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Neuropeptide S: Anatomy, Pharmacology, Genetics and Physiological Functions

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Abstract Neuropeptide S (NPS) is one of the most recent examples of a neurotransmitter identified by the orphan receptor strategy. Impressive progress has been made in the short time since its identification to determine physiological functions modulated by NPS. The anatomical distribution of NPS and its receptor, NPSR, suggests possible functions in the regulation of vigilance states and modulation of emotional behaviors. Early studies provided evidence that NPS induces behavioral arousal and promotes wakefulness by suppressing all stages of sleep. NPS was also found to produce anxiolytic-like effects in behavioral paradigms that measure fear or responses to novelty. Recent studies have demonstrated that NPS can modulate energy and endocrine homeostasis. Differential regulation of NPS and NPSR transcripts was observed after caffeine or nicotine treatment, indicating complex interactions with adenosine and cholinergic systems. NPS has been found co-localized with other excitatory transmitters such as glutamate, acetylcholine, or corticotropine-releasing factor. Activation of NPSR triggers mobilization of intracellular Ca^{2+} and stimulation of cAMP synthesis, therefore increasing cellular excitability. A functional polymorphism in NPSR has been identified that produces a gain-of-function phenotype by increasing agonist potency up to tenfold. Finally, a gender-specific association of this NPSR polymorphism with panic disorder was found in male patients, indicating that the NPS system might be involved in modulating anxiety responses in humans. Further studies about interactions of the NPS system with other transmitter systems might help to discover additional functions of NPS and define its role within complex neural networks.

Keywords Anxiety · Arousal · Asthma · Feeding · Panic disorder · Sleep · Stress

Abbreviations

CRF	Corticotropin-releasing factor
GPCR	G protein-coupled receptor
HPA axis	Hypothalamus–pituitary–adrenal axis
NPS	Neuropeptide S
NPSR	neuropeptide S receptor
SNP	Single-nucleotide polymorphism

1 Identification and Structure of NPS

Using the orphan receptor strategy, Neuropeptide S (NPS) was identified as the endogenous ligand of the orphan G protein-coupled receptor GPR154

(Sato et al. 2002). NPS is a peptide of 20 amino acids, encoded by a relatively short precursor protein (only 89 amino acids in human). Comparing the primary structures of NPS from various species, it became evident that serine (single amino acid code "S") is always found as the amino terminal residue in all species (Fig. 1) and therefore this structural feature was used to name the peptide accordingly. The NPS precursor protein displays the typical structural characteristics of other neuropeptide precursors with a hydrophobic signal peptide at the amino terminus of the protein and a pair of basic amino acids immediately preceding the immature peptide sequence (Xu et al. 2004; Reinscheid and Xu 2005; Reinscheid 2007). The primary structure of NPS itself shows no homology to any other known neuropeptide. DNA sequences

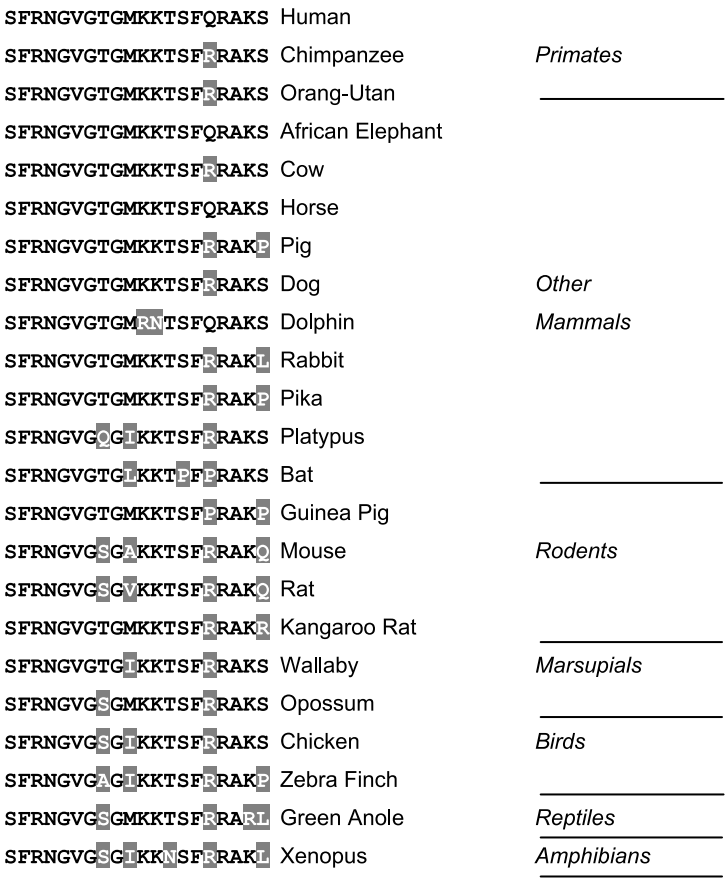

Fig. 1 Alignment of NPS peptide sequences deduced from cDNA and genomic DNA sources of representative tetrapods (Reinscheid 2007). The seven amino terminal residues of NPS are perfectly conserved across all species. Amino acid residues different from the human NPS sequence are *shaded*

encoding putative NPS precursor proteins are highly conserved among vertebrates, but are absent from fish or invertebrate genomes (Reinscheid 2007). This indicates that the NPS gene is a relatively recent neuropeptide gene in vertebrate evolution and it is thus far the only example of a neuropeptide that specifically occurs in tetrapods. This peculiar evolutionary distribution suggests that NPS might serve specialized physiological functions in tetrapod vertebrates. Fish might either lack these functions or use alternative transmitter systems. The NPS receptor (NPSR) is a typical G protein-coupled receptor with moderate homology to other peptide receptors. The two most closely related sequences are the V1a and V2 vasopressin receptors, albeit with only 21–23% amino acid identity. NPSR was first identified as the orphan receptor GPR154 and is also known as vasopressin receptor-related receptor 1 (VRR1) (Gupte et al. 2004) or G protein-coupled receptor for asthma susceptibility (GPRA) (Laitinen et al. 2004).

2 Anatomy and Neurochemistry of the NPS System

Distribution of NPS and NPSR gene expression was mapped in detail by in-situ hybridization (Xu et al. 2004, 2007). In the rat brain, expression of NPS precursor is remarkably restricted to only three brainstem structures. A few scattered NPS-expressing cells are found in amygdala and hypothalamus. Among the brainstem structures, a prominent cluster of NPS-expressing cells was found in close proximity to the noradrenergic locus coeruleus (LC). This group of cells in the LC area is also distinct from the neighboring Barrington's nucleus that expresses corticotropin-releasing factor (CRF) as a marker. The NPS-expressing neurons therefore define a previously uncharacterized nucleus in the pericoerulear region. NPS precursor is also expressed in the lateral parabrachial nucleus and the sensory principle 5 nucleus (Pr5) of the rat brainstem.

Analysis of neurochemical markers revealed that NPS appears to be co-localized with other excitatory transmitters (Xu et al. 2007). In the LC area, the majority of NPS-expressing neurons co-express vesicular glutamate transporter mRNA and are thus glutamatergic neurons. In addition, co-expression of choline acetyltransferase was detected in a few cells in this structure. In the lateral parabrachial nucleus, most NPS-producing cells co-express CRF while all NPS-synthesizing neurons in Pr5 are glutamatergic neurons. NPS was never co-localized with markers for GABAergic, noradrenergic, or dopaminergic neurons. NPS precursor mRNA expression appears to be very restricted, with probably less than 200 cells in the rat brain synthesizing NPS.

