Neuropeptide S increases motor activity and thermogenesis in the rat through sympathetic activation

Takuya Ensho[a], Keiko Nakahara[a]*, Yoshihiro Suzuki[b] and Noboru Murakami[a]

[a]*Department of Veterinary Physiology, Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192, Japan*

[b]*Laboratory of Animal Health Science, School of Veterinary Medicine, Kitasato University, Aomori 034-8628, Japan*

* To whom correspondence should be addressed:
Keiko Nakahara PhD
Department of Veterinary Physiology
Faculty of Agriculture
University of Miyazaki, Miyazaki 889-2155, Japan
Tel. and Fax: +81-985-58-7265
E-mail: a0d209u@cc.miyazaki-u.ac.jp

Abstract

The central role of neuropeptide S (NPS), identified as the endogenous ligand for GPR154, now named neuropeptide S receptor (NPSR), has not yet been fully clarified. We examined the central role of NPS for body temperature, energy expenditure, locomotor activity and adrenal hormone secretion in rats. Intracerebroventricular (icv) injection of NPS increased body temperature in a dose-dependent manner. Energy consumption and locomotor activity were also significantly increased by icv injection of NPS. In addition, icv injection of NPS increased the peripheral blood concentration of adrenalin and corticosterone. Pretreatment with the β1- and β2-adrenergic receptor blocker timolol inhibited the NPS-induced increase of body temperature. The expression of both NPS mRNA in the brainstem and NPSR mRNA in the hypothalamus showed a nocturnal rhythm with a peak occurring during the first half of the dark period. To examine whether the endogenous NPS is involved in regulation of body temperature, NPSR antagonist SHA68 was administered one hour after darkness. SHA68 attenuated the nocturnal rise of body temperature. These results suggest that NPS contributes to the regulation of the sympathetic nervous system.

Key Words: neuropeptide S, thermoregulation, sympathetic nervous system, energy expenditure

1. Introduction

Neuropeptide S (NPS), comprising 20 amino acid residues, was originally identified as an endogenous ligand for the orphan receptor GPR154, now named neuropeptide S receptor (NPSR), in 2004 (Koob and Greenwell, 2004; Xu et al., 2004). The structure of NPS has been highly conserved in terrestrial vertebrates (Reinscheid, 2007). Expression of mRNA for the NPS precursor has been recognized mainly in the peri locus ceruleus, lateral parabrachial nucleus and trigeminal nucleus (Clark et al., 2011; Xu et al., 2007; Xu et al., 2004). On the other hand, it has been reported that expression of mRNA for NPSR is widely distributed in the brain, suggesting that NPS and the NPSR system may be involved in multiple central nervous functions (Clark et al., 2011; Liu et al., 2011; Xu et al., 2007).

In vitro studies have shown that NPS acts through an increase of intracellular cyclic AMP and mobilization of intracellular Ca^{2+} (Xu et al., 2004), and an increase of glutamatergic synaptic transmission to intercalated GABAergic neurons in the amygdala via presynaptic NPSR on connected principal neurons (Jüngling et al., 2008). NPS has also been reported to behave as a potent inhibitor of serotonin and noradrenalin release from mouse frontal cortex synaptosomes (Raiteri et al., 2009). *In vivo* studies, on the other hand, have examined the effects of intracerebroventricular (icv) injection of NPS on behavior in mice and rats: icv injection of NPS stimulates locomotor activity (Fendt et al., 2011; Mochizuki et al., 2010; Pañeda et al., 2009; Rizzi et al., 2008; Roth et al., 2006; Smith et al., 2006; Xu et al., 2004), increases plasma ACTH and corticosterone (Smith et al., 2006), increases wakefulness and decreases sleeping time (Xu et al., 2004; Zhao et al., 2012), increases the urge for ethanol (Badia-Elder et al., 2008) or cocaine (Kallupi et al., 2010; Pañeda et al., 2009) use, and inhibits or stimulates food intake (Beck et al., 2005; Fedeli et al., 2009; Niimi, 2006). In addition,

icv injection of NPS improves spatial memory (Lukas and Neumann, 2012; Okamura et al., 2011; Zhao et al., 2010), time in center entries in an open field (Jüngling et al., 2008; Xu et al., 2004), time on open arms in an elevated plus maze (Jüngling et al., 2008; Rizzi et al., 2008; Xu et al., 2004), and time in the light area in a light/dark box (Pañeda et al., 2009; Xu et al., 2004).

