

NEUROPEPTIDE S PROMOTES WAKEFULNESS THROUGH ACTIVATION OF THE POSTERIOR HYPOTHALAMIC HISTAMINERGIC AND OREXINERGIC NEURONS

P. ZHAO,¹ Y. F. SHAO,¹ M. ZHANG, K. FAN, X. P. KONG, R. WANG AND Y. P. HOU*

Departments of Neuroscience, Anatomy, Histology, and Embryology, Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, 199 Donggang Xi Road, Lanzhou 730000, PR China

Abstract—In spite of the initial and pivotal findings that the newly identified neuropeptide S (NPS) promotes arousal associated with locomotor and anxiolytic-like effects, the mechanisms through which NPS acts to modulate sleep-waking states remain unclear. The present study was undertaken to investigate in the rat the effects of i.c.v. injection of NPS on the EEG, sleep-wake cycle, and brain c-Fos expression. NPS at 0.1 and 1 nmol increased significantly wakefulness (W) during the first 2 h (54.7 ± 3.2 and 64.9 ± 2.1 min, respectively, vs. 41.4 ± 2.5 min seen with saline injections, $P < 0.01$ and $P < 0.001$), accompanied by an increase in EEG high frequency activities (14.5–60 Hz). In the meanwhile, slow wave sleep (SWS) and paradoxical sleep (PS) decreased significantly. *Ex-vivo* Fos immunohistochemistry in the posterior hypothalamus revealed that, as compared with saline-treated rats, NPS enhanced c-Fos expression in histaminergic neurons by 76.0% in the ventral tuberomammillary nucleus (TMN) and 57.8% in the dorsal TMN, and in orexinergic neurons by 28.2% in the perifornical nucleus (PeF), 24.3% in the dorsomedial hypothalamic nucleus (DMH), and 13.7% in the lateral hypothalamic area (LH) of the posterior hypothalamus. The NPS-induced c-Fos expression in histaminergic neurons and orexinergic neurons where NPS receptor (NPSR) mRNA is highly expressed, suggests that NPS activates histaminergic and orexinergic neurons to promote W. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: neuropeptide S, sleep-waking states, c-Fos, histamine neuron, orexin neuron, cortical activation.

Neuropeptide S (NPS) is a newly identified neuromodulator located in the brainstem. NPS selectively binds with high affinity to Gs and Gq protein-coupled receptors, identified as GPR154 previously and now referred to as NPSR,

¹ These authors contributed equally to this work.

*Corresponding author. Tel: +86-931-8915886; fax: +86-931-8915886. E-mail address: houyiping@lzu.edu.cn (Y. P. Hou).

Abbreviations: ANOVA, analysis of variance; Arc, arcuate hypothalamic nucleus; DAB, 3,3' diaminobenzidine; DMH, dorsomedial hypothalamic nucleus; DTMN, dorsal tuberomammillary nucleus; EEG, electroencephalogram; EMG, electromyogram; FFT, fast Fourier transform; HA, histamine; ir, immunoreactive; LH, lateral hypothalamic area; LSD, least significant difference; NPS, neuropeptide S; NPSR, neuropeptide S receptor; Ox/Hcrt, orexin/hypocretin; PAG, periaqueductal gray; PBS, phosphate buffer saline; PeF, perifornical nucleus; PFA, paraformaldehyde; PS, paradoxical sleep; REM, rapid eye movement; SWS, slow wave sleep; VTMN, ventral tuberomammillary nucleus; W, wakefulness.

producing mobilization of intracellular Ca^{2+} and increases in cAMP levels (Xu et al., 2004). NPS precursor mRNA in the rat is expressed in a group of neurons located between the locus coeruleus and Barrington's nucleus, the principle sensory trigeminal nucleus, and the lateral parabrachial nucleus (Xu et al., 2004). In contrast, NPSR mRNA was found widely distributed in the brain, mainly in the cortex, thalamus, hypothalamus, amygdala, and subiculum (Xu et al., 2004, 2007).

This profile of receptor expression suggests the involvement of NPS-NPSR system in the regulation of multiple central functions. Indeed, activation of NPSR by i.c.v. injection of NPS enhances locomotor activity, evokes anxiolytic-like effects in mice, and promotes wakefulness in rats (Xu et al., 2004). NPS also produces antinociception in mice (Li et al., 2009), facilitates relapse to cocaine seeking in rats (Kallupi et al., 2010), controls fear expression and extinction (Jungling et al., 2008), and is involved in memory processes in mice (Han et al., 2009; Okamura et al., 2011).

The NPS-NPSR system is proposed as a newly identified arousal system (Adamantidis et al., 2010), and its interaction with other arousal systems is presumed to promote wakefulness (Guerrini et al., 2010). Although NPS has been shown to promote arousal in rats (Xu et al., 2004), its effects on the architectures of sleep-waking states and the mechanisms involved remain unclear. NPSR mRNA has been reported to be highly expressed in several structures of the rat posterior hypothalamus known to play a major role in the regulation of arousal, including the histaminergic tuberomammillary nucleus (TMN), and the orexinergic perifornical nucleus (PeF), dorsomedial hypothalamic nucleus (DMH), and lateral hypothalamic area (LH) (Xu et al., 2007). More recent evidence indicates that NPS-immunoreactive fibers overlap with NPSR mRNA expression in the mouse posterior hypothalamus and suggests that the brainstem NPS-producing nuclei project to the hypothalamic regions involved in arousal (Clark et al., 2011). It is well documented that histamine (HA)-containing neurons and orexin/hypocretin (Ox/Hcrt)-containing neurons send widespread projections to most brain areas and contribute to tonic cortical activation during wakefulness (Lin, 2000; Lin et al., 2011). We then hypothesized that NPS activates HA and Ox neurons to promote brain arousal.

The present study was designed to characterize the sleep-wake effects of NPS in the rat following its i.c.v. injection. Attempts were also made to identify the potential neuronal targets of NPS in the posterior hypothalamus involved in arousal by analyzing c-Fos expression using *ex vivo* Fos immunohistochemistry.

EXPERIMENTAL PROCEDURES

Animals and surgical implantation

Adult male Sprague–Dawley rats, weighing 250–300 g (8–10 weeks old, $n=30$), were purchased from the Experimental Animal Center of Lanzhou University (Lanzhou, PR China). They were housed at an ambient temperature (22 ± 1 °C) with a relative humidity of 50% on an automatically controlled 12:12-h light/dark cycle (lights on 8:00–20:00 h, illumination intensity ≈ 100 lx). Food and water were available *ad libitum*. All animals were cared for, and experiments were conducted in accordance with the European Community guidelines for the use of experimental animals (86/609/EEC). The experimental protocol was approved by the Ethics Committee of Lanzhou University.

Under chloral hydrate anesthesia (350 mg/kg, i.p.), four stainless-steel screws ($\varnothing=1$ mm) as electroencephalogram (EEG) electrodes were screwed into the skull, and three silver wire as electromyogram (EMG) electrodes were inserted into the dorsal cervical neck muscles for polysomnographic recordings. The cortical electrodes were inserted into the dura through two pairs of holes located, respectively, in the frontal (1 mm lateral and anterior to the bregma) and parietal (1 mm lateral to the lambda) cortices. A guide cannula ($\varnothing=0.6$ mm, length=28 mm) was stereotaxically implanted into the right lateral ventricle (AP -0.9 , ML $+1.5$, DV -3.3 , according to the atlases of Paxinos and Watson, 1998) for i.c.v. injection. The free ends of the electrode leads were soldered into a pedestal socket. The pedestal socket and cannula were chronically fixed to skull with dental cement.

Polygraphic recordings and drug administrations

After surgery, the animals were housed singly in transparent barrels and monitored by an infrared video camera in the recording chamber during both the light and dark phases, and allowed to recover for 1 week. After the rats were acclimated to the recording cable for 2 days, a 24-h basal sleep-wake cycle was performed. EEG and EMG signals were amplified and digitalized with a resolution of 256 and 128 Hz, respectively, using a CED 1401 MK II (Cambridge Electronic Design Limited, London, UK).

The mouse NPS (SFRNGVSGAKKTSFRRAKQ), a gift from Prof. Wang, was synthesized by Department of Biochemistry and Molecular Biology, School of Life Science, Lanzhou University (Chang et al., 2005; Peng et al., 2010). The rat NPS (SFRNGVSGVKKTSFRRAKQ) was purchased from Phoenix Pharmaceuticals (005-88, Burlingame, CA, USA). Fresh NPS (0.1–1 nmol) dissolved in saline was dosed at 10 h (i.e. 2 h after lights on) with 2.5 μ l per rat to detect awakening effect, because rats exhibit maximal sleep in this period. NPS or saline i.c.v. injection was carried out through the guide cannula with the flow rate 1 μ l/min after a baseline recording of sleep-wake cycle. In our preliminary experiments, we found central administration of both mouse and rat NPS evoked equally the alteration of sleep-waking states. The data shown in the present study were the effect of the mouse NPS on sleep-waking states.

Immunohistochemistry

Tissue preparation. One hour and a half after the NPS (1 nmol, $n=5$) or saline ($n=4$) i.c.v. administration, the animals were deeply anesthetized with chloral hydrate and perfused via the ascending aorta with 200 ml saline followed by 300 ml ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). Their brains were removed, postfixed in 4% PFA for 24 h, and immersed in 30% sucrose solution in PBS overnight at 4 °C and coronally sectioned (30 μ m) on a cryostat (CM1900, Leica Microsystems, Heidelberg, Germany) at -20 °C. One set of sections was used for Fos immunostaining, whereas the other set for double immunostaining of Fos and histidine decarboxylase (HDC,

the histamine-synthesizing enzyme) or of Fos and orexin-A (Ox-A).

Fos immunostaining. The floating sections were rinsed in 0.01 M PB saline (PBS, pH 7.4), treated 30 min in 3% H_2O_2 in PBS, and incubated in blocking solution (10% bovine serum in PBS) for 1 h. Sections were incubated with a rabbit polyclonal antibody against c-Fos (1:5000, sc-253, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS containing 1% bovine serum for 48 h at 4 °C on an agitator. After rinsing in PBS, sections were incubated with a biotinylated goat anti-rabbit IgG (1:1000, AP132B, Millipore, Temecula, CA, USA), then with horseradish peroxidase conjugated streptavidin (1:1000, SA202, Millipore, Temecula, CA, USA). Both incubations were at 4 °C overnight on an agitator. Following rinsing, the sections were immersed in 0.05 M Tris–HCl buffer, pH 7.6, containing 0.05% 3,3' diaminobenzidine (DAB), 0.01% H_2O_2 , and 0.6% nickel ammonium sulfate for 2–5 min at room temperature.

Double immunostaining for Fos and HDC/Ox-A. After Fos immunostaining in which Fos-immunoreactive (-ir) neurons were visualized as a black reaction product, sections were incubated with guinea pig anti-HDC (1:4000, 16046, Progen Biotechnik GmbH, Heidelberg, Germany) or rabbit anti-Ox-A (1:2000, AB3704, Millipore, Temecula, CA, USA) in PBS containing 1% bovine serum for 48 h at 4 °C on an agitator. The specificity of the anti-HDC antibody has been demonstrated in several species in previous studies (Murata et al., 2005; Agis et al., 2006), including rats (Umehara et al., 2011). After several rinses in PBS, sections were respectively incubated with biotinylated goat anti-guinea pig IgG (1:1000, BA-7000, Vector Laboratories, Burlingame, CA, USA) or goat anti-rabbit IgG (1:1000, AP132B, Millipore) overnight at 4 °C, and followed by incubation with horseradish peroxidase conjugated streptavidin overnight at 4 °C. Finally, HDC- and Ox-ir neurons were visualized as a brown reaction product after 2-min detection in a Tris–HCl buffer containing 0.05% 3,3' DAB and 0.01% H_2O_2 .

Data analysis

Demarcation of the sleep-waking states and analysis of cortical EEG power spectra. The sleep-waking states were defined by the EEG and EMG signal recordings and behaviors monitored with video camera. Using a Spike 2 (CED, London, UK) script and with the assistance of spectral analysis by the fast Fourier transform (FFT), polygraphic records were visually scored by 30-s epochs for wakefulness (W), slow wave sleep (SWS), and paradoxical (or rapid eye movement, REM) sleep (PS) according to previously described criteria validated for rats (Maloney et al., 1997).

Cell counting. In rats treated with NPS and saline, Fos-ir, HDC-ir, Ox-A-ir, Fos-ir+HDC-ir, and Fos-ir+Ox-A-ir neurons were counted bilaterally on four evenly spaced sections per animal throughout HA-containing neural populations in the ventral TMN (VTMN) and the dorsal TMN (DTMN), and Ox-containing neural populations in the PeF, LH, and DMH. Counting was performed on sections from identical retrocaudal levels, and a mean value for the two sides was calculated.

Statistical analysis. All data were expressed as means \pm SEM. Different parameters were analyzed using one-way analysis of variance (ANOVA) and post hoc Fisher's least significant difference (LSD) test: total amounts of sleep-wake stages and their mean episode number and duration (Fig. 2), sleep-wake amounts per 2 h (Fig. 3). One-way ANOVA was also used to compare the number of Fos-ir neurons or the percentage of HDC-ir and Ox-A-ir neurons expressing Fos-ir between NPS and saline administrated groups. In all statistical comparisons, the criterion for significance was set at $P<0.05$.

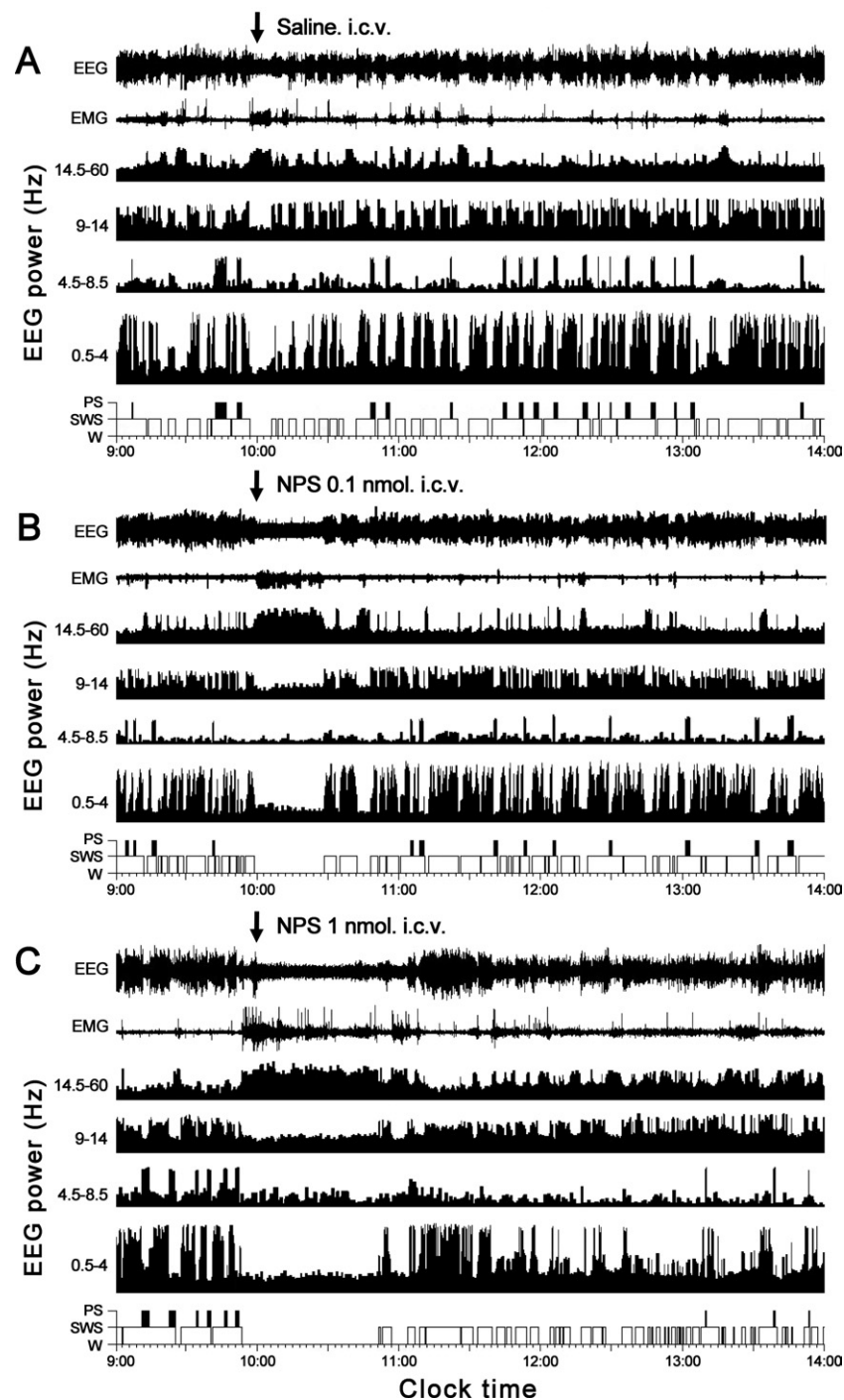


Fig. 1. Effects of i.c.v. injection of NPS on the EEG and sleep-wake cycle in the rat. Representative 5-h (9:00–14:00 h) EEG, EMG, hypnograms, and cortical power spectra at 0.5–4, 4.5–8.5, 9.0–14.0, and 14.5–60 Hz in rats administrated respectively with saline (A), NPS 0.1 nmol (B), and 1 nmol (C) at 10:00 h are shown. Note that NPS induced an increase in wakefulness accompanied by an increase in cortical beta and gamma (14.5–60 Hz) activity and a decrease in SWS and PS and suppression of cortical alpha (9.0–14.0 Hz), theta (4.5–8.5 Hz), and delta (0.5–4.0 Hz) activity.

RESULTS

Effects of NPS on sleep-waking states and cortical EEG

Typical examples of the EEG, EMG, cortical power spectra, and the corresponding hypnograms from rats respec-

tively given saline, 0.1, and 1 nmol of NPS were presented in Fig. 1. As shown, i.c.v. administration of 0.1 and 1 nmol of NPS immediately induced a cortical EEG of fast and low-voltage activity and a dense EMG activity. The effects lasted for 1 (0.1 nmol) to 3 h (1 nmol) dependent on the doses used. During these periods, time spent in W in-

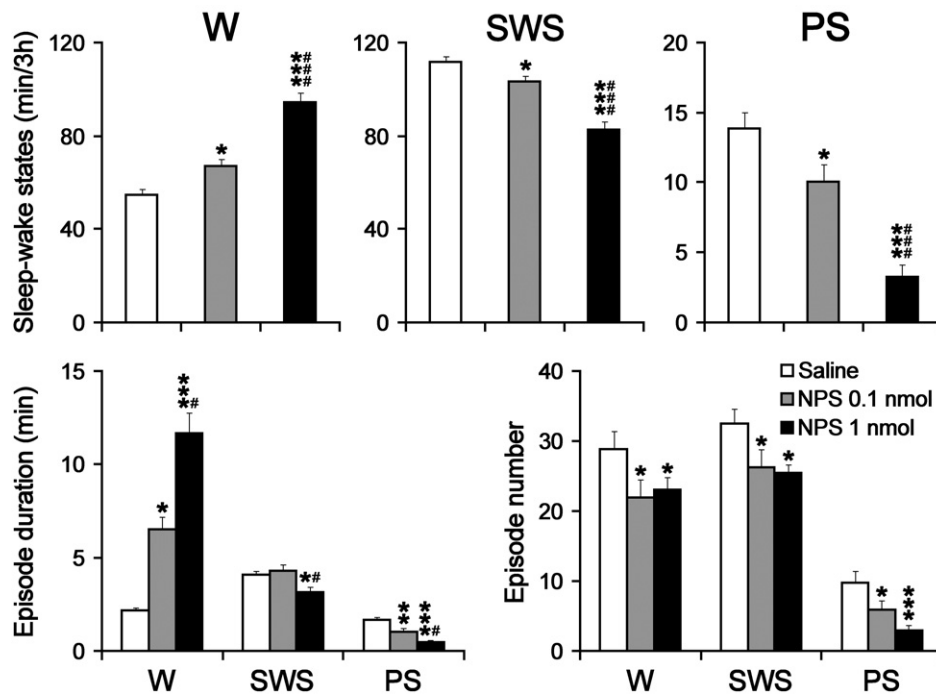


Fig. 2. Total amount and episode duration and number of sleep-wake states for 3 h postinjection of NPS and saline. Open, gray, and black bars respectively show the profiles for saline-, 0.1, and 1 nmol of NPS-treated rats. Values are means \pm SEM ($n=10$ rats in each group). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with saline; # $P<0.05$, ### $P<0.001$, dose of 1 nmol NPS compared with dose of 0.1 nmol NPS. Statistics were analyzed by one-way ANOVA and followed by Fisher's LSD test.

increased significantly, whereas SWS and PS were suppressed or decreased (Figs. 1–3).

Cortical EEG power spectral analysis showed that NPS caused promptly a marked increase in beta and gamma (14.5–60 Hz) activities, a suppression of spindles (9–14 Hz), theta (4.5–8.5 Hz), and delta activities (0.5–4 Hz) (Fig. 1C) compared with that of saline treatment (Fig. 1A). The effects lasted for nearly 1 h at the dose of 0.1 nmol (Fig. 1B) and more than 1 h for the dose of 1 nmol (Fig. 1C). The recovery of each cortical EEG band after the activating effect of NPS occurred during the 2nd–4th hour post dosing (Fig. 1C).

An analysis of sleep-wake amounts during 3 h post NPS injection showed that the arousing and sleep-suppressing effects of NPS were greater using 1 nmol than using 0.1 nmol. In addition to the quantitative analysis shown in Fig. 2, we found in fact that W was increased up to 173% of the control level (saline) using 1 nmol vs. 123% using 0.1 nmol, whereas SWS and PS were decreased by 26% and 77%, respectively, vs. 8% and 27% (Fig. 2, upper). Thus, the effects of centrally administrated NPS appeared to be dose related. Moreover, the increase in W was because of prolonged wake episode duration and not to an increased episode number. In fact, the wake episode number decreased after NPS injection. With regard to SWS and PS, their decrease was because of a reduction of both episode number and duration (Fig. 2, lower).

An analysis of sleep-wake amounts per 2 h over 12 h further revealed that the main effects of NPS on W, SWS, and PS occurred during the first 2 h section post injection using 0.1 nmol, whereas those using 1 nmol extended to

the second 2 h section. Thus, the arousing and sleep-suppressing effects of NPS were longer lasting using 1 nmol than using 0.1 nmol. Starting from the 5th hour, all sleep wake amounts returned to their control level (saline) (Fig. 3).

NPS-induced c-Fos labeling in the posterior hypothalamus

Following central administration of NPS (1 nmol), moderate to dense Fos-ir neurons were seen in the cerebral cortices including motor and somatosensory cortex and the amygdala and the periaqueductal gray (PAG) of the midbrain, the number was greater than that seen with saline injection. In the posterior hypothalamus, a great number of Fos-ir neurons were found in the TMN, arcuate hypothalamic nucleus (Arc) (Fig. 4A–C), PeF, DMH, and LH (Fig. 5A–C). In comparison with saline administration, NPS significantly increased the number of Fos-ir neurons by 6.6-fold (403.1 ± 5.1 vs. 61.4 ± 3.5) in the VTMN, 5.9-fold (219.0 ± 15.7 vs. 37.4 ± 5.6) in the DTMN (Fig. 4D), 2.6-fold (1120.6 ± 71.9 vs. 439.0 ± 67.2) in the DMH, 5.2-fold (604.5 ± 33.4 vs. 117.0 ± 16.9) in the PeF, and 3.6-fold (282.4 ± 13.1 vs. 79.3 ± 13.7) in the LH (Fig. 5D).

To determine the Fos expression in HA-containing neurons in the TMN and in Ox-containing neurons in the PeF, LH, and DMN, Fos-ir staining combined with HDC-ir and Ox-A-ir staining were performed (Table 1; Fig. 4A–C, A'–C'; Fig. 5A–C, A'–C'). NPS central administration increased the number of dual labeling of Fos-ir+HDC-ir neurons in HDC-ir cells by 76% (i.e. $308.5\pm 9.0/353.8\pm 11.4$ vs.

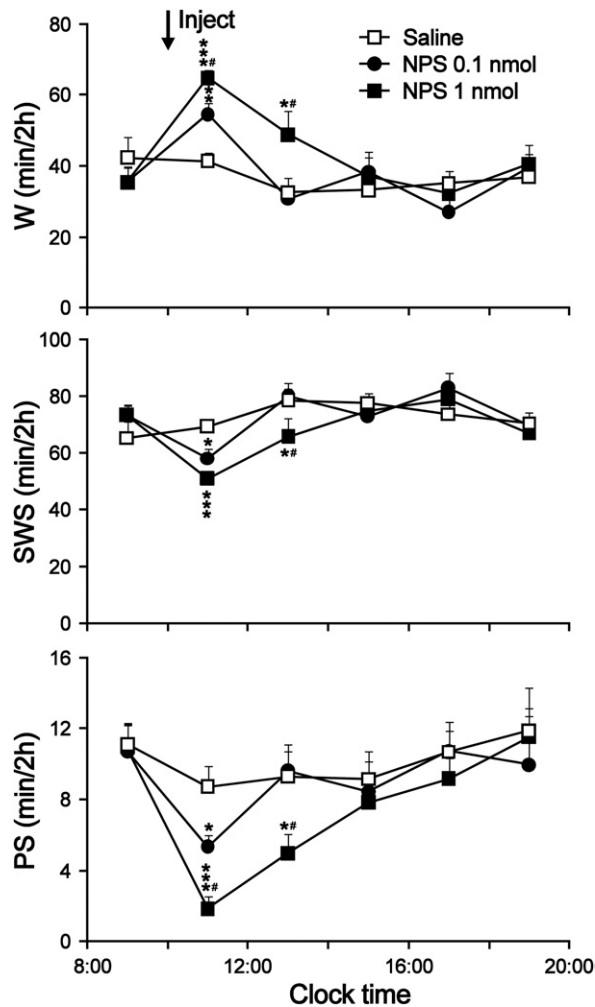


Fig. 3. Analysis (per 2 h) of sleep-wake amounts before and after NPS i.c.v. injection during the lights-on phase. Note that NPS significantly increased wakefulness (W) and decreased slow wave sleep (SWS) and paradoxical sleep (PS). At dose of 1 nmol, the effect of NPS was greater and longer lasting than those induced at dose of 0.1 nmol. Values represent means \pm SEM ($n=10$ rats in each group). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with saline; # $P<0.05$, dose of 1 nmol NPS compared with dose of 0.1 nmol NPS. Statistics were analyzed by one-way ANOVA and followed by Fisher's LSD test.

$40.0\pm 1.5/350.5\pm 16.6$, Table 1) in the VTMN and 57.8% in the DTMN (Fig. 4D'), and the number of dual labeling of Fos-ir+Ox-A-ir neurons in Ox-A-ir cells by 28.2% in the PeF, by 24.3% in the DMH, and 13.7% in the LH (Fig. 5D'), compared respectively to the corresponding nucleus in rats administrated with saline (Figs. 4D' and 5D').

DISCUSSION

The present study showed that NPS i.c.v. administration increased significantly wakefulness and decreased concomitantly SWS and PS. The effects were accompanied by signs of EEG activation such as enhancement of cortical fast rhythms and decrease in spindles and slow activities. The W-increasing effect was because of a significant increase in the mean duration of wake episode (Figs. 1–3).