The restricted distribution pattern of NPS precursor is in contrast to a much broader presence of NPS receptor mRNA throughout the brain. Highest expression of NPSR transcripts is found in the cortex, olfactory nuclei,

thalamus, hypothalamus, amygdala, and parahippocampal formation, such as the subiculum. Only low levels of NPSR expression are detected in the brainstem and no NPSR transcripts were found in NPS precursor-expressing cells (Xu et al. 2007). The pattern of NPSR expression in the central nervous system suggests possible functions in emotional and sensory processing, arousal, stress, energy homeostasis, endocrine regulation, or learning and memory. NPS and NPSR transcripts were also found in peripheral tissues, including thyroid, salivary, and mammary glands, which might indicate additional endocrine functions (Xu et al. 2004), but so far no detailed analysis of these transcripts in peripheral tissues has been reported.

3 Pharmacology and Genetics of NPSR

Human and mouse NPS receptors were studied in heterologous expression systems, showing that NPS induces mobilization of Ca^{2+} and stimulates synthesis of cAMP at EC_{50} values of 4–10 nM. These results imply that NPSR couples to both G_q and G_s proteins and might increase cellular excitability (Xu et al. 2004; Reinscheid et al. 2005). NPSR displays high-affinity saturable and displaceable binding in the subnanomolar range using a radioiodinated NPS analog. High affinity binding and receptor activation in the low nanomolar range are typical hallmarks for neuropeptides and their receptors. Structure–activity relationship studies of NPS have revealed the importance of amino terminal residues for receptor activation, while carboxy-terminal deletions of the last eight amino acids only gradually affect agonist activity (Reinscheid et al. 2005). Alanine- and D-amino acid scanning analogs of NPS demonstrated that residues 2, 3, 4, 6, and 7 contribute critically to NPS agonist activity (Roth et al. 2006; Bernier et al. 2006). These results nicely complement the phylogenetic data because the first seven amino acids of NPS are perfectly conserved across all species analyzed so far (Fig. 1; Reinscheid 2007).

Multiple single-nucleotide polymorphisms (SNPs) and several splice variants have been identified in the human NPSR gene that is located on chromosome 7p14-15. Genetic linkage studies suggested that some SNPs were associated with an increased risk of developing asthma or other allergic diseases characterized by high serum IgE levels (Laitinen et al. 2004). The association study did not provide physiological evidence for any of the NPSR variants. Therefore, we analyzed pharmacological features of two NPSR variants: (i) a SNP that encodes an amino acid change ($Asn^{107}Ile$) in the first extracellular loop of the receptor protein (SNP591694 A>T; ref SNP ID: rs324981), and (ii) a C-terminal splice variant of the receptor that was reportedly overexpressed in human asthmatic airway tissue (Laitinen et al. 2004). We found that the $Asn^{107}Ile$ polymorphism results in a gain-of-function characterized by a five- to tenfold increase in agonist potency at NPSR Ile^{107} compared to

NPSR Asn^{107}. The C-terminal splice variant of NPSR, however, did not appear to cause measurable differences in the pharmacological profile of the receptor (Reinscheid et al. 2005; Bernier et al. 2006).