Recently it has been shown that icv injection of several neuronal peptides increases or decreases body temperature, locomotor activity, heart rate and energy consumption through activation of the sympathetic or parasympathetic nervous system (Billington et al., 1991; Currie and Coscina, 1995; Inoue et al., 2013; Lawrence et al., 2002; Mondal et al., 2003; Nakahara et al., 2016; Nakazato et al., 2000; Monda et al., 2006; Messina et al., 2016a). Thermogenesis can be either exercise-induced or non-exercise-induced. Non-exercise-induced thermogenesis includes all forms of energy expenditure not associated with formal exercise and includes spontaneous physical activity as well as thermogenesis via basal metabolism and brown adipose tissue (Argyropoulos and Harper, 2002; Riley et al., 2016; De Luca et al., 2008; Messina et al., 2012; Messina et al., 2013; Messina et al., 2016c; Messina et al., 2016b). Basal metabolism is regulated by sympathetic activity, whereas thermogenesis by brown adipose tissue is under the control of the sympathetic nervous system and endocrine system (Whittle and Vidal-Puig, 2012). When we preliminarily examined the expression of NPSR mRNA in the brain, relatively high expression was observed in the hypothalamus, which is an important region for homeostasis of body temperature and energy metabolism. Therefore, we speculated that NPS would be involved in thermogenesis or energy consumption. Therefore, in the present study, we examined whether NPS is involved in regulation of body temperature, and if so, whether NPS influences the autonomic

nervous system.

2. Materials and Methods

2.1. Animals

Male wistar rats (Charles River Japan, Inc., Yokohama, Japan) weighing 300-350 g were housed in individual plexiglas cages (420×250×200 mm) in an animal room maintained under a constant light-dark cycle (lights on 7:00-19:00 h) and temperature (22 ± 1˚C). Food and water were provided *ad libitum.* All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care, and the experiments were authorized by Miyazaki University Animal Experiment Committee (authorization number: 2012-006-5).

2.2. ICV injection of NPS

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (Kyoritsu Seiyaku Corporation, Tokyo, Japan; 50 mg/kg BW) and were then mounted in a Narushige brain stereotaxic instrument (Narushige, Tokyo). A stainless steel cannula (guide cannula, 23-gauge; insert, 27-gauge) was then implanted into the lateral left ventricle. The cannula tip was placed at the following stereotaxic coordinates: 8 mm anterior to the interaural; 1.5 mm lateral to the midline; 3.0 mm below the dura. The guide cannula was anchored to the skull with machine screws and dental acrylic. During a 4-day postoperative recovery period, the rats became accustomed to the handling procedure. NPS (Sigma-Aldrich Co. LLC) was dissolved in saline (0.01, 0.1, 0.5 and 1.0, 5.0 nmol/10 μl), and then injected into each free-moving rat through a 27-gauge injection cannula connected to a 50-μl Hamilton syringe.

2.3. Measurement of body temperature

Rectal temperature was measured with a rectal probe thermocouple thermometer (Unique Medical Co., Ltd., Tokyo, Japan). Until the experiment day, rats habituated to the measurement of rectal temperature well. The experiment began at 7:30 and NPS (0.5, 1 nmol /10 μl) or saline was administered at 8:00, measurements being conducted for the following 60 minutes. The average value during the 30 minutes before administration was assumed to be that at zero min, and the values obtained thereafter were indicated as increases or decreases. Room temperature was maintained at subthermoneutrality (i.e., 22 ± 1˚C).

Back surface temperature in free-moving animals was recorded using infrared thermographic imaging (FLIR SC620, FLIR Systems, Danderyd, Sweden) as described in our previous paper (Inoue et al., 2013). We started infrared thermographic imaging of the back surface temperature from 7:30 h. Images taken at 1-min intervals were saved during the following 30 min. Thereafter, NPS was administered icv, and measurements were conducted for the following 120 min. Room temperature was maintained at subthermoneutrality (i.e., 22 ± 1˚C). The FLIR SC620 has a thermal resolution lower than 0.04 °C, an accuracy of ± 2%, and a picture resolution of 640 × 480 pixels. The average value during the 10 min before administration was assumed to be that at zero min, and the values obtained thereafter were indicated as increases or decreases.

2.4. Measurement of energy expenditure and calorie consumption

Rats were placed individually in Oxymax (Columbus Instrument, Columbus, OH, USA) recording cages, and oxygen consumption and carbon dioxide emission were measured continuously at 10-min intervals for 2 days. Calorie consumption per hour (kcal/hr) was then calculated. Calorie consumption in 60 min (kcal) was calculated as

the average calorie consumption per hour (kcal/hr) from 10 minutes to 60 minutes after administration. Room temperature was maintained at subthermoneutrality (i.e., 22 ± 1˚C). Body weight and daily food intake did not change throughout the measurement period.

2.5. Measurement of locomotor activity

Locomotor activity was measured using a rat locomotor activity recording system (Muromachi Co. Ltd., Tokyo, Japan) comprising infrared sensors, an interface and a computer. The infrared sensors were placed above the cages and measured all locomotor activity (e.g. eating, walking and grooming). After the surgery, rats were housed in the cage in order to habituate to the test chamber in four days. Each cage with its infrared sensor was placed in an isolated chamber. Data were collected at 10-min intervals and analyzed using CompactACT AMS software (Muromachi Co.). NPS or saline was administered icv at 9:00 h. After the injections, the rats were immediately returned to their individual cages. Locomotor activity counts were determined every 10 min and summed for the 3-h period following administration.

2.6. Measurement of the plasma concentrations of adrenalin and corticosterone

Rats were killed by decapitation 15 min after icv injection of NPS (1.0 nmol) at 8:00 h. Plasma adrenalin and corticosterone levels were then measured using EIA kits (CUSABIO, China: Catalog number CSB-E08678r for adrenalin, Cayman Chemical Company, USA, Catalog number 500655 for corticosterone).

2.7. Effect of sympathetic nerve blocker on NPS-induced change of body temperature

To examine whether the sympathetic nervous system is involved in the effect of NPS on thermoregulation, an antagonist of the β1- and β2-adrenergic receptors, timolol (Sigma–Aldrich Co.) was pre-administered 30 min before NPS treatment at 08:00 h. Timolol (0.5 mg/kg BW) was administered intraperitoneally. The doses were decided on the basis on our previous study (Nakahara et al., 2016).

2.8. Analysis of mRNA expression

Each of the tissues removed were immediately dissected out and homogenized in TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) to extract the total RNA, which was then purified using a RNeasy Micro Kit (QIAGEN GmbH, Hilden, Germany). Real-time quantitative PCR was carried out by the method reported previously (Nakahara et al., 2016) using TaqMan Universal Master Mix II (Applied Biosystems) with primers to amplify the target genes. For those genes, probe/primer kits were purchased from Applied Biosystems (TaqMan Gene Expression Assay ID: Rn01524824_ml, GenBank NM: NM_001106808.1 for NSPR, Assay ID: Rn04339527_ml, GenBank NM: NM_001271113.1 for NPS, Assay ID: Rn00565393_ml, GenBank NM: NM_013108.2 for *β*3-adrenergic receptor, Assay ID: Rn00562126_ml, GenBank NM: NM_012682.2 for UCP1). The expression level of each mRNA was evaluated as a ratio relative to that of *β*2-microglobulin mRNA.

2.9. Effect of NPSR antagonist on thermoregulation

To examine whether the NPSR is involved in the effect of NPS on thermoregulation, SHA68 (Tocris Bioscience, USA), an antagonist of the NPSR, was administered. After having dissolved it in DMSO, SHA68 diluted 1,000 times in DW. SHA68 (0.5 nmol/5 μl) was injected into the lateral ventricle at 20:00 h, and then 60 minutes after the

injection, we measured the back surface temperature for 10 minutes and calculated the average. Manipulations under dark conditions were performed using night vision infrared binoculars. Before the experiment, we checked the pretreatment of SHA68 (0.5 nmol/5 μl) prevent the increase of back surface temperature induced by NPS (0.5nmol).

2.10. Statistical analysis

The data (mean ± SEM) were analyzed statistically by Student's *t* test or two-way repeated measures ANOVA using GraphPad Prism software (v.5.0.1). The t-value and P-value for data analyzed by Student's *t* test are shown in the figure legend, as are F-value and P-value for data analyzed by two-way repeated measures ANOVA. Diurnal rhythmicity of daily variation was analyzed by the cosinor method. Differences at $P<0.05$ were considered statistically significant.

3. Results

3.1. Effects of icv injection of NPS on body temperature

To evaluate the effects of NPS on body temperature, we carried out infrared thermographic measurement of back surface temperature and measurement of rectal temperature with a rectal probe thermocouple thermometer. When the changes in rectal temperature and those in back surface temperature were compared after treatment with NPS, the results were almost the same for the two parameters (Fig. 1 A vs 1 C), i.e. NPS increased body temperature (both rectal and back surface), and this effect was dependent on the dose of NPS (Fig. 1 B). The NPS (1 nmol)-induced increase in the back surface temperature continued for at least 1 hour, and then returned to the basal level after 2 hours (data not shown).

3.2. Effects of icv injection of NPS on locomotor activity

Icv injection of both saline and NPS increased the locomotor activity, because rats might be stimulated by the injection of these. Although injections stimulated rats, there was a significant difference between the saline- and NPS-treated groups, the increase in the NPS group being greater (Fig. 2 A, B).

3.3. Effects of icv injection of NPS on energy expenditure

Icv injection of both saline and NPS increased the energy expenditure, because rats might be stimulated by the injection of these. Although injections stimulated rats, there was a significant difference between the saline- and NPS-treated groups, the increase in the NPS group being greater (Fig. 2 C, D).

3.4. Effects of icv injection of NPS on plasma concentrations of adrenalin

Icv injection of NPS caused a significant increase in the plasma concentrations of adrenalin and corticosterone 15 minutes after injection. The concentration of adrenalin was increased approximately three-fold after icv injection of NPS (Fig. 2 E, F).

3.5. Effects of sympathetic nerve blocker on NPS-induced hyperthermia

Pretreatment with the sympathetic β1- and β2-adrenergic receptor blocker timolol followed by saline treatment had no effect on back surface temperature during the light period. Pretreatment with timolol prevented the increase of back surface temperature induced by NPS (Fig. 3 A, B).

3.6. Nocturnal rhythm of NPS and NPSR mRNA expression in the hypothalamus and brainstem

Expression of NPS mRNA in the brainstem, which includes the locus ceruleus, showed diurnal variation (Fig. 4 A). Analysis of diurnal rhythmicity using the cosinor method revealed a nocturnal rhythm of NPS mRNA expression in the brainstem (amplitude: 0.256, mesor: 23:02±165 min, $p<0.05$). We examined the expression of mRNA for NPSR in the central nervous system including the olfactory bulb, cerebral cortex, hippocampus, hypothalamus and brainstem during the light and dark periods. NPSR mRNA expression was confirmed in all regions except for the olfactory bulb. The highest expression of NPSR mRNA was observed in the hypothalamus, being higher in the dark period than in the light period (Fig. 4 C). When the difference of NPSR mRNA expression in the hypothalamus was examined in more detail, it showed a nocturnal rhythm with a peak just after lights off (Fig. 4 B).

3.7. Effect of NPSR antagonist on thermoregulation

To examine whether the endogenous NPS contributes to the thermoregulation, we measured the back surface temperature after icv injection of NPSR antagonist (SHA68). The temperature of SHA68-treated group showed lower than that of saline-treated group in the dark phase (Fig. 5).

4. Discussion

The present study is the first report that NPS contributes to the regulation of the sympathetic nervous system. We demonstrated that icv injection of NPS increased the body temperature, locomotor activity and energy consumption of rats. At least with

regard to the rise in body temperature, sympathetic nerves appeared to be activated by NPS, since pretreatment with the sympathetic β1- and β2-adrenergic receptor blocker timolol abrogated the NPS-induced increase in back surface temperature, and the plasma concentration of adrenalin was increased approximately three-fold after icv injection of NPS. The expression of NPS mRNA in the brainstem and NPSR mRNA in the hypothalamus showed a diurnal variation with peaks in the dark period. This diurnal variation of NPS mRNA and NPSR mRNA suggest that NPS signaling is activated in the dark phase. Inhibition of endogenous NPS in the dark phase caused a decrease of back surface temperature. These results suggest that endogenous NPS related to the regulation of body temperature.

There is a possibility that NPS might have increased body temperature, locomotor activity and energy expenditure through activation of sympathetic tone and/or increasing wakefulness, since NPS has been shown to induce arousal in rats and mice (Xu et al., 2004; Zhao et al., 2012). Icv injection of NPS prolongs the period of wakefulness and reduces the length of all stages of sleep (Xu et al., 2004; Zhao et al., 2012). In contrast, it has been reported that icv injection of a NPSR antagonist shortens waking time and lengthens non-REM sleep time (Oishi et al., 2014). NPSR-*KO* mice show lower activity during the dark period (Duangdao et al., 2009; Fendt et al., 2011). In addition, the present findings indicate that icv injection of NPS increased sympathetic tone, since the plasma concentration of adrenalin was increased approximately three-fold after icv injection of NPS.

Icv injection of NPS increased locomotor activity and plasma concentration of corticosterone, in agreement with previous reports (Fendt et al., 2011; Mochizuki et al., 2010; Pañeda et al., 2009; Rizzi et al., 2008; Roth et al., 2006; Smith et al., 2006; Xu et al., 2004). Two previous studies have indicated that icv injection of NPS reduced the

stress-induced hyperthermia response (Leonard et al., 2008; Rizzi et al., 2008). In these experiments, the rectal temperature was measured for the baseline temperature. One report showed that icv injection of NPS increased the baseline temperature (Leonard et al., 2008). Another report showed that icv injection of NPS did not increase the baseline temperature (Rizzi et al., 2008). However, in those studies, the rectal temperature was measured 60 minutes after injection. In the present study, at 60 minutes after icv injection, rectal temperature did not differ significantly between saline and NPS. We were able to detect the effect of NPS on thermogenesis in the present study because we measured rectal temperature at 15-min intervals and back surface temperature at 1-min intervals. In addition, it seems likely that our measurement of rectal temperature would impose less stress on animals than the previous reports, because rats were habituated well for the measurement of rectal temperature.

Some neuronal peptides have been shown to exert effects similar to those of autonomic nerves. Central administration of some anorexigenic neuronal peptides, such as neuropeptide W (Mondal et al., 2003), neuromedin U (Nakazato et al., 2000) and neuromedin S (Nakahara et al., 2016) increases body temperature, heart rate, energy consumption and locomotor activity. The thermogenesis induced by neuromedin U and neuromedin S is inhibited by blockers of the sympathetic nervous system (Nakahara et al., 2016). On the other hand, central administration of some orexigenic peptides, such as neuropeptide Y (Billington et al., 1991; Currie and Coscina, 1995) and ghrelin (Inoue et al., 2013; Lawrence et al., 2002), decreases body temperature through activation of the parasympathetic nervous system. Like such peptides, NPS may also exert autonomic nerve-like effects.

5. Conclusions

Central administration of NPS increases body temperature, energy consumption, locomotion activity and blood adrenalin release, through activation of the sympathetic nervous system. Expression of NPS mRNA in the brainstem and NPSR mRNA in the hypothalamus showed a nocturnal rhythm with peaks in the dark period. Central administration of NPSR antagonist attenuates the rise of body temperature. These results suggest that NPS stimulates motor activity, energy expenditure and body temperature through sympathetic activation.

Acknowledgements

This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan, and by AMED-CREST to NM.

Reference

Argyropoulos, G., Harper, M.E., 2002. Uncoupling proteins and thermoregulation. J Appl Physiol (1985) 92, 2187-2198. http://dx.doi.org/10.1152/japplphysiol.00994.2001

Badia-Elder, N.E., Henderson, A.N., Bertholomey, M.L., Dodge, N.C., Stewart, R.B., 2008. The effects of neuropeptide S on ethanol drinking and other related behaviors in alcohol-preferring and -nonpreferring rats. Alcohol. Clin. Exp. Res. 32, 1380-1387. http://dx.doi.org/10.1111/j.1530-0277.2008.00713.x

Beck, B., Fernette, B., Stricker-Krongrad, A., 2005. Peptide S is a novel potent inhibitor of voluntary and fast-induced food intake in rats. Biochem. Biophys. Res. Commun. 332, 859-865. http://dx.doi.org/10.1016/j.bbrc.2005.05.029

Billington, C.J., Briggs, J.E., Grace, M., Levine, A.S., 1991. Effects of intracerebroventricular injection of neuropeptide Y on energy metabolism. Am. J.

Physiol. 260, R321-327.

Clark, S.D., Duangdao, D.M., Schulz, S., Zhang, L., Liu, X., Xu, Y.L., Reinscheid, R.K., 2011. Anatomical characterization of the neuropeptide S system in the mouse brain by in situ hybridization and immunohistochemistry. J. Comp. Neurol. 519, 1867-1893. http://dx.doi.org/10.1002/cne.22606

Currie, P.J., Coscina, D.V., 1995. Dissociated feeding and hypothermic effects of neuropeptide Y in the paraventricular and perifornical hypothalamus. Peptides 16, 599-604.

De Luca, V., Viggiano, E., Messina, G., Viggiano, A., Borlido, C., Viggiano, A., Monda, M., 2008. Peripheral amino Acid levels in schizophrenia and antipsychotic treatment. Psychiatry Investig. 5, 203-208. http://dx.doi.org/10.4306/pi.2008.5.4.203

Duangdao, D.M., Clark, S.D., Okamura, N., Reinscheid, R.K., 2009. Behavioral phenotyping of neuropeptide S receptor knockout mice. Behav. Brain Res. 205, 1-9. http://dx.doi.org/10.1016/j.bbr.2009.07.024

Fedeli, A., Braconi, S., Economidou, D., Cannella, N., Kallupi, M., Guerrini, R., Calò, G., Cifani, C., Massi, M., Ciccocioppo, R., 2009. The paraventricular nucleus of the hypothalamus is a neuroanatomical substrate for the inhibition of palatable food intake by neuropeptide S. Eur. J. Neurosci. 30, 1594-1602. http://dx.doi.org/10.1111/j.1460-9568.2009.06948.x

Fendt, M., Buchi, M., Bürki, H., Imobersteg, S., Ricoux, B., Suply, T., Sailer, A.W., 2011. Neuropeptide S receptor deficiency modulates spontaneous locomotor activity and the acoustic startle response. Behav. Brain Res. 217, 1-9. http://dx.doi.org/10.1016/j.bbr.2010.09.022

Inoue, Y., Nakahara, K., Maruyama, K., Suzuki, Y., Hayashi, Y., Kangawa, K.,

Murakami, N., 2013. Central and peripheral des-acyl ghrelin regulates body temperature in rats. Biochem. Biophys. Res. Commun. 430, 278-283. http://dx.doi.org/10.1016/j.bbrc.2012.10.137

Jüngling, K., Seidenbecher, T., Sosulina, L., Lesting, J., Sangha, S., Clark, S.D., Okamura, N., Duangdao, D.M., Xu, Y.L., Reinscheid, R.K., Pape, H.C., 2008. Neuropeptide S-mediated control of fear expression and extinction: role of intercalated GABAergic neurons in the amygdala. Neuron 59, 298-310. http://dx.doi.org/10.1016/j.neuron.2008.07.002

Kallupi, M., Cannella, N., Economidou, D., Ubaldi, M., Ruggeri, B., Weiss, F., Massi, M., Marugan, J., Heilig, M., Bonnavion, P., de Lecea, L., Ciccocioppo, R., 2010. Neuropeptide S facilitates cue-induced relapse to cocaine seeking through activation of the hypothalamic hypocretin system. Proc. Natl. Acad. Sci. U. S. A. 107, 19567-19572. http://dx.doi.org/10.1073/pnas.1004100107

Koob, G.F., Greenwell, T.N., 2004. Neuropeptide S: a novel activating anxiolytic? Neuron 43, 441-442. http://dx.doi.org/10.1016/j.neuron.2004.08.007

Lawrence, C.B., Snape, A.C., Baudoin, F.M., Luckman, S.M., 2002. Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. Endocrinology 143, 155-162. http://dx.doi.org/10.1210/endo.143.1.8561

Leonard, S.K., Dwyer, J.M., Sukoff Rizzo, S.J., Platt, B., Logue, S.F., Neal, S.J., Malberg, J.E., Beyer, C.E., Schechter, L.E., Rosenzweig-Lipson, S., Ring, R.H., 2008. Pharmacology of neuropeptide S in mice: therapeutic relevance to anxiety disorders. Psychopharmacology (Berl.) 197, 601-611. http://dx.doi.org/10.1007/s00213-008-1080-4

Liu, X., Zeng, J., Zhou, A., Theodorsson, E., Fahrenkrug, J., Reinscheid, R.K., 2011.

Molecular fingerprint of neuropeptide S-producing neurons in the mouse brain. J. Comp. Neurol. 519, 1847-1866. http://dx.doi.org/10.1002/cne.22603

Lukas, M., Neumann, I.D., 2012. Nasal application of neuropeptide S reduces anxiety and prolongs memory in rats: social versus non-social effects. Neuropharmacology 62, 398-405. http://dx.doi.org/10.1016/j.neuropharm.2011.08.016

Messina, A., De Fusco, C., Monda, V., Esposito, M., Moscatelli, F., Valenzano, A., Carotenuto, M., Viggiano, E., Chieffi, S., De Luca, V., Cibelli, G., Monda, M., Messina, G., 2016a. Role of the Orexin System on the Hypothalamus-Pituitary-Thyroid Axis. Front Neural Circuits 10, 66. http://dx.doi.org/10.3389/fncir.2016.00066

Messina, G., Chieffi, S., Viggiano, A., Tafuri, D., Cibelli, G., Valenzano, A., Triggiani, A.I., Messina, A., De Luca, V., Monda, M., 2016b. Parachute Jumping Induces More Sympathetic Activation Than Cortisol Secretion in First-Time Parachutists. Asian J. Sports Med. 7, e26841. http://dx.doi.org/10.5812/asjsm.26841

Messina, G., De Luca, V., Viggiano, A., Ascione, A., Iannaccone, T., Chieffi, S., Monda, M., 2013. Autonomic nervous system in the control of energy balance and body weight: personal contributions. Neurol. Res. Int. 2013, 639280. http://dx.doi.org/10.1155/2013/639280

Messina, G., Di Bernardo, G., Viggiano, A., De Luca, V., Monda, V., Messina, A., Chieffi, S., Galderisi, U., Monda, M., 2016c. Exercise increases the level of plasma orexin A in humans. J. Basic Clin. Physiol. Pharmacol. 27, 611-616. http://dx.doi.org/10.1515/jbcpp-2015-0133

Messina, G., Vicidomini, C., Viggiano, A., Tafuri, D., Cozza, V., Cibelli, G., Devastato, A., De Luca, B., Monda, M., 2012. Enhanced parasympathetic activity of sportive

women is paradoxically associated to enhanced resting energy expenditure. Auton. Neurosci. 169, 102-106. http://dx.doi.org/10.1016/j.autneu.2012.05.003

Mochizuki, T., Kim, J., Sasaki, K., 2010. Microinjection of neuropeptide S into the rat ventral tegmental area induces hyperactivity and increases extracellular levels of dopamine metabolites in the nucleus accumbens shell. Peptides 31, 926-931. http://dx.doi.org/10.1016/j.peptides.2010.02.006

Monda, M., Viggiano, A., Viggiano, A., Viggiano, E., Messina, G., Tafuri, D., De Luca, V., 2006. Quetiapine lowers sympathetic and hyperthermic reactions due to cerebral injection of orexin A. Neuropeptides 40, 357-363. http://dx.doi.org/10.1016/j.npep.2006.07.003

Mondal, M.S., Yamaguchi, H., Date, Y., Shimbara, T., Toshinai, K., Shimomura, Y., Mori, M., Nakazato, M., 2003. A role for neuropeptide W in the regulation of feