Neuron, Vol. 43, 487–497, August 19, 2004, Copyright ©2004 by Cell Press

Neuropeptide S: A Neuropeptide Promoting Arousal and Anxiolytic-like Effects

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Summary

Arousal and anxiety are behavioral responses that involve complex neurocircuitries and multiple neurochemical components. Here, we report that a neuropeptide, neuropeptide S (NPS), potently modulates wakefulness and could also regulate anxiety. NPS acts by activating its cognate receptor (NPSR) and inducing mobilization of intracellular Ca^{2+}. The NPSR mRNA is widely distributed in the brain, including the amygdala and the midline thalamic nuclei. Central administration of NPS increases locomotor activity in mice and decreases paradoxical (REM) sleep and slow wave sleep in rats. NPS was further shown to produce anxiolytic-like effects in mice exposed to four different stressful paradigms. Interestingly, NPS is expressed in a previously undefined cluster of cells located between the locus coeruleus (LC) and Barrington's nucleus. These results indicate that NPS could be a new modulator of arousal and anxiety. They also show that the LC region encompasses distinct nuclei expressing different arousal-promoting neurotransmitters.

Introduction

Sleep disorders and anxiety affect millions of people. Identifying and understanding the molecular regulators and neurocircuitries that are involved in sleep/wake cycles or arousal and anxious states are keys to the development of therapeutic targets for these diseases. Neurochemically, it has been shown that classical neurotransmitters such as noradrenaline (NA) (Aston-Jones et al., 1991a; Berridge and Waterhouse, 2003), acetycholine (Jones, 1991; Millan, 2003), serotonin (Millan, 2003; Ursin, 2002), glutamate (Chojnacka-Wojcik et al., 2001; Jones, 2003), and GABA (Gottesmann, 2002) are important transmitters of arousal systems and also play important roles in regulating emotional states as they relate to anxiety-like behavior. In addition, various neuropeptides such as hypocretin/orexin (Hcrt/Ox) (Sutcliffe and de Lecea, 2002), neuropeptide Y (Silva et al., 2002), galanin (Bing et al., 1993; Holmes et al., 2003; Saper et al., 2001), or nociceptin/orphanin FQ (Reinscheid and Civelli, 2002) are also modulators of arousal and/or anxiety. Anatomically, the dorsolateral pontine tegmental region is one of the important areas that have been implicated in both sleep regulation and stress-related behaviors. The dorsolateral tegmental region contains several distinct nuclei, such as Barrington's nucleus and the locus coeruleus (LC), and also comprises unidentified neurons outside of the LC proper, such as the peri-LC region (Rizvi et al., 1994; Sutin and Jacobowitz, 1988). The LC is the primary source of noradrenergic input to the cortex, and the NA-LC system plays important roles in regulating arousal and anxiety (Berridge and Waterhouse, 2003; Swanson and Hartman, 1975). The firing of LC neurons correlates with vigilance states. Tonic discharge of LC neurons is virtually absent during rapid eye movement (REM) sleep, low during slow wave sleep (SWS stages 1 and 2), and highest during wakefulness (Foote et al., 1980; Hobson et al., 1975). Barrington's nucleus, the pontine micturition reflex center, expresses corticotrophin-releasing factor (CRF) as its peptidergic neurotransmitter (Sutin and Jacobowitz, 1988; Swanson et al., 1983; Valentino et al., 1995).

In addition to these known neurotransmitters and neurocircuitries that are involved in arousal and anxiety, there could be other important regulators and structures in the CNS that have not yet been uncovered. Novel neurotransmitters or modulators can be found by using orphan G protein-coupled receptors (GPCRs) as targets. Orphan GPCRs are cloned receptor proteins whose endogenous ligands have not yet been identified. Identification of the natural ligands (deorphanization) of orphan GPCRs leads to the discovery of novel neurotransmitters or modulators. Using orphan GPCRs, several novel neuropeptides have recently been discovered which ultimately have shed new insights on our understanding of particular brain functions and helped to reveal novel therapeutic targets for mental disorders (Civelli et al., 2001; Wilson et al., 1998).

We describe here the physiological functions of such a newly deorphanized GPCR system, neuropeptide S (NPS), and its cognate GPCR. The sequence of the GPCR (GenBank accession numbers BD183774, BD183814, BD183773) was first disclosed in a patent published in April 2002 (WO 02/31145 A1; Sato et al., 2002). The patent also reported the isolation of its endogenous peptide ligand without providing further information about pharmacological characteristics or physiological functions. Here, we report that NPS is a novel neuropeptide that potently modulates arousal and could also regulate anxiety-related behavior. We further analyze the distribution of the NPS precursor mRNA expression and describe the existence of a previously uncharacterized population of cells that are adjacent to the noradrenergic LC neurons.

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SFRNGVGTGMKKTSFQRAKS	human
SFRNGVGTGMKKTSF**R**RAKS	chimpanzee
SFRNGVG**S**G**A**KKTSF**R**RAK**Q**	mouse
SFRNGVG**S**G**V**KKTSF**R**RAK**Q**	rat
SFRNGVGTGMKKTSF**R**RAKS	dog
SFRNGVG**S**G**I**KKTSF**R**RAKS	chicken

Figure 1. Primary Structures of Neuropeptide S from Human, Chimpanzee, Rat, Mouse, Dog, and Chicken

Amino acids divergent from the human sequence are shown in bold type. Sequences were deduced from GenBank entries BD168686 (human), BD168712 (rat), BD168690 (mouse), BU293859 (chicken), and genome sequencing traces 231487919 (chimpanzee) and 250468833 (dog).

Results

Evolutionary Conservation of NPS Primary Structures

The human, rat, and mouse NPS precursor proteins contain a hydrophobic signal peptide and a pair of basic amino acid residues preceding the unprocessed peptide. Searching public DNA databases, we identified a chicken EST clone and partial genomic sequences for the chimpanzee and canine precursor proteins. Alignment of the deduced primary structures of the mature peptide shows that the amino-terminal residue in all species is a conserved serine (Figure 1). According to the nomenclature that has been used most recently (Shimomura et al., 2002), we propose to name this peptide "Neuropeptide S" (NPS).

Pharmacological Profiles of NPS and NPS Receptor

Cell lines stably expressing human NPS receptor (NPSR) in both Chinese hamster ovary (CHO) cells and human embryonic kidney 293 T cells (HEK293 T) were used to define the pharmacological characteristics of NPS. Human, rat, and mouse NPS induce dose-dependent elevations in intracellular $[Ca^{2+}]_i$ in both HEK293 T (Figure 2A) and CHO (data not shown) cell lines, indicating that the NPS receptor couples to G_q proteins. Half-maximal effective concentrations (EC_{50}) for mobilization of $[Ca^{2+}]_i$ were 9.4 ± 3.2 nM, 3.2 ± 1.1 nM, and 3.0 ± 1.3 nM for human, rat, and mouse NPS, respectively.

Since position 10 of NPS is not conserved among the different species, we decided to substitute the corresponding amino acid with tyrosine (Y) in order to develop an analog suitable for radioiodination. Human Y^{10}-NPS retains full agonist activity with an EC_{50} of 6.7 ± 2.4 nM (data not shown). The monoiodinated form of Y^{10}-NPS was used as a radioligand in receptor binding experiments. Binding of [^{125}I] Y^{10}-hNPS to CHO cells stably expressing hNPSR is saturable with high affinity (K_d = 0.33 ± 0.12 nM; B_{max} = 3.2 ± 0.4 fmol/150.000 cells, Figure 2B) and displaceable by increasing concentrations of human NPS (IC_{50} = 0.42 ± 0.12 nM) (Figure 2C). No specific binding was detected in mock-transfected CHO cells. These results demonstrate that NPS binds and activates its cognate receptor with high potency and specificity.

Distribution of NPS Precursor and Receptor mRNA Expression

We next examined the sites of synthesis of the NPS precursor and receptor mRNA in rats. Quantitative RT-PCR shows that NPS and its receptor are expressed in various tissues, the highest levels being found in brain, thyroid, salivary, and mammary glands (Figure 3).