The pharmacological data demonstrate that the coding polymorphism at Asn^{107}Ile in the NPSR gene causes significantly altered pharmacology of the encoded receptor and might therefore suggest phenotypical changes that could be associated with inheritable disorders. Genetic linkage of the NPSR gene locus with asthma and atopy has been replicated so far in several independent cohorts (Kormann et al. 2005; Melen et al. 2005; Feng et al. 2006; Malerba et al. 2007), although two other studies of asthma patients failed to confirm linkage on chromosome 7p (Immervoll et al. 2001; Shin et al. 2004). In addition, two studies in Northern European individuals found no association of the NPSR risk haplotypes with atopic dermatitis, a skin disorder that is characterized by high serum IgE levels (Söderhäll et al. 2005; Veal et al. 2005). A potential role of NPSR in the pathophysiology of asthma was further questioned by a study using NPSR knockout mice that found no evidence for NPSR function in normal airway physiology or asthma pathophysiology in mice (Allen et al. 2006). In the original paper, Laitinen et al. (2004) reported upregulation of NPSR mRNA in bronchial tissue of human asthma patients and in a mouse model of airway inflammation. However, recent studies could not replicate the presence of NPSR mRNA in either normal or inflamed mouse airway tissue (Allen et al. 2006). The same study also found no evidence for NPSR mRNA expression in human airway tissue. Our own unpublished data confirm the findings of Allen et al. At present, the functional role of NPSR in asthma is therefore unclear. It remains to be seen whether the NPSR chromosomal region contains other thus-far unrecognized genes or regulatory elements that could be involved in asthma or other allergic diseases. Indeed, the original report identified an alternative transcript of unknown function encoded by the complementary DNA strand (Laitinen et al. 2004). Since functional evidence for an involvement of NPSR in airway function has not been found, alternative genes or genetic mechanisms need to be considered to account for the genetic linkage data, indicating that this chromosomal region might confer asthma susceptibility.

Based on the spectrum of behavioral effects produced by NPS (see below), we investigated possible associations of the functional Asn^{107}Ile polymorphism in NPSR with disorders affecting arousal, attention, or anxiety. Analysis of DNA samples from patients diagnosed with schizophrenia, attention-deficit/hyperactivity disorder, or panic disorder revealed a gender-specific association of NPSR genotypes with panic disorder in male patients (Okamura et al. 2007). Homozygous NPSR Asn^{107} carriers were found to be significantly under-represented among male panic disorder patients, indicating that this allele might have protective effects. Female panic disorder patients, as well as the schizophrenia cohort, showed a distribution of NPSR alleles that were indistinguishable from healthy controls. No preferred transmission of specific

NPSR alleles was detected in families with children diagnosed with attention-deficit/hyperactivity disorder. Interestingly, the chromosomal region 7p14-15 had been linked to panic disorder before in two independent studies using genome-wide linkage analysis (Knowles et al. 1998; Crowe et al. 2001). Together with the earlier findings these data suggest that NPSR might therefore be a candidate gene for panic disorder susceptibility. Replication of these findings in independent cohorts will, of course, be necessary to confirm this hypothesis.

4 Modulation of Arousal and Wakefulness by NPS

The regional distribution of NPS and NPSR expression in the brain suggested that activation of the NPS system might influence behavioral arousal and possibly modulate sleep–wakefulness cycles. Studies in our laboratory demonstrated that central administration of NPS produces arousal independent of novelty (Xu et al. 2004). Mice injected with low doses of NPS display

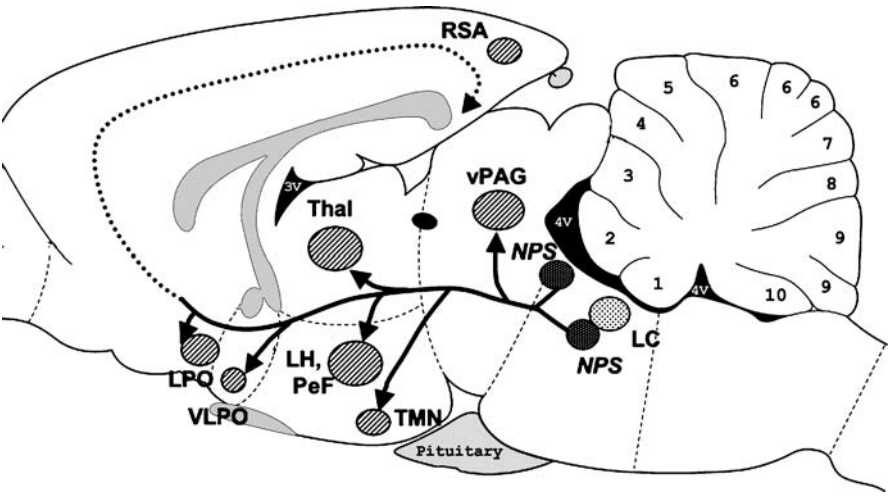

Fig. 2 Schematic drawing of a sagittal section of the rat brain showing possible connections of NPS synthesizing nuclei (*NPS*, *dark shading*) in the brainstem with arousal centers in the brain that express NPS receptor mRNA (*diagonal shading*). The noradrenergic locus coeruleus (*LC*) is depicted for orientation. The *dotted line* represents hypothetical projections of NPS producing neurons to cortical structures, such as the retrosplenial agranular cortex (*RSA*). Cerebral ventricles are shown in *black* and major fiber tracts are *shaded in grey*. Other abbreviations: *LH* lateral hypothalamic area, *LPO* lateral preoptic area, *PeF* perifornical nucleus, *Thal* thalamus, *TMN* tuberomammillary nucleus, *VLPO* ventrolateral preoptic nucleus, *vPAG* ventral part of periaqueductal gray, *3V* third ventricle, *4V* fourth ventricle. The drawing was adapted according to the rat brain atlas of Paxinos and Watson (1997)

increased horizontal and vertical activities for up to 60 min after central administration of 1 nmol NPS. Electroencephalographic (EEG) recordings in rats revealed that NPS produces cortical activation, indicative of enhanced wakefulness. NPS was found to suppress all stages of sleep for up to 1 h when EEG was recorded during the normal time of inactivity in rats (Xu et al. 2004). The NPS-induced state of forced wakefulness was followed later by a rebound in slow wave sleep and REM sleep. These observations suggest an important role of NPS in the modulation of sleep–wakefulness.

A possible anatomical substrate for NPS-induced arousal might be the thalamic midline nuclei that express high levels of NPSR mRNA and have a well-established role as integrators of arousal between the brainstem reticular formation and the cortex (Jones 2003; van der Werf et al. 2002). NPS receptors are ideally located in these thalamic relay nuclei to modulate arousal signals, but are also found in a number of additional brain centers that have been associated with arousal and sleep–wakefulness regulation (Fig. 2). Electrophysiological recordings will be necessary to further examine the functional role of NPSR in these thalamic structures and their contribution to behavioral arousal.

5 Anxiolytic-Like Effects of NPS

Strong NPSR mRNA expression in amygdala, the dorsal endopiriform nucleus, and various hypothalamic nuclei suggest that the NPS system might influence emotional behaviors such as stress or anxiety responses. Behavioral studies using validated paradigms to measure anxiety, including open field, light–dark box and elevated plus maze, demonstrated that central administration of NPS produces anxiolytic-like effects (Xu et al. 2004). These tests are based on the natural aversion of rodents for open or unfamiliar spaces and anxiolytic drugs increase exploration of these exposed areas. Therefore, these tests are also sensitive to confounding interference from agents that increase locomotor activity. The marble-burying test was used to control for such potentially confounding effects of NPS. In this paradigm, anxiolytic drugs reduce a naturally defensive behavior, i.e., burying of marbles in bedding material. Central administration of NPS reduced burying of marbles in a dose-dependent pattern, indicating that NPS produces potent anxiolytic-like effects in addition to its arousing effects (Xu et al. 2004). The anxiolytic-like properties of NPS have been confirmed independently in the four-plate test (Leonard et al. 2005). Together, these data show that NPS appears to produce robust anxiolytic effects across five different tests of anxiety-like behavior and might therefore be involved in the modulation of emotional responses to stress, such as fear and anxiety. The observation that specific NPSR alleles appear to be associated with panic disorder, which is considered a spe-

cific form of anxiety disorder, adds further notion to the hypothesis that the NPS system might also modulate emotional behaviors in humans (Okamura et al. 2007).

6 Modulation of Feeding Behavior by NPS

Studies from two independent groups indicate that NPS can transiently inhibit food intake in a dose-dependent manner (Beck et al. 2005; Smith et al. 2006). However, another paper challenged this hypothesis recently and reported orexigenic effects after NPS administration (Niimi 2006). Interpretation of these conflicting results is difficult, but should not hinder further studies in this direction. The NPS doses that were found to produce anorectic effects in the study by Beck et al. (2005) also promote significant hyperlocomotion. They reported that NPS injected intracerebroventricularly (ICV) at 1 and 10 μg (corresponding to 0.45 and 4.5 nmol NPS, respectively), attenuated food intake in fasted rats during the first hour, but cumulative 24-h food intake was not affected (Beck et al. 2005). In contrast, Smith et al. (2006) found no effect on food intake in fasted rats after ICV administration of NPS doses up to 10 nmol. According to their study, only local injections of NPS (0.1–1 nmol) into the paraventricular hypothalamic nucleus (PVN) attenuated food intake in fasted rats during the first hour. They also reported that intra-PVN administration of NPS did not increase locomotion but instead promoted rearing behavior accompanied by decreased grooming activity. From these data it is difficult to conclude whether NPS has a direct anorectic effect or whether the suppression of food intake occurs secondary to the behavioral arousal induced by the peptide.

Anatomical substrates for NPS-mediated effects on feeding behavior might be the arcuate nucleus or lateral hypothalamic areas that express high levels of NPS receptors (Xu et al. 2007). Both brain structures have well-established roles in feeding and satiety circuits (Vettor et al. 2002). Data presented in the study by Niimi (2006) indicate NPS-mediated activation of c-fos expression in orexin/hypocretin-positive neurons of the lateral hypothalamus. Although a direct activation of these neurons by NPS still needs to be established, the observation might indicate a functional link between the two systems. The orexin/hypocretin system is critically important for the maintenance of awake states (Chemelli et al. 1999; Lin et al. 1999) and has also been implicated in the coordination of arousal with food intake (Saper 2006). Under certain conditions, orexin/hypocretin can produce orexigenic effects (Sakurai 2006) and it has been shown that orexin/hypocretin-synthesizing neurons are activated by ghrelin but inhibited by glucose and leptin (Yamanaka et al. 2003). Interaction between NPS and orexin/hypocretin systems might also explain a functional link between the arousal effects that have been reported for both systems.

Detailed anatomical and physiological studies are required to explore this hypothesis.

7 The NPS System as Part of Neural Networks

The expression of NPSR transcripts in various parts of the hypothalamus indicates that NPS might also influence endocrine functions. Indeed, it was found that central administration of nanomolar doses of NPS can modulate the hypothalamus–pituitary–adrenal (HPA) axis (Smith et al. 2006). NPS increased plasma levels of corticosterone and adrenocorticotropine (ACTH) after central administration in rats in a time- and dose-dependent manner. NPS was also shown to stimulate release of CRF and vasopressin, but not neuropeptide Y, from hypothalamic explants, indicating that NPS might have a direct effect on these systems. Since elevated plasma corticosterone and ACTH levels or release of CRF and vasopressin are commonly associated with increased levels of stress, these observations are in apparent contrast with the anxiolytic-like behavioral effects produced by NPS. It is, however, possible that increased physical activity and elevated behavioral arousal produced by NPS administration could subsequently trigger HPA activation and might thus not be related to the primary effects of NPSR activation. On the other hand, data showing NPS-stimulated CRF release from hypothalamic slices indicate a rather direct effect on the HPA system. Further experiments to investigate interactions of the NPS system with the anatomical and neurochemical components of the HPA axis in the context of its behavioral effects are obviously needed.

Two recent reports demonstrated regulation of NPS precursor and NPS receptor mRNA expression by caffeine or nicotine treatment, respectively (Lage et al. 2006, 2007). Two hours after treatment with a single dose of the adenosine A_1 antagonist caffeine, a decrease of NPS precursor transcripts in the brainstem was detected while no change was observed after chronic caffeine treatment for 48 h. Conversely, no acute effect on hypothalamic NPSR mRNA levels was observed after caffeine treatment with a single dose while chronic treatment for 48 h induced NPSR expression in the hypothalamus. These observations indicate that adenosine neurotransmission might provide differential input to the NPS system, depending on the duration of the stimulus and the location of its anatomical substrate. Caffeine has well-known stimulating and wakefulness-enhancing effects and is also used clinically to provoke panic attacks in susceptible patients (Bourin et al. 1995). It is therefore intriguing to hypothesize that caffeine might exert at least part of its stimulating effects by influencing gene expression of NPS and NPSR.

Nicotine is another exogenous substance with well-known effects on arousal, wakefulness, and anxiety in both humans and animal models. In add-

ition, nicotine administration is known to reduce food intake. The parallels between the behavioral effects of nicotine and NPS had been outlined already in an editorial that accompanied the original report on NPS (Koob and Greenwell 2004). A recent study by Lage et al. (2007) found that acute nicotine treatment only increased NPSR mRNA levels in the brainstem but did not affect NPS precursor mRNA expression. In contrast, chronic nicotine treatment for 48 h increased both NPS and NPSR transcript levels in the brainstem and also induced increased NPSR expression in the hypothalamus. Although the study did not address mechanistic questions of direct versus indirect interactions between the two systems, these data suggest that nicotine might produce at least some of its effects on wakefulness, emotional behavior, and feeding by regulating components of the NPS system. Further anatomical and functional studies of these proposed interactions are certainly necessary to substantiate the two hypotheses.

Studies on the neurochemical properties of NPS-synthesizing neurons have shown that NPS is abundantly co-localized with other excitatory transmitters such as glutamate, acetylcholine, and CRF (Xu et al. 2007). Based on contemporary models of neural function, the data indicate that NPS might be co-released together with these excitatory transmitters and could exert modulatory or cooperative functions at the postsynaptic level. For example, ionotropic glutamate receptors have been shown to interact with GPCRs with profound consequences for postsynaptic responses (Lin et al. 2001; Lee et al. 2002). Electrophysiological and biochemical studies may help to investigate such a role for NPSR.

8 Conclusions

Due to its novelty, characterization of the NPS system and its physiological functions is still in its infancy. The many brain structures that express NPSR transcripts suggest that additional functions will be discovered. In addition, genetic mouse models that have been targeted for either over-expression or absence of NPS or NPSR, respectively, will certainly become useful tools to study this system further. For pharmacological studies, the availability of selective small molecule agonists and antagonists will be critically important.

Substantial progress has already been made to establish NPS as a potent modulator of arousal, sleep–wakefulness, and anxiety behaviors. New results are pointing at additional roles for NPS in energy homeostasis and endocrine regulation. The combination of arousal-enhancing and anxiolytic effects describes a unique pharmacological spectrum for NPS: Psychostimulants such as amphetamine or cocaine are known to produce anxiogenic responses in the behavioral anxiety models (Hascoet and Bourin 1998; Paine et al. 2002) whereas traditional anxiolytic drugs such as benzodiazepines induce sedation

and hypolocomotion (Haefely 1989; Chaouloff et al. 1997). The pharmacological spectrum of NPS also appears to be different from other endogenous neurotransmitters or neuropeptides, where arousal appears to be generally associated with increased anxiety (Okamura and Reinscheid 2007). The similarity between NPS and nicotine with respect to their effects on arousal, sleep, anxiety, and feeding deserves further attention and might yield clues about functional interactions at the systems level. Finally, these early studies imply that the NPS system might be an interesting candidate for development of new therapeutics to target sleep, anxiety, and metabolic disorders.

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