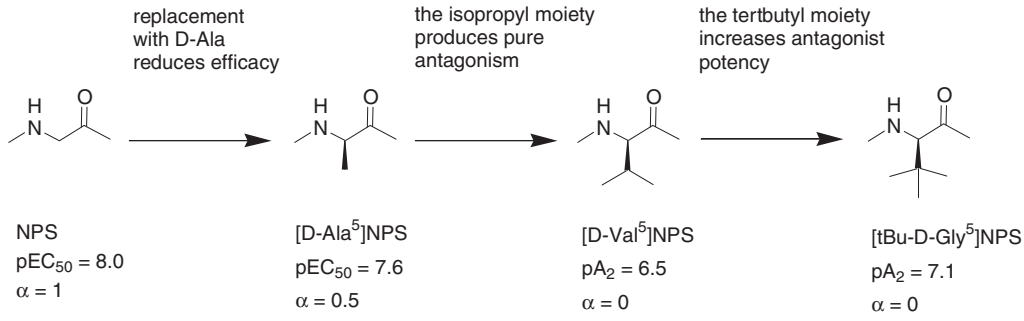

Figure 4. Chemical modifications of NPS Gly[5]: critical steps toward the identification of NPSR peptide antagonists.

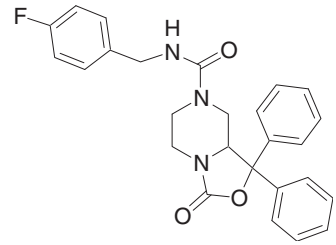

Figure 5. Chemical structure of the nonpeptide NPSR antagonist SHA 68.

5. NPSR NONPEPTIDE LIGANDS

The first example of nonpeptide molecules able to interact with the NPSR was reported in the patent literature by Takeda researchers.[68] 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 (see Fig. 5) i.e. the racemic mixture (9*R*/*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.[68]) has been selected by Okamura et al.[24] 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 Ile107 (7.6) and Asn107 (7.8) receptor isoforms. Moreover, superimposable results (pA_2 8.0, Camarda et al., unpublished) were obtained in our laboratories using mNPSR expressing cells (see Table III). SHA 68 appears to be selective for NPSR since it did not affect signaling at 14 NPSR unrelated G-protein coupled receptors.[24] 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.[24] In a separate study SHA 68 given into the amygdala exerted functionally opposing responses compared to NPS.[19] Thus, available data (see Table III) demonstrated that SHA 68 behaves as a potent and selective NPSR pure antagonist.

Structure–activity studies performed on position 7 of SHA 68[69] 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 α-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.[24,69]

6. CONCLUSIONS

In the present article we summarized the biological features of the novel peptidergic system NPS/NPSR. Most of the studies performed to date analyzed the biological actions of exogenously applied NPS. Thus, little is known about control, by the endogenous system, of these biological functions. This may likely change in the near future since NPSR(−/−) mice are now available[42] and the first generation of NPSR antagonists has been identified.[24,28,31,67] These are both nonpeptide and peptide molecules. SHA 68 is the first nonpeptide antagonist selective for the NPSR. Although SHA 68 is currently the only NPSR nonpeptide ligand reported in literature it is likely that novel molecules will be soon identified since screening campaigns on NPSR are underway in industrial laboratories.[70]

Peptide NPSR antagonists were identified in the context of the series of structure–activity studies summarized in Figure 6.

Classical peptide structure–activity studies i.e. Ala- and D-scan together with *N*- and *C*-terminal truncation allowed identification of the *N*-terminus of NPS as the bioactive core of the molecule. In particular, the sequence Phe^2-Arg^3-Asn^4 may act as message domain crucial for receptor binding and activation while the sequence Gly^5-Val^6-Gly^7 may have importance in shaping the bioactive conformation of the peptide. Focused structure–activity studies on Phe^2, Arg^3, and Asn^4 revealed the chemical characteristics needed for high potency binding to NPSR. Conformation activity studies demonstrated that helicity is not required for bioactivity rather it is not tolerated around position 7. In the context of this conformation activity study it has been observed that [D-Ala^5]NPS behaved as a NPSR partial agonist. This finding was critical in the identification of NPSR peptide antagonists. In fact, subsequent structure–activity studies focused on Gly^5 demonstrated that its substitution with D-amino acids with a short lipophilic-branched side chain generates fairly potent, pure, and selective NPSR antagonists.