Since both NPS and NPS receptor mRNA are expressed highly in CNS among all the tissues examined, we next studied the localization of NPS and its receptor mRNA in rat brains by in situ hybridization. These experiments revealed that the rat NPS precursor mRNA is expressed discretely in a few brain areas, with strongest expression in the LC area (Figure 4B), principle sensory 5 nucleus, and lateral parabrachial nucleus (Figures 5B and 5C). Moderate expression was also found in a few scattered cells of the dorsomedial hypothalamic nucleus (Figures 5E and 5F) and the amygdala (Figures 5H and 5I).

To describe the NPS-expressing neurons in the LC area more precisely, double in situ hybridization with antisense probes for NPS precursor and tyrosine hydroxylase (TH) was carried out. As shown in Figures

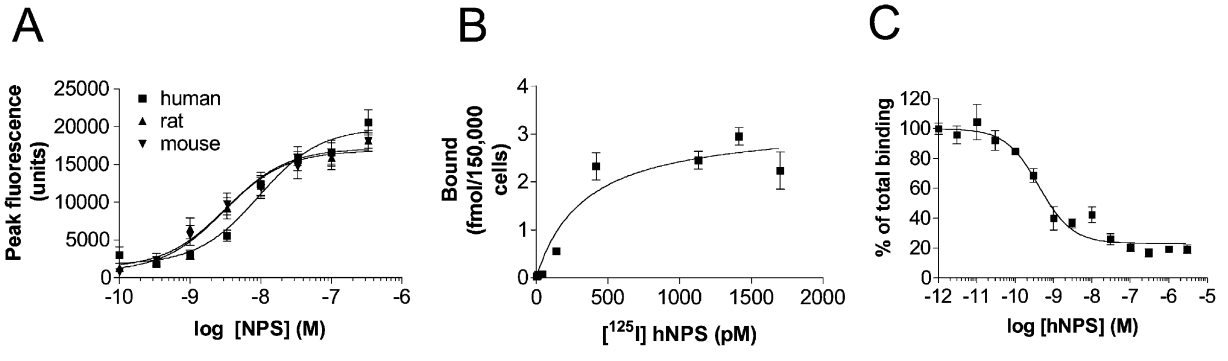

Figure 2. Pharmacological Characterization of the Human NPS Receptor

(A) Dose response curve of $[Ca^{2+}]_i$ mobilization induced by human, rat, and mouse NPS in an HEK cell line stably expressing human NPS receptor.
(B) Saturation binding of [^{125}I] Y^{10}-NPS (4 pM to 1.7 nM) to CHO cells stably expressing human NPS receptor.
(C) Displacement of 0.15 nM [^{125}I] Y^{10}-NPS by increasing concentrations of unlabeled human NPS. Data from triplicate experiments are shown as means ± SEM.

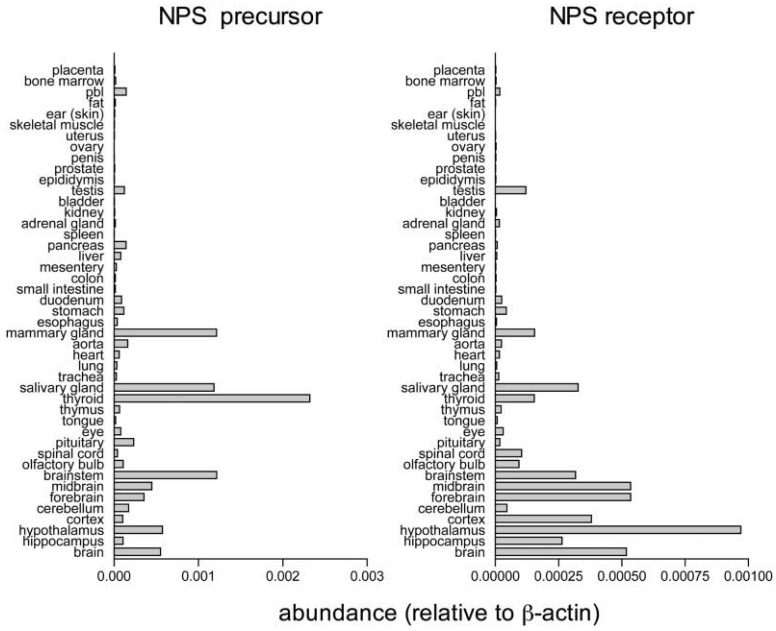

Figure 3. Tissue Distribution of NPS Precursor and NPS Receptor mRNA in Rat Tissues

Quantitative RT-PCR was used to measure transcript levels of NPS precursor (left) and NPS receptor mRNA (right) in 45 rat tissues. Transcript levels were normalized to β-actin. pbl, peripheral blood leucocytes.

4C–4E, NPS does not colocalize with TH. The majority of NPS-positive cells were observed at midpontine levels, ventromedial to the noradrenergic LC neurons. Few NPS-expressing neurons were found intermingled with TH-positive cells at the ventral pole of LC proper. We conclude that the NPS-expressing neurons in the LC area form a cluster of cells that do not produce NA and intermingle with LC proper neurons along the medial and ventral border of LC, extending just medially into the peri-LC area.

Within that area and ventromedial to the LC lies Barrington's nucleus, the micturition reflex center, which is a well-studied ovoid-shaped nucleus located at the rostral pole of LC. It has been shown that Barrington's nucleus is negative for TH and choline acetyltransferase and most of its neurons express CRF (Rizvi et al., 1994; Valentino et al., 2000). Double in situ hybridization with NPS and CRF antisense riboprobes revealed that NPS does not colocalize with CRF (Figures 4F–4H). At the level of highest NPS neuron density, only a few scattered neurons were found expressing CRF that were located ventrally to the NPS-expressing neurons. At a more rostral level, densely packed CRF-positive neurons were observed as the ovoid-shaped Barrington's nucleus. Only a few NPS-expressing neurons were found along the dorsal border of Barrington's nucleus at this level. We conclude that the NPS-expressing neurons lie caudally to Barrington's nucleus and at the mid-level of LC. They extend ventromedially from the LC proper, caudodorsally to Barrington's nucleus. This unique anatomical pattern of NPS-expressing neurons defines a previously unrecognized population of cells located in-between the noradrenergic LC proper and Barrington's nucleus.

The NPSR mRNA is widely expressed in many brain regions. The strongest expression signals were found in several discrete nuclei or regions, such as anterior olfactory nucleus (Figures 6B and 6C), dorsal and ventral endopiriform nucleus (Figures 6B, 6C, 6E, 6H, 6I, and 6K), amygdala (Figures 6H and 6I), precommissural nucleus, paraventricular thalamic nucleus, and subiculum (Figures 6K and 6L). High levels of expression were also observed in cortical regions. Motor cortex 2 and retrosplenial agranular cortex are distinct areas in cortex that show strong expression of NPS receptor mRNA (Figures 6E, 6H, 6K, 6M, and 6O). Medium levels of expression are also found in dispersed neurons in other cortical regions, such as somatosensory cortex (Figure 6N). High levels of expression was found in multiple nuclei of the hypothalamus (Figures 6H and 6K). Moderate NPSR expression was also found in midbrain. Pons and medulla are brain regions that express NPSR mRNA only weakly (data not shown).

These data suggest that NPS could be involved in a variety of brain functions. Interestingly, NPS receptor mRNA is not detected in LC area. However, significant NPSR expression is also found in thalamic midline nuclei, such as central medial thalamic nucleus, interanteriomedial thalamic nucleus, reuniens and rhomboid thalamic nucleus (Figure 6E), which relay extensive inputs from brain stem reticular formation to diffuse cortical fields and are involved in the regulation of arousal and wakefulness (Van der Werf et al., 2002).

NPS Increases Locomotor Activity and Promotes Wakefulness

In view of the NPSR sites of expression and the prominent expression of the NPS precursor in LC area, we hypothesized that NPS may be involved in arousal and anxiety. To start this investigation, we tested the effects of NPS on locomotor activity in both naive and habituated mice (Figure 7A). NPS (0.1 nmole or 1 nmole) administered intracerebroventricularly (i.c.v.) caused a significant increase in locomotor activity in both naive and habituated mice ($p < 0.01$) during the 60 min observation period, while 10 pmoles NPS did not. The total distance traveled, percentage of time moving, number of rearing events, and center entries were also significantly increased in mice injected with 0.1 and 1 nmole NPS

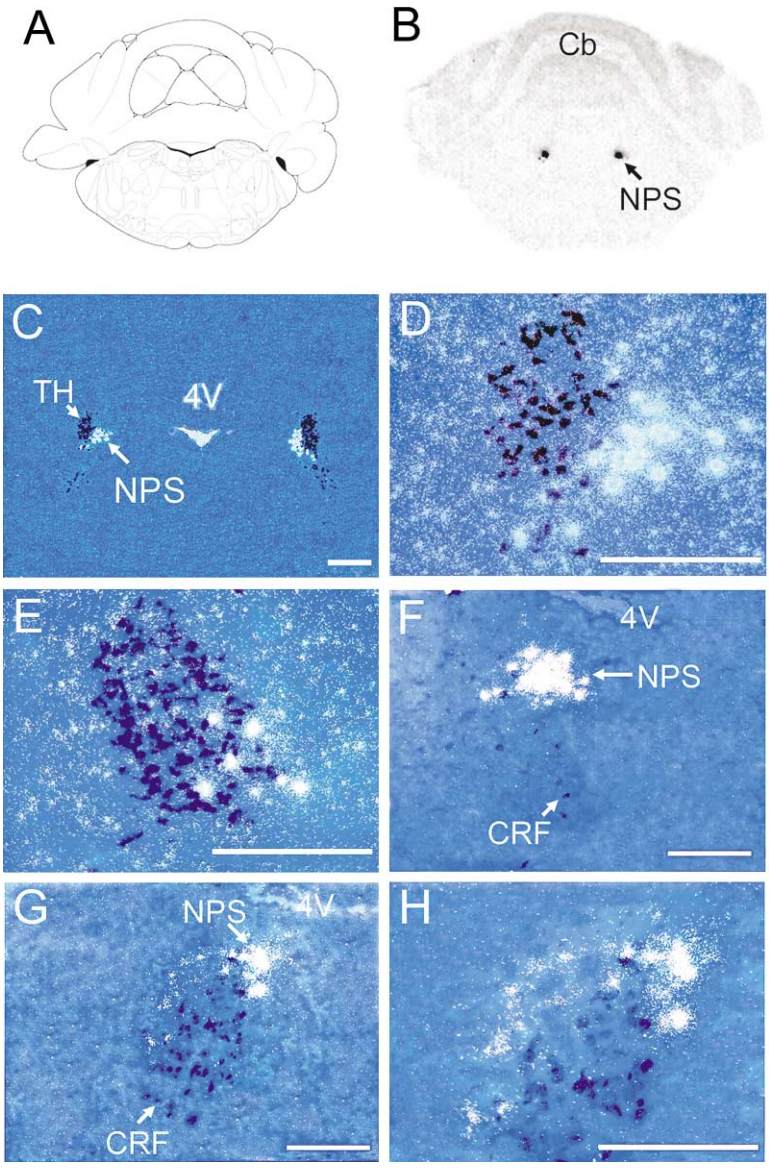

Figure 4. Expression of NPS Precursor mRNA in the Pontine Area of the Rat Brain

(A) Schematic drawing of the section shown in (B). The level is at bregma −9.80 mm (Paxinos and Watson, 1997, reprinted with permission from Elsevier). (B) Representative autoradiogram of NPS mRNA expression in LC area. (C–E) Dark-field images of double in situ hybridization of NPS precursor mRNA (white) and TH mRNA (dark blue) in LC area. (D) Higher magnification of the area indicated by an arrow in (C). (E) Higher magnification of a more caudal section. (F–H) Dark-field images of double in situ hybridization of NPS precursor mRNA (white) and CRF mRNA (dark blue) at mid-level of LC area (F) and rostral LC (G). (H) Higher magnification of the area indicated by an arrow in (G). TH, tyrosine hydroxylase; NPS, neuropeptide S; CRF, corticotropin-releasing factor. Landmarks: Cb, cerebellum; 4V, fourth ventricle. Scale bar, 500 μm in (C), 250 μm in all other pictures.

(data not shown). The elevation of locomotor activity in habituated animals indicates that NPS may produce behavioral arousal independent of novelty or stress.

The effects of NPS on locomotor activity suggest a possible role of NPS in modulating sleep-wake patterns. Rats were implanted with a standard set of electrodes and electroencephalograms (EEG) and electromyograms (EMG) were recorded after i.c.v. administration of NPS. Polygraphic recordings of vigilance states indicate that rats treated with 0.1 nmole and 1.0 nmole of NPS spent up to 69% and 87%, respectively, of the first hour of recording in wakefulness, compared to 45% for saline treatment ($F_{2,21} = 16.80$; $p < 0.01$) (Figure 7B). In contrast, the amount of slow wave sleep stage 1 (SWS1) ($F_{2,21} = 9.69$; $p < 0.01$), stage 2 (SWS2) ($F_{2,21} = 11.859$; $p < 0.01$), and REM sleep ($F_{2,13} = 12.29$; $p < 0.01$) in NPS-treated rats was significantly reduced compared with saline-treated animals. The increase in wakefulness was due to a significant increase in the mean duration of the episodes ($F_{2,21} = 7.22$; $p < 0.01$) compared to the saline group. Interestingly, the increase in wakefulness during the first hour post-NPS injection was followed by a rebound in the amount of non-REM sleep at the second hour (20% increase versus saline ($F_{2,21} = 5.44$; $p < 0.01$)) and fourth hour (48% increase compared to saline-treated animals [$F_{2,21} = 12.22$; $p < 0.01$]). Together, these data show that NPS can promote arousal and might be involved in the induction of wakefulness or suppression of sleep.

NPS Attenuates Anxiety-like Behavior

The expression of NPS receptor in several brain regions that are known to be involved in anxiety, such as amygdala, thalamus, and hypothalamic regions, indicates that the NPS system could also play a role in the behavioral response to stress (Charney and Deutch, 1996; Redmond and Huang, 1979; Sah et al., 2003). Naive mice were tested in the open field, a paradigm of free exploratory behavior in a novel environment. It was found that NPS significantly increased the number of entries in the

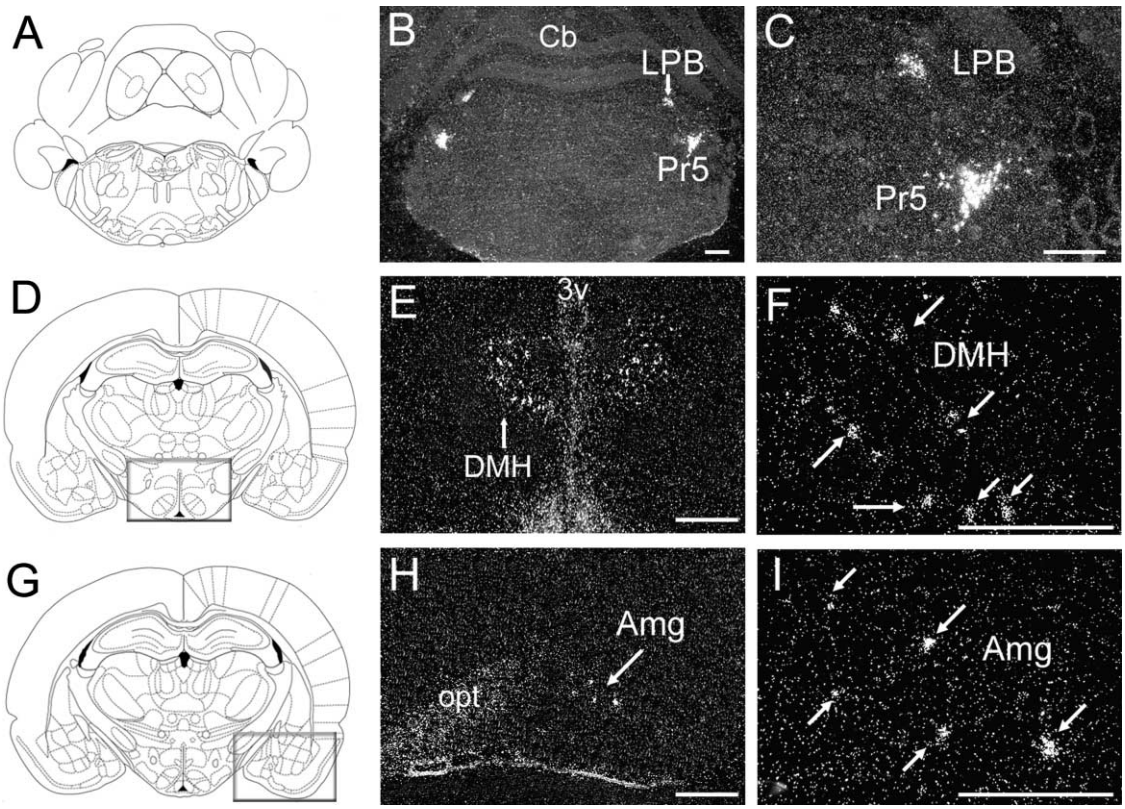

Figure 5. Distribution of NPS Precursor mRNA Expression in Rat Brain

(A, D, and G) Drawings of the sections illustrated in (B) and (C) (Bregma −9.68 mm), (E) and (F) (Bregma −2.80 mm), and (H) and (I) (Bregma −3.14 mm), respectively (Paxinos and Watson, 1997). (B, C, E, F, H, and I) Dark-field images of NPS precursor mRNA expression in coronal sections of rat brain. (E and H) Expression of NPS precursor mRNA in boxed regions in (D) and (G), respectively. (C, F, and I) Higher magnification of the area indicated by an arrow in (B), (E), and (H), respectively. Arrows in (F) and (I) indicate single cells showing hybridization signals for NPS precursor mRNA. LPB, lateral parabrachial nucleus; Pr5, principle sensory 5 nucleus; DMH, dorsomedial hypothalamic nucleus; Amg, amygdala. Landmarks: Cb, cerebellum; 3V, third ventricle; opt, optic tract. Scale bar, 500 μm.

central zone during the first 10 min, which could indicate an anxiolytic-like effect ($p < 0.05$; Figure 8A). However, the same doses of NPS also increased ambulations in the outer zones of the open field, consistent with the arousal-promoting effect of the peptide. In order to further study NPS effects on stress as it relates to anxiety, two additional tests were performed that were based on the natural aversion of rodents to open or unprotected spaces: the light-dark box and the elevated plus maze (Figures 8B and 8C). Mice injected with NPS exhibited a dose-dependent reduction in anxiety-like behavior in both paradigms.

In the light-dark box, mice injected with NPS at a dose range of 0.03–3 nmole, but not at 0.01 nmole, spent a prolonged time in the light area ($p < 0.05$–0.01, Figure 8B) and showed a higher percentage of entries in the light area (data not shown). The latency until the first exit from the protected dark compartment was significantly reduced by NPS at doses between 0.3 and 3 nmole. General activity was also enhanced as the number of transitions between the two compartments significantly increased at doses between 0.1 and 3 nmole. In the elevated plus maze, mice injected with 0.1 and 1 nmole NPS, but not at 0.01 nmole, spent significantly more time on the open arms ($p < 0.05$, Figure 8C) and showed a higher number of transitions from closed to the open arms ($p < 0.05$–0.01). The average number of transitions between the two closed arms of the elevated plus maze (closed-closed transitions) was increased at all doses but did not reach statistical significance. Closed-closed transitions are a measure of general activity in this behavioral paradigm, so our data indicate that in the elevated plus maze NPS may not produce significant hyperlocomotion. Together, the increased number of entries and prolonged time spent in the unprotected zones of both paradigms (open arm/light area) suggest that central administration of NPS produces an anxiolytic-like effect. However, consistent with the hyperlocomotor effect of NPS as described above, these NPS doses (>0.1 nmole) also significantly increased the total activity in both tests.

Most anxiolytic drugs increase exploratory activity in the open field, light-dark box, or elevated plus maze paradigms. However, compounds stimulating locomotion could produce false-positive effects in these tests because the enhanced exploration could be secondary to the increase in general activity. In order to validate the observed anxiolytic-like effects of NPS, we tested increasing doses of NPS in the marble-burying paradigm. Mice tend to bury objects such as glass marbles

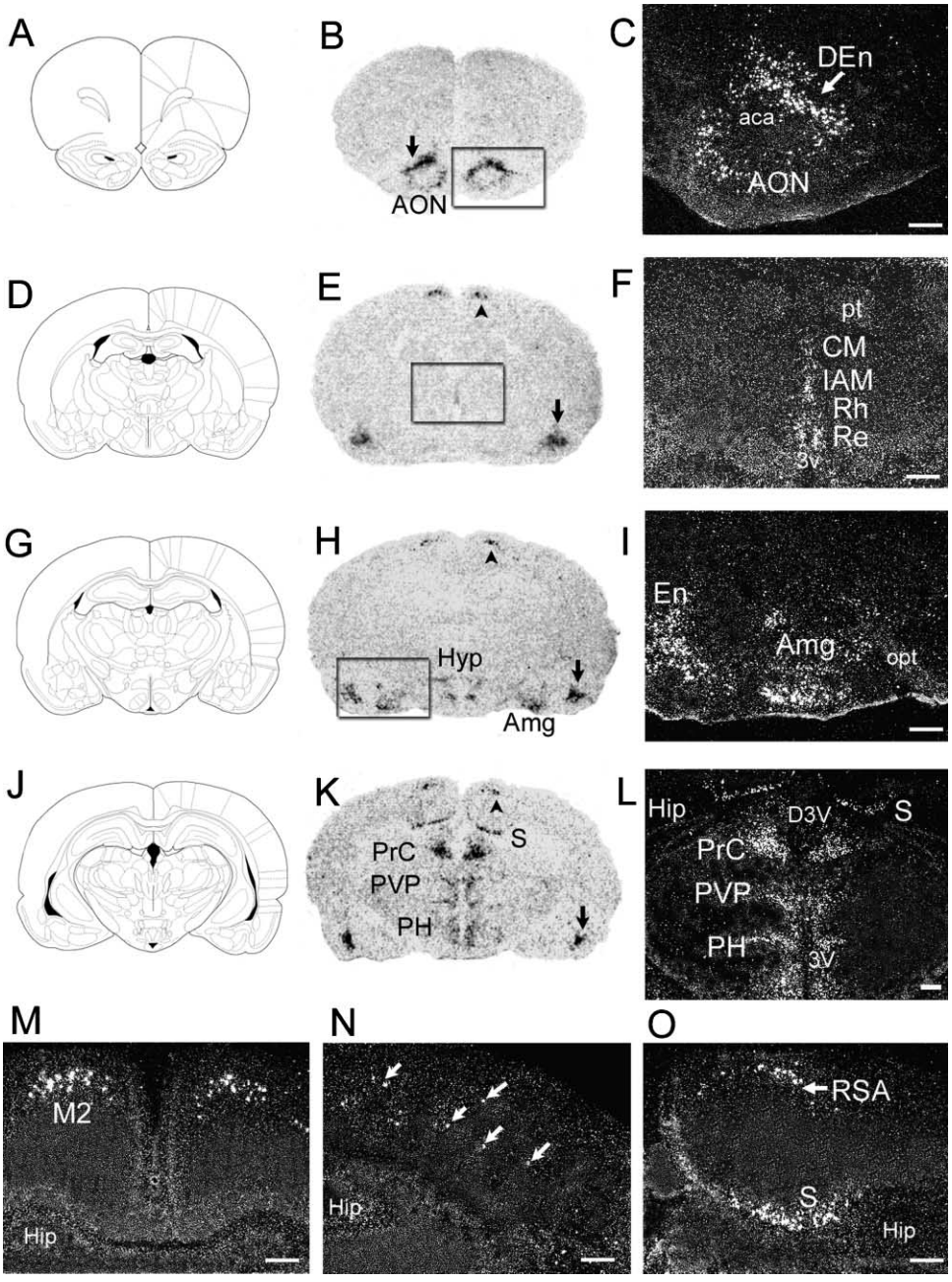

Figure 6. Distribution of NPS Receptor mRNA Expression in Rat Brain

(A, D, G, and J) Schematic drawings of the sections shown in (B) and (C) (Bregma, 3.20 mm), (E) and (F) (Bregma −1.80 mm), (H) and (I) (Bregma −2.80 mm), and (K) and (L) (Bregma −4.52 mm), respectively (Paxinos and Watson, 1997). (B, E, H, and K) Autoradiograms of NPSR mRNA expression in coronal rat brain sections. Arrows in (B), (E), (H), and (K) indicate endopiriform nucleus (En). Arrowheads in (E), (H), and (K) refer to secondary motor cortex (M2), retrosplenial agranular cortex (RSA)/M2, and RSA, respectively. (C, F, and I) Dark-field images of boxed regions in (B), (E), and (H), respectively. (L) Dark field image of midline thalamic regions of section (K). (M and N) Dark-field image of cortical regions in section (E). Arrows in (N) indicate scattered cells expressing NPSR mRNA in somatosensory cortex. (O) Dark-field image of cortical and subicular regions in section (K). AON, anterior olfactory nucleus; DEn, dorsal endopiriform nucleus; CM, central medial thalamic nucleus; IAM, interanteromedial thalamic nucleus; Rh, rhomboid thalamic nucleus; Re, reuniens thalamic nucleus; Amg, amygdala; Hyp, hypothalamus; S, subiculum; Prc, precommissural nucleus; PVP, paraventricular thalamus nucleus, posterior; PH, posterior hypothalamus. Landmarks: aca, anterior commissure, anterior part; pt, paratenial thalamic nuclei; opt, optic tract; D3V, dorsal third ventricle; 3V, third ventricle; Hip, hippocampus. Scale bar, 500 μm.