These findings are complementary to the earlier observation that NPS promotes wakefulness (Xu et al., 2004). The arousal effect of NPS has also been demonstrated by using the test of righting reflex. NPS dose dependently (0.01–1 nmol) reduced the proportion of mice losing the righting reflex induced by a hypnotic dose of diazepam in response to the benzodiazepine and their sleep time (Rizzi et al., 2008). It also reduced the ketamine- and thiopental-induced anesthesia time in rats (Kushikata et al., 2011). In humans, evidence for a role of the NPS and NPSR system in arousal and sleep regulation has been also provided by a recent genetic epidemiology study performed on 749 subjects showing a clear association between the Asn/Ile 107 NPSR polymorphism and mean bedtime delay (Gottlieb et al., 2007). These findings support the hypothesis that the NPS and NPSR system constitutes one of the brain arousal systems (Adamantidis et al., 2010; Guerrini et al., 2010).

We found that at 1 nmol, NPS dosing enhanced wakefulness promptly, and the effect lasted for 2–3 h (Fig. 3), longer than that of about 1 h reported by Xu et al. (2004). This difference could be because of the use of NPS from different species. In fact, the present study used mouse and rat NPS, whereas Xu et al. (2004) used a human NPS. We also noted that the arousing effect of NPS dosing at 10 nmol was similar to that of 1 nmol (not shown), suggesting that 1 nmol NPS would probably already activate maximally its brain targets.

In the present study, high dose of NPS i.c.v. injection induced a suppression of PS for 3 h, and that was not followed by any rebound of PS (Figs. 1–3). The NPS precursor mRNA has been identified by *in situ* hybridization in a previously undefined group of neurons located between the locus coeruleus (LC) and Barrington's nucleus (Xu et al., 2004). The majority of NPS-expressing neurons in the LC area and the principal sensory trigeminal nucleus are glutamatergic neurons, whereas many NPS-positive neurons in the lateral parabrachial nucleus coexpress corticotropin-releasing factor (CRF) (Xu et al., 2007). NPS seems to be coexpressed 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 (Xu et al., 2007). Based on the observation that NPS induces quite long-lasting PS suppression, it was postulated that NPS neurons, like other brainstem arousal system including noradrenergic/serotonergic neurons, act as PS-Off neurons to suppress PS in part by lifting inhibition upon the cholinergic PS-On cells (Jones, 2004).

More importantly, our study aims at the identification of the potential targets of NPS promoting arousal through examination of neurons expressing Fos, the product of the immediate early gene that is expressed in association with neuronal activation (Morgan and Curran, 1986; Dragunow and Faull, 1989). Our results showed that central administration of NPS enhanced the number of Fos-ir neurons in several posterior hypothalamic structures including the TMN, PeF, LH, DMH, and Arc (Figs. 4 and 5), as well as in the cerebral cortices including motor and somatosensory

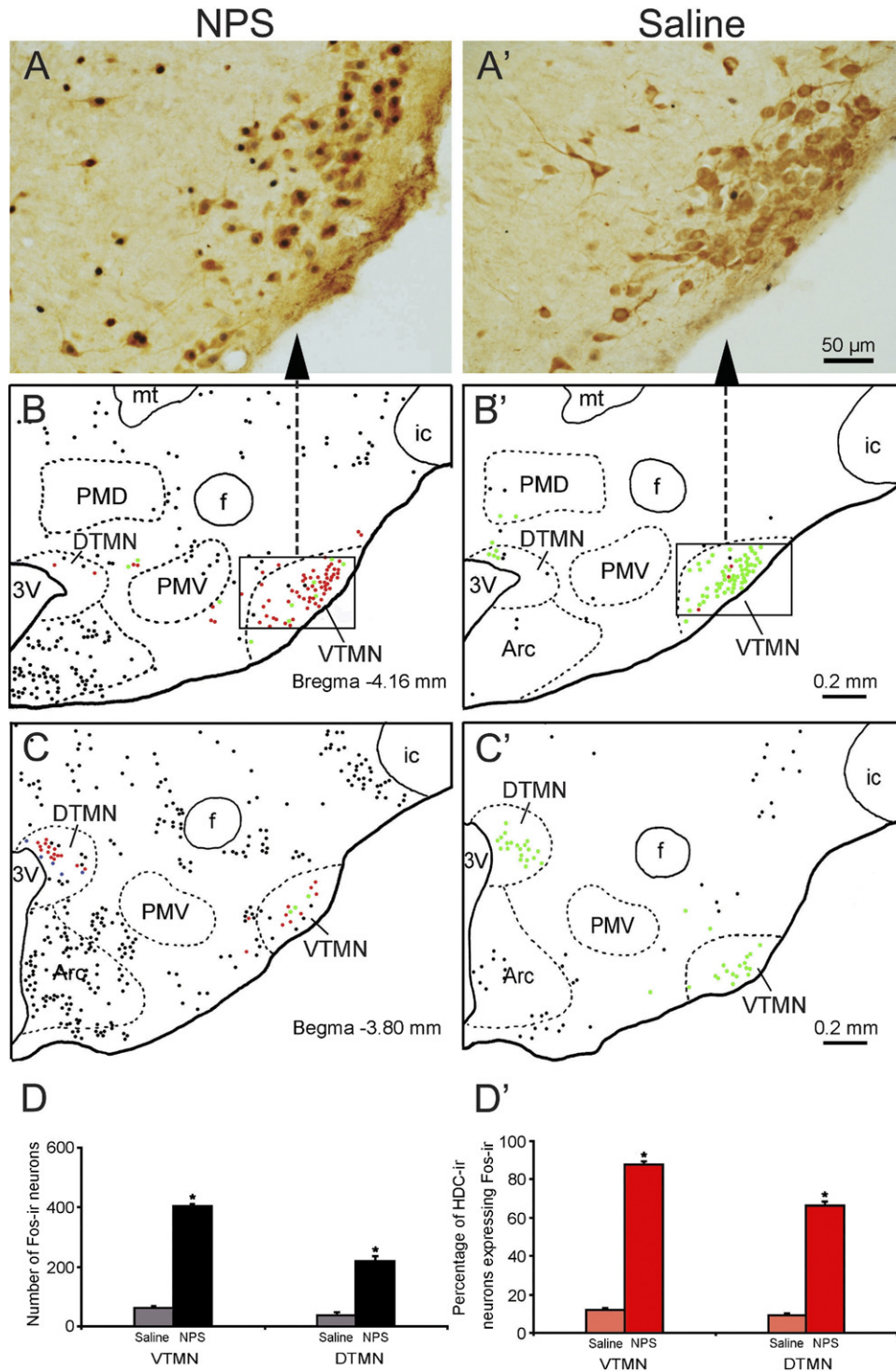


Fig. 4. Effects of i.c.v. injection of NPS on Fos immunoreactivity in histaminergic neurons of the TMN and adjacent areas in the rat. (A, A') Photomicrographs show Fos-ir and HDC-ir neurons in the VTMN. (B-C, B'-C') Camera lucida drawings of frontal sections show the distributions of Fos-ir (black dots), HDC-ir (green dots), and double staining of Fos-ir+HDC-ir neurons (red dots) in the VTMN and DTMN. (D, D') Histograms show quantitative analysis of the number of Fos-ir neurons and the percentage of Fos-ir+HDC-ir in HDC-ir neurons in the VTMN and DTMN following NPS ($n=5$) and saline ($n=4$) i.c.v. injection. Values represent means \pm SEM. * $P < 0.001$ compared with saline group. Statistics were analyzed by one-way ANOVA and followed by Fisher's LSD test. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

cortex and the amygdala and the PAG of the midbrain (not shown) compared with that of saline.

It is well known that TMN HAergic neurons and orexinergic neurons in the lateral hypothalamic areas, where

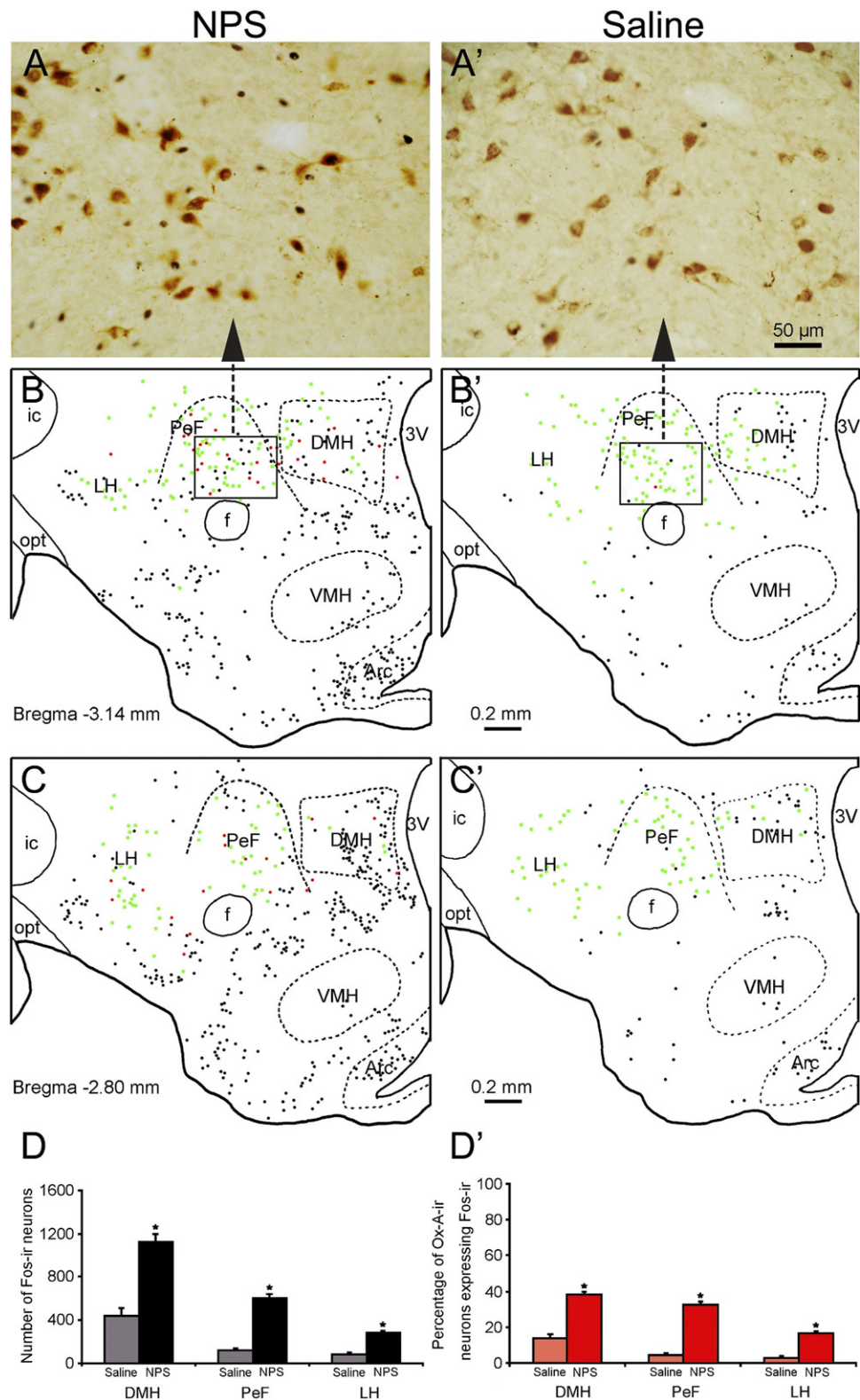


Fig. 5. Effects of i.c.v. injection of NPS on Fos immunoreactivity in orexinergic neurons in the PeF, DMH, and LH in the rat. (A, A') Photomicrographs show Fos-ir and Ox-A-ir neurons of the PeF. (B–C, B'–C') Camera lucida drawings of frontal sections show the distributions of Fos-ir (black dots), Ox-A-ir (green dots), and dual staining of Fos-ir+Ox-A-ir neurons (red dots) in the DMH, PeF, and LH. (D, D') Histograms show quantitative analysis of the number of Fos-ir neurons and the percentage of Fos-ir+Ox-A-ir in Ox-A-ir neurons in the DMH, PeF, and LH following NPS ($n=5$) and saline ($n=4$) i.c.v. injection. Values represent means \pm SEM. * $P<0.001$ compared with saline group. Statistics were analyzed by one-way ANOVA and followed by Fisher's LSD test. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Table 1. Number of HDC-ir and dual labeling of Fos-ir+HDC-ir neurons in the VTMN and DTMN, and Ox-A-ir and dual labeling of Fos-ir+Ox-A-ir neurons in the DMH, PeF, and LH after NPS or saline i.c.v. injection

	HDC-ir		Fos-ir+HDC-ir		Ox-A-ir			Fos-ir+Ox-A-ir		
	VTMN	DTMN	VTMN	DTMN	DMH	PeF	LH	DMH	PeF	LH
NPS (n=5)	353.8±11.4	142.8±9.9	308.5±9.0*	95.2±8.9*	134.6±5.5	461.6±11.8	209±8.5	51.2±1.7*	151.7±3.2*	33.8±1.6*
Saline (n=4)	350.5±16.6	138.0±7.2	40.0±1.5	11.8±1.3	143.0±5.3	469.8±3.1	208.8±11.3	19.8±3.6	22.0±1.4	5.5±0.3

Values expressed as means±SEM. Cells were counted bilaterally on four evenly spaced sections per animal. **P*<0.001 compared with saline-treated rats. Statistics were analyzed by one-way ANOVA and followed by Fisher's LSD test.

NPSR mRNA is highly expressed (Xu et al., 2007; Kallupi et al., 2010), play a major role in the regulation of arousal (see Lin, 2000; Lin et al., 2011). Our results showed that dual staining of Fos-ir+HDC-ir neurons in HDC-ir neurons, respectively represented 87.4% and 66.4% in the histaminergic VTMN and DTMN, and that dual staining of Fos-ir+Ox-A-ir neurons in Ox-A-ir neurons represented 38.1%, 32.8%, and 16.1% in the orexinergic DMH, the PeF, and LH after NPS central administration (Table 1, Figs. 4 and 5). HAergic and orexinergic neurons send widespread projections to most brain areas (Lin, 2000; Sakurai, 2007; Haas et al., 2008). HAergic neurons discharge tonically during waking in the cat and mouse (Vanni-Mercier et al., 2003; Takahashi et al., 2006), constituting the most wake-selective firing pattern so far identified in the brain. Pharmacological dosing impairing brain HA neurotransmission increases cortical slow activity. Conversely, enhancement of HA transmission promotes wakefulness (Monti, 1993; Lin, 2000; Passani et al., 2004; Parmentier et al., 2007). A large body of evidence indicates that an orexin deficiency is responsible for the pathogenesis of human and animal narcolepsy (Chemelli et al., 1999; Lin et al., 1999). Orexin neurons increase their activity during active waking and in close association with locomotion and postural changes, and they virtually cease firing during SWS and PS (Lee et al., 2005; Takahashi et al., 2008). Anatomical/functional interactions between HAergic and orexinergic neurons, particularly, a direct excitation of HA neurons by Ox, have been identified (Bayer et al., 2001; Eriksson et al., 2001; Lin et al., 2002; Yamanaka et al., 2002). Taken together, these data indicate that NPS activates both HAergic and orexinergic neurons in the posterior hypothalamus through NPSR to promote W. The fact that NPS induced more Fos-ir expression in HA neurons than in Ox neurons suggests a stronger activation of HAergic neurons than orexinergic neurons. Recent studies indicate that Ox, but not HA, promotes wakefulness through enhanced locomotion and suggest that HAergic and orexinergic neurons exert a distinct, but complementary and synergistic control of wakefulness. Orexin would be more involved in the behavioral aspects of W, notably locomotion, food intake, and emotional activities, whereas HA would be mainly responsible for the qualitative and cognitive aspects during W, notably maintaining a tonic cortical EEG activation (Parmentier et al., 2002; Anacleto et al., 2009; Sakurai, 2007; Lin et al., 2011 for reviews).

CONCLUSIONS

Central administration of NPS enhanced significantly wakefulness and reduced SWS and PS. These effects were accompanied by a marked enhancement of c-Fos expression in the posterior hypothalamus, notably histaminergic neurons in the TMN and, to a less extent, orexinergic neurons in the PeF, LH, and DMH. These results suggest that NPS promotes wakefulness and cortical activation through activation of histaminergic and orexinergic neurons.

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