Thus, after five years of research we may count on the following knowledge and tools instrumental for investigating this novel peptidergic system: (i) a raw pharmacophoric model of NPS possibly useful for the design of novel NPSR peptide ligands, (ii) moderate potency peptide antagonists, (iii) a potent nonpeptide antagonist, (iv) NPSR(−/−) mice. For performing more detailed and systematic studies in this field, other tools and knowledge need now to be identified and developed. These include (i) an NPSR molecular model useful for ligand/receptor docking studies that may give indication for the rationale design of novel ligands, (ii) more potent peptide as well as chemically unrelated nonpeptide antagonists, (iii) selective and potent synthetic agonists possibly of nonpeptide structure and able to elicit their effects after peripheral administration, (iv) an in vitro NPS sensitive pharmacological preparation expressing native NPSR, (v) ppNPS(−/−) animals. The availability of these tools will allow investigating in detail which biological functions and how they are controlled by the NPS/NPSR system and the neurobiological mechanisms of NPS actions. Collectively, this information will permit the therapeutic potential of novel NPSR ligands as innovative drugs to be explored. From the limited information currently available it is possible to suggest that NPSR agonists (and in some instances partial agonists) may be useful in the treatment of sleep, anxiety states, memory, and possibly food intake disorders, while NPSR antagonists might represent novel therapeutics for

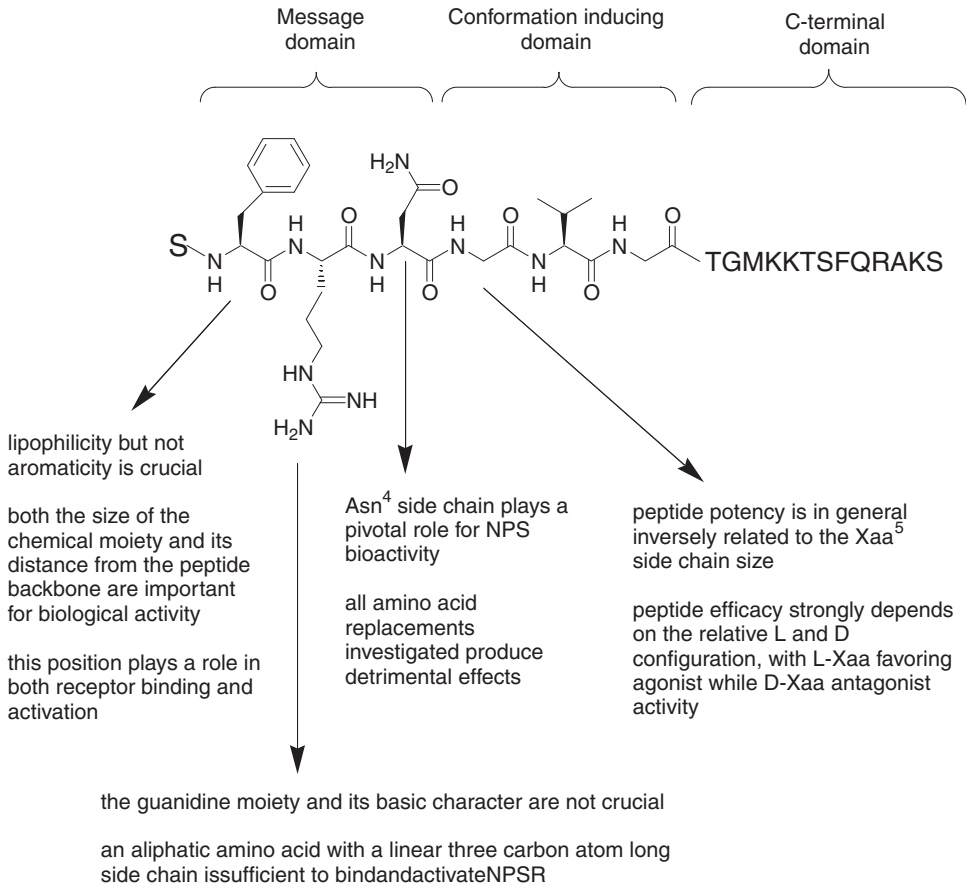

Figure 6. Summary of structure–activity studies on NPS. The message domain (Phe^2-Arg^3-Asn^4) is crucial for NPSR binding and activation. The conformation inducing domain (Gly^5-Val^6-Gly^7) plays a critical role in shaping the bioactive conformations of NPS: changing the chirality of the Cα carbon of position 5 has huge impact on peptide efficacy. The *C*-terminal domain is required for in vivo biological activity.

the control of drug addiction. However, before the first clinical trials with NPSR selective ligands can be planned, several physiological and pharmacological studies should be performed to firmly define the therapeutic potential of the NPS/NPSR receptor system and medicinal chemistry efforts should be successful for the identification of drug-like NPSR selective molecules.

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