present in their environment. Anxiolytic drugs such as benzodiazepines reduce the number of marbles buried over a fixed period of time. It has been suggested that the inhibition of marble-burying behavior is correlated with anxiolytic-like activity (Njung'e and Handley, 1991). As shown in Figure 8D, mice injected with saline covered about 50% of the marbles during the 30 min observation period (total of 18 marbles per cage). NPS dose-depen-

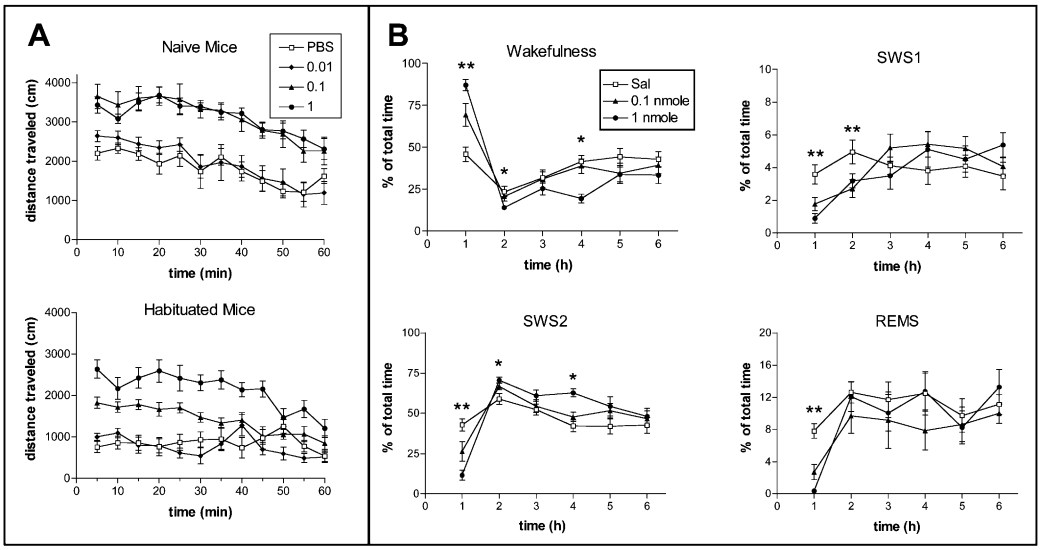

Figure 7. Central Administration of NPS Produces Behavioral Arousal and Wakefulness

(A) Hyperlocomotion effects of NPS in naive and habituated mice. Naive mice were new to the test chamber, while habituated animals were acclimatized for 1 hr prior to the injection. In naive mice, 0.1 and 1 nmole NPS induce significant hyperlocomotion ($F_{3,324} = 92.83$, $p < 0.0001$, two-way ANOVA for repeated measures). The same doses of NPS also produced significant effects in habituated animals ($F_{3,336} = 135.59$, $p < 0.0001$).

(B) Arousal promoting effects of NPS in rats. NPS increases the amount of wakefulness and decreases SWS1, SWS2, and REM sleep in rats ($n = 8$ for each dose). **$p < 0.01$, 0.1 nmole and 1.0 nmole compared with saline; *$p < 0.01$, 1.0 nmole compared with saline (ANOVA followed by Scheffe's post hoc test).

dently reduced the number of marbles buried. NPS-injected mice were actively exploring the marbles and eventually engaged in burying them, however, at significantly lower numbers as compared to mice injected with saline ($p < 0.05$–0.01). In summary, the combined results of all four paradigms measuring anxiety-like behavior suggest that NPS might produce anxiolytic-like effects in the presence of increased arousal.

Discussion

We provide evidence that the neuropeptide NPS is a novel modulator of arousal and possibly anxiety-related behavior. The effects of NPS on inducing wakefulness are rapid (during the first hour after injection) and potent, since low doses of NPS are sufficient to reduce all sleep stages, such as REM, SWS1, and SWS2, suggesting a profound change in sleep architecture. Recently, the neuropeptide hypocretin 1/orexin A (Hcrt/Ox) has also been demonstrated to induce arousal, and genetic analysis has provided compelling evidence that the absence of Hcrt/Ox or its receptor(s) produces narcolepsy in mice, dogs, and humans (Sutcliffe and de Lecea, 2002). Single i.c.v. injection of Hcrt/Ox produces arousal lasting for 2–3 hr (Bourgin et al., 2000; Hagan et al., 1999), whereas comparable NPS administrations show a more short-term effect within the first hour postinjection. Both peptides appear to increase wakefulness while suppressing REM sleep and deep sleep (SWS stage 2) (Bourgin et al., 2000), although one study could not detect a significant effect of Hcrt/Ox on deep sleep duration (Hagan et al., 1999). Hctr/Ox appears to exert its effects partially by directly activating noradrenergic LC neurons, since orexin 1 receptors are found to be colocalized with TH in LC neurons and electrophysiological recordings from LC neurons show excitatory effects of exogenously applied Hcrt/Ox (Bourgin et al., 2000). However, the arousal-promoting effect of NPS is unlikely mediated by direct activation of noradrenergic systems, since our anatomical data show that NPS-expressing neurons do not produce NA and no NPSR mRNA was detected in LC neurons. However, we cannot rule out an indirect activation of noradrenergic systems. Electrophysiological recording will be necessary to confirm a possible link between NPS and monoaminergic transmitter systems that have been implicated in the neurochemistry of wakefulness and arousal.

One unexpected outcome of this study is the discovery of a cluster of NPS-expressing neurons that do not produce NA or CRF and are localized in close proximity to the LC proper and Barrington's nucleus. The cluster of NPS-expressing neurons is likely to be a previously uncharacterized population of cells in the peri-LC area. Noteworthy, it has been reported before that a large number of uncharacterized neurons are found in the peri-LC area (Aston-Jones et al., 1991b; Rizvi et al., 1994), and our present data suggest that the NPS neuronal cluster could be a subset of these neurons.

It is well documented that the noradrenergic LC is involved in the regulation of an aroused state of wakefulness (Berridge and Waterhouse, 2003). On the other hand, several studies found no major disruption of EEG activity after selective cytotoxic lesions of TH-positive LC neurons or genetic ablation of the noradrenaline-synthesizing enzyme dopamine β-hydroxylase (Cirelli et al., 1996; Hunsley and Palmiter, 2003), underscoring the fact that arousal is modulated by multiple neuronal sys-

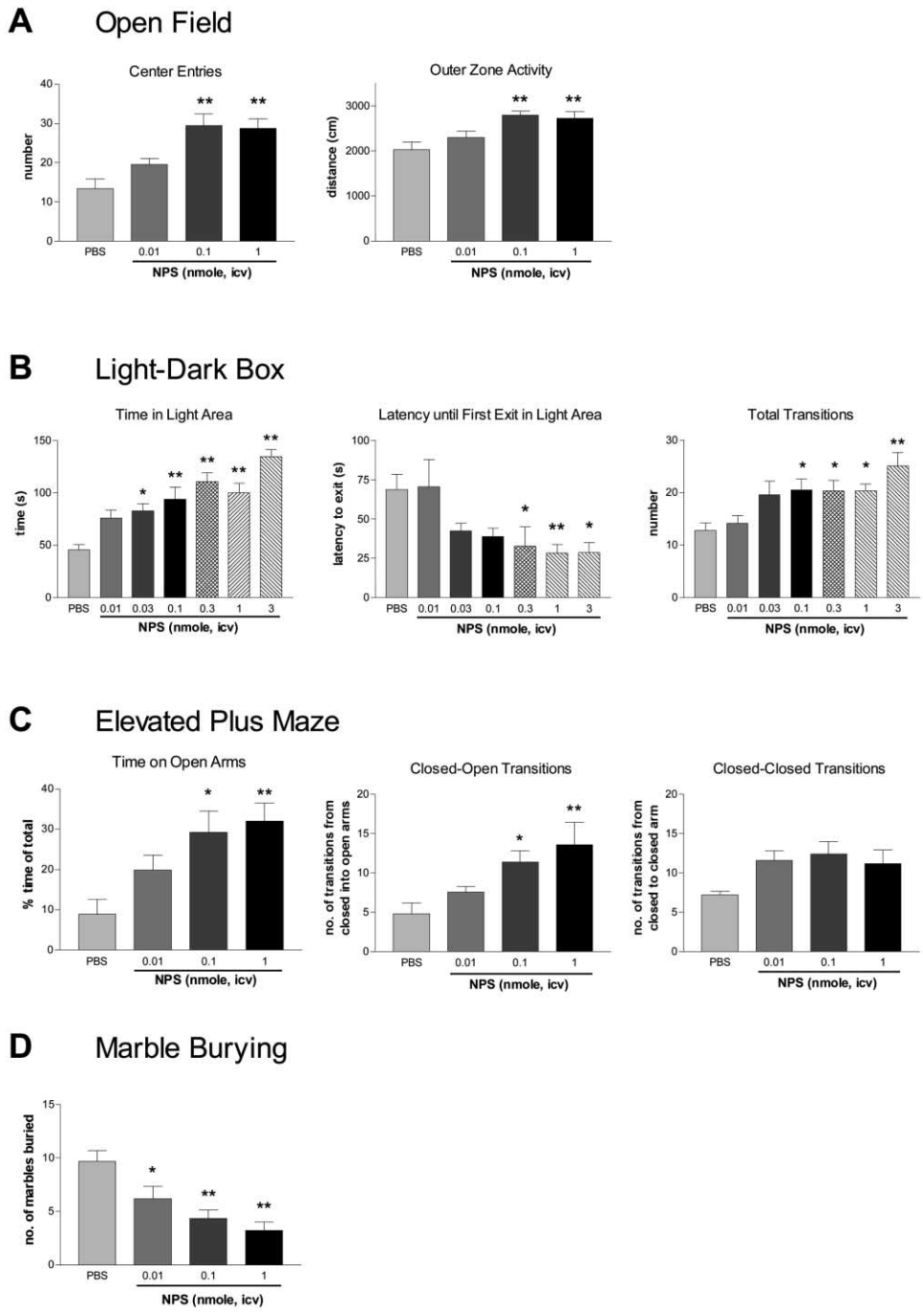

Figure 8. Anxiolytic-like Effects of NPS in Mice

NPS produces dose-dependent anxiolytic-like effects in C57Bl/6 mice exposed to the open field (A), light-dark box (B), elevated plus maze (C), and marble burying paradigm (D). Doses and groups: all doses are in nmole per animal; open field ($n = 8$ for each dose); light-dark box (PBS, $n = 10$; 0.01 nmole, $n = 5$; 0.03 nmole, $n = 5$; 0.1 nmole, $n = 5$; 0.3 nmole, $n = 11$; 1 nmole, $n = 5$; 3 nmole, $n = 8$); elevated plus maze ($n = 5$ for all doses); marble burying (PBS and 0.01 nmole, $n = 10$; 0.1 and 1 nmole, $n = 9$). $^{**}p < 0.01$, $^{*}p < 0.05$ compared to PBS control, ANOVA followed by Dunnett's test for multiple comparisons. All data are presented as means ± SEM.

tems. Our present data provide evidence that NPS could be a previously uncharacterized arousal-modulating transmitter system. Interestingly, the close vicinity of NPS-producing neurons and the noradrenergic neuronal cluster in LC indicate that this brainstem area might contain two independent transmitter systems that regulate vigilance states.

Central administration of NPS produces anxiolytic-like effects but also increases locomotor activity at similar doses. In the open field, elevated plus maze, and light-dark box paradigms, increases in exploration are generally interpreted as an anxiolytic effect, but the interpretation might be confounded by hyperlocomotion. Factor analysis, however, has shown that the behavioral

parameters monitored in these tests can be divided into two components—an activity component (total distance traveled, number of transitions) and an anxiety component (number of entries in unprotected zone, time spent in unprotected zone) (Rodgers and Johnson, 1995)—and that these two components show poor correlation. For example, both the psychostimulants amphetamine and cocaine produce hyperlocomotion yet increase anxiety-like behavior, i.e., they are anxiogenic (Hascoet and Bourin, 1998; Paine et al., 2002). On the other hand, the wake-promoting neuropeptide Hcrt/Ox enhances arousal and hyperlocomotion and suppresses REM sleep but has no effect on anxiety-like behavior in rodents (Hagan et al., 1999). Moreover, typical anxiolytic drugs, such as benzodiazepines, have either no effect or reduce locomotor activity, depending on the doses used (Chaouloff et al., 1997). Therefore, although we acknowledge the possible confounding effects of hyperlocomotion, we suggest that the increased exploratory activity observed in mice after NPS administration may indicate an anxiolytic-like profile in these three paradigms. To further investigate the possible anxiolytic-like effects of NPS, we used the marble burying test as an alternative behavioral paradigm. In this test, the selective suppression of marble burying behavior is suggested to correlate with anxiolytic activity, in contrast to the other three paradigms where increases of natural behaviors are an index of anxiolytic-like effects. Numerous drugs clinically effective in the treatment of anxiety disorders, such as benzodiazepines or selective serotonin reuptake inhibitors, reduce marble burying behavior in rodents (Borsini et al., 2002). Our data demonstrate that NPS also inhibits this natural behavior at doses which increase locomotion. Altogether, central administration of NPS reduces behavioral signs of anxiety in four different anxiety tests. These findings indicate that NPS could be involved in modulating anxiety responses.

In conclusion, we have characterized the neuropeptide NPS. Central administration of NPS produces a unique behavioral profile by increasing locomotor activity and wakefulness in rodents. NPS could also exert anxiolytic-like effects. In addition, we identify a previously undescribed group of neurons adjacent to the noradrenergic LC that express NPS. The discovery of this previously uncharacterized transmitter system that modulates sleep-wake cycles and anxiety might help to further our understanding of sleep disorders, such as insomnia, and pathological states of anxiety. It should be noted that excessive anxiety and disruption of sleep patterns are often observed in patients suffering from depression (Ohayon et al., 1998).

Experimental Procedures

Molecular Cloning of Human NPS Receptor and Rat NPS Precursor

Human NPS receptor was cloned into pcDNA3.1(+) from human brain cDNA (Clontech, Carlsbad, CA) using nested PCR. Primers were 5′-AGGAGCAAGGACAGTGAGGCTCAA-3′ and 5′-TGCCCAA GCAGGTGACAAGGACCT-3′ for first round amplification and 5′-ATACTCGAGCCATGCCAGCCAACTTCACAGAGGGCA-3′ and 5′-GCT TCTAGAGCTCAGCCTAGCACTGGCACTGCCCTA-3′ for the second round. Rat NPS precursor cDNA was cloned into pBluescript from a rat total brain cDNA library (Clontech). First round amplification primers were 5′-CAGATTTTGGGAAGTCCA-3′ and 5′-AGATTAATT CCCCGAGTC-3′; second round primers were 5′-GTTTCTAGAAAT GATTAGCTCAGTAAAACTCAA-3′ and 5′-GCAGAATTCGTCATGAT TTTGCTCTTTGAAAGG-3′. The cloned DNAs were sequenced on both strands.

Cell Transfection and Intracellular Ca^{2+} Measurement

HEK293 T cells and CHO dhfr(−) cells were transfected with the human NPS receptor cDNA cloned into pcDNA 3.1(+) using LipofectAmine. Stable clones were selected with 800 mg/l G418 and tested for mobilization of intracellular Ca^{2+} with 100 nM NPS (generous gift of Phoenix Pharmaceuticals, Belmont, CA). Changes in intracellular Ca^{2+} were measured in a fluorometric imaging plate reader system (FLIPR, Molecular Devices) as described before (Saito et al., 1999). Dose response curves for agonist activation were calculated from peak fluorescence values of triplicate incubations, and EC_{50} values were calculated with Prism software (GraphPad, San Diego, CA).

Radioligand Binding Assay

Y^{10}-NPS was labeled with ^{125}I using the chloramine T method and purified by reversed-phase HPLC in a collaboration with NEN Perkin Elmer (Boston, MA). CHO cells stably expressing human NPSR were seeded into 24-well plates and cultured for 48 hr. For saturation binding experiment, [^{125}I] Y^{10}-NPS at concentrations from 4 pM to 1.7 nM were used. For displacement binding, increasing concentrations of unlabeled human NPS (1 pM to 3 μM) were used to compete with 0.15 nM [^{125}I] Y^{10}-NPS. Nonspecific binding was determined in the presence of 1 μM unlabeled human NPS. The binding assay was carried out as described (Sakurai et al., 1998). In brief, cells were washed with PBS first and then incubated with radioligand with or without unlabeled NPS peptide in DMEM medium containing 0.1% bovine serum albumin at 20°C for 1.5 hr. Cells were washed five times with cold PBS and lysed with 1 N NaOH. Bound radioactivity was counted in a MicroBeta liquid scintillation counter (EG&G Wallac, Gaithersburg, MD) and corrected for counting efficiency. Data from triplicate incubations were analyzed using PRISM.

Quantitative Real-Time PCR

Tissue was collected from male and female adult Sprague-Dawley rats, and RNA was extracted with Trizol. PolyA$^+$ RNA was prepared using OligoTex (Qiagen) and converted to cDNA using oligo dT and random primers with Superscript reverse transcriptase (Invitrogen). Primers for NPSR (5′-TGCAGGGAGCAAAGATCACA-3′ and 5′-AAT CTGCATCTCATGCCTCTCA-3′), NPS precursor (5′-TGTCGCTGTCC ACAATGCAT-3′ and 5′-AATCAGATTTTCCAGACACCTTAGAAG-3′), and β-actin (5′-CACGGCATCGTCACCAACT-3′ and 5′-AGCCACAC GCAGCTCATTG-3′) were predicted using ABI Prism Primer Select software and tested for linearity of amplification using cloned cDNAs as template. Quantitative real-time PCR was performed in an ABI Prism 7000 using SYBR Green PCR Master Mix (Applied Biosystems).

In Situ Hybridization

A 326 bp fragment of the rat NPS receptor (corresponding to nt 408–734) was amplified by PCR and subcloned into pBluescript SK. A fragment of the rat NPS precursor (corresponding to nt 92–276) was cloned into the same vector. Sense and antisense riboprobes were labeled with ^{35}S-UTP. Rat tyrosine hydroxylase (TH) cDNA was a gift from Dr. Francis Leslie (UCI) and cloned in pBluescript. Rat corticotropin-releasing factor (CRF) cDNA was a gift from Dr. Christine Gall (UCI) and cloned in the same vector. For double in situ hybridization, antisense TH riboprobes or CRF riboprobes were labeled with digoxigenin using DIG RNA labeling kit (Roche Applied Science). In situ and double in situ hybridization to 20 μm coronal sections of adult Sprague-Dawley rat brains was carried out as described before (Clark et al., 2001).

Behavioral Studies

Male C57Bl/6 mice (National Cancer Institute, Bethesda, MD), age 10–14 weeks, were group housed (four animals per cage) under controlled conditions (temperature 21°C ± 2°C; relative humidity 50%–60%; 12 hr light-dark cycle, lights on 6:00 AM) with free access to food and water. Prior to drug injections, mice were briefly anesthetized with halothane. NPS was dissolved in phosphate-buffered saline (PBS, pH 7.4) and injected i.c.v. (total volume: 2 μl) as described

before (Laursen and Belknap, 1986). Mice were allowed to recover for 5 min and then placed in the observation apparatus.

For sleep studies, adult male Sprague-Dawley rats (250-300 g) were implanted under halothane anesthesia (1%–2%) with a stainless steel cannula for i.c.v. administration and a standard set of stainless steel screw electrodes for chronic sleep recordings as reported previously (Bourgin et al., 2000). Rats were injected with NPS or vehicle (5 μl) at the beginning of the light cycle, and cortical activity was recorded over 6 hr. All animal experiments had been approved by the local IACUC committee and were done in accordance with federal regulations and guidelines on animal experimentation.

Locomotion was monitored in rectangular plexiglass boxes (60 × 40 × 50 cm). Horizontal activity was measured over 60 min by 18 × 12 infrared sensors placed 2 cm above the floor. A second row of sensors at 8 cm above the floor was used to record rearing events. The imaginary central zone was defined as a 30 × 20 cm rectangle in the middle of each observation area. Data were collected using MatLab (Mathworks, Natick, MA). Experimental procedures for open field, elevated plus maze, and light-dark box were described previously (Köster et al., 1999). Marble burying was measured in mice placed individually in polypropylene cages (30 × 18 × 12 cm) containing 18 glass marbles (1.5 cm diameter) evenly spaced on 5 cm deep rodent bedding (bed-o'cob, The Andersons Inc., Maumee, OH) (Njung'e and Handley, 1991). No food or water was present during the observation period. Cages were covered with a metal grid, and the number of marbles covered at least two-thirds was counted after 30 min.

Acknowledgments

This work was supported in part by grants from NIH (R.K.R., O.C.) and the Stanley Medical Research Institute (O.C.). We thank Hans-Peter Nothacker for helpful discussions; Christine Gall for critical review of in situ hybridization results; Gary Lynch for help with locomotor experiments; and Valerie Jackson, Hua Zeng, and Alanna Pei Sun for technical assistance.

Received: March 16, 2004
Revised: July 6, 2004
Accepted: July 30, 2004
Published: August 18, 2004

References

Aston-Jones, G., Chiang, C., and Alexinsky, T. (1991a). Discharge of noradrenergic locus coeruleus neurons in behaving rats and monkeys suggests a role in vigilance. Prog. Brain Res. *88*, 501–520.

Aston-Jones, G., Shipley, M.T., Chouvet, G., Ennis, M., van Bockstaele, E., Pieribone, V., Shiekhattar, R., Akaoka, H., Drolet, G., Astier, B., et al. (1991b). Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. Prog. Brain Res. *88*, 47–75.

Berridge, C.W., and Waterhouse, B.D. (2003). The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. Brain Res. Brain Res. Rev. *42*, 33–84.

Bing, O., Moller, C., Engel, J.A., Soderpalm, B., and Heilig, M. (1993). Anxiolytic-like action of centrally administered galanin. Neurosci. Lett. *164*, 17–20.

Borsini, F., Podhorna, J., and Marazziti, D. (2002). Do animal models of anxiety predict anxiolytic-like effects of antidepressants? Psychopharmacology (Berl.) *163*, 121–141.

Bourgin, P., Huitron-Resendiz, S., Spier, A.D., Fabre, V., Morte, B., Criado, J.R., Sutcliffe, J.G., Henriksen, S.J., and de Lecea, L. (2000). Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons. J. Neurosci. *20*, 7760–7765.

Chaouloff, F., Durand, M., and Mormede, P. (1997). Anxiety- and activity-related effects of diazepam and chlordiazepoxide in the rat light/dark and dark/light tests. Behav. Brain Res. *85*, 27–35.

Charney, D.S., and Deutch, A. (1996). A functional neuroanatomy of anxiety and fear: implications for the pathophysiology and treatment of anxiety disorders. Crit. Rev. Neurobiol. *10*, 419–446.

Chojnacka-Wojcik, E., Klodzinska, A., and Pilc, A. (2001). Glutamate receptor ligands as anxiolytics. Curr. Opin. Investig. Drugs *2*, 1112–1119.

Cirelli, C., Pompeiano, M., and Tononi, G. (1996). Neuronal gene expression in the waking state: a role for the locus coeruleus. Science *274*, 1211–1215.

Civelli, O., Nothacker, H.P., Saito, Y., Wang, Z., Lin, S.H., and Reinscheid, R.K. (2001). Novel neurotransmitters as natural ligands of orphan G-protein-coupled receptors. Trends Neurosci. *24*, 230–237.

Clark, S.D., Nothacker, H.P., Wang, Z., Saito, Y., Leslie, F.M., and Civelli, O. (2001). The urotensin II receptor is expressed in the cholinergic mesopontine tegmentum of the rat. Brain Res. *923*, 120–127.

Foote, S.L., Aston-Jones, G., and Bloom, F.E. (1980). Impulse activity of locus coeruleus neurons in awake rats and monkeys is a function of sensory stimulation and arousal. Proc. Natl. Acad. Sci. USA *77*, 3033–3037.

Gottesmann, C. (2002). GABA mechanisms and sleep. Neuroscience *111*, 231–239.

Hagan, J.J., Leslie, R.A., Patel, S., Evans, M.L., Wattam, T.A., Holmes, S., Benham, C.D., Taylor, S.G., Routledge, C., Hemmati, P., et al. (1999). Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc. Natl. Acad. Sci. USA *96*, 10911–10916.

Hascoet, M., and Bourin, M. (1998). A new approach to the light/dark test procedure in mice. Pharmacol. Biochem. Behav. *60*, 645–653.

Hobson, J.A., McCarley, R.W., and Wyzinski, P.W. (1975). Sleep cycle oscillation: reciprocal discharge by two brainstem neuronal groups. Science *189*, 55–58.

Holmes, A., Kinney, J.W., Wrenn, C.C., Li, Q., Yang, R.J., Ma, L., Vishwanath, J., Saavedra, M.C., Innerfield, C.E., Jacoby, A.S., et al. (2003). Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze. Neuropsychopharmacology *28*, 1031–1044.

Hunsley, M.S., and Palmiter, R.D. (2003). Norepinephrine-deficient mice exhibit normal sleep-wake states but have shorter sleep latency after mild stress and low doses of amphetamine. Sleep *26*, 521–526.

Jones, B.E. (1991). The role of noradrenergic locus coeruleus neurons and neighboring cholinergic neurons of the pontomesencephalic tegmentum in sleep-wake states. Prog. Brain Res. *88*, 533–543.

Jones, B.E. (2003). Arousal systems. Front. Biosci. *8*, s438–s451.

Köster, A., Montkowski, A., Schulz, S., Stübe, E.M., Knaudt, K., Jenck, F., Moreau, J.L., Nothacker, H.P., Civelli, O., and Reinscheid, R.K. (1999). Targeted disruption of the orphanin FQ/nociceptin gene increases stress susceptibility and impairs stress adaptation in mice. Proc. Natl. Acad. Sci. USA *96*, 10444–10449.

Laitinen, T., Polvi, A., Rydman, P., Vendelin, J., Pulkkinen, V., Salmikangas, P., Makela, S., Rehn, M., Pirskanen, A., Rautanen, A., et al. (2004). Characterization of a common susceptibility locus for asthma-related traits. Science *304*, 300–304.

Laursen, S.E., and Belknap, J.K. (1986). Intracerebroventricular injections in mice. Some methodological refinements. J. Pharmacol. Methods *16*, 355–357.

Millan, M.J. (2003). The neurobiology and control of anxious states. Prog. Neurobiol. *70*, 83–244.

Njung'e, K., and Handley, S.L. (1991). Evaluation of marble-burying behavior as a model of anxiety. Pharmacol. Biochem. Behav. *38*, 63–67.

Ohayon, M.M., Caulet, M., and Lemoine, P. (1998). Comorbidity of mental and insomnia disorders in the general population. Compr. Psychiatry *39*, 185–197.

Paine, T.A., Jackman, S.L., and Olmstead, M.C. (2002). Cocaine-induced anxiety: alleviation by diazepam, but not buspirone, dimenhydrinate or diphenhydramine. Behav. Pharmacol. *13*, 511–523.

Paxinos, G., and Watson, C. (1997). The Rat Brain in Stereotaxic Coordinates, Compact Third Edition (San Diego: Academic Press).

Redmond, D.E., Jr., and Huang, Y.H. (1979). Current concepts. II.

New evidence for a locus coeruleus-norepinephrine connection with anxiety. Life Sci. *25*, 2149–2162.

Reinscheid, R.K., and Civelli, O. (2002). The orphanin FQ/nociceptin knockout mouse: a behavioral model for stress responses. Neuropeptides *36*, 72–76.

Rizvi, T.A., Ennis, M., Aston-Jones, G., Jiang, M., Liu, W.L., Behbehani, M.M., and Shipley, M.T. (1994). Preoptic projections to Barrington's nucleus and the pericoerulear region: architecture and terminal organization. J. Comp. Neurol. *347*, 1–24.

Rodgers, R.J., and Johnson, N.J. (1995). Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol. Biochem. Behav. *52*, 297–303.

Sah, P., Faber, E.S., Lopez De Armentia, M., and Power, J. (2003). The amygdaloid complex: anatomy and physiology. Physiol. Rev. *83*, 803–834.

Saito, Y., Nothacker, H.P., Wang, Z., Lin, S.H., Leslie, F., and Civelli, O. (1999). Molecular characterization of the melanin-concentrating-hormone receptor. Nature *400*, 265–269.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richarson, J.A., Kozlowski, G.P., Wilson, S., et al. (1998). Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell *92*, 573–585.

Saper, C.B., Chou, T.C., and Scammell, T.E. (2001). The sleep switch: hypothalamic control of sleep and wakefulness. Trends Neurosci. *24*, 726–731.

Sato, S., Shintani, Y., Miyajima, N., and Yoshimura, K. (2002). Novel G protein-coupled receptor protein and DNA thereof. World Patent Application. WO 02/31145 A1.

Shimomura, Y., Harada, M., Goto, M., Sugo, T., Matsumoto, Y., Abe, M., Watanabe, T., Asami, T., Kitada, C., Mori, M., et al. (2002). Identification of neuropeptide W as the endogenous ligand for orphan G-protein-coupled receptors GPR7 and GPR8. J. Biol. Chem. *277*, 35826–35832.

Silva, A.P., Cavadas, C., and Grouzmann, E. (2002). Neuropeptide Y and its receptors as potential therapeutic drug targets. Clin. Chim. Acta *326*, 3–25.

Sutcliffe, J.G., and de Lecea, L. (2002). The hypocretins: setting the arousal threshold. Nat. Rev. Neurosci. *3*, 339–349.

Sutin, E.L., and Jacobowitz, D.M. (1988). Immunocytochemical localization of peptides and other neurochemicals in the rat laterodorsal tegmental nucleus and adjacent area. J. Comp. Neurol. *270*, 243–270.

Swanson, L.W., and Hartman, B.K. (1975). The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. J. Comp. Neurol. *163*, 467–505.

Swanson, L.W., Sawchenko, P.E., Rivier, J., and Vale, W.W. (1983). Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. Neuroendocrinology *36*, 165–186.

Ursin, R. (2002). Serotonin and sleep. Sleep Med. Rev. *6*, 55–69.

Valentino, R.J., Pavcovich, L.A., and Hirata, H. (1995). Evidence for corticotropin-releasing hormone projections from Barrington's nucleus to the periaqueductal gray and dorsal motor nucleus of the vagus in the rat. J. Comp. Neurol. *363*, 402–422.

Valentino, R.J., Kosboth, M., Colflesh, M., and Miselis, R.R. (2000). Transneuronal labeling from the rat distal colon: anatomic evidence for regulation of distal colon function by a pontine corticotropin-releasing factor system. J. Comp. Neurol. *417*, 399–414.

Van der Werf, Y.D., Witter, M.P., and Groenewegen, H.J. (2002). The intralaminar and midline nuclei of the thalamus. Anatomical and functional evidence for participation in processes of arousal and awareness. Brain Res. Brain Res. Rev. *39*, 107–140.

Wilson, S., Bergsma, D.J., Chambers, J.K., Muir, A.I., Fantom, K.G., Ellis, C., Murdock, P.R., Herrity, N.C., and Stadel, J.M. (1998). Orphan G-protein-coupled receptors: the next generation of drug targets? Br. J. Pharmacol. *125*, 1387–1392.

Note added in proof

During the preparation of this manuscript a mutant form of the NPS receptor was identified as a candidate gene for asthma and termed GPRA (G protein-coupled receptor for asthma susceptibility) (Laitinen et al., 2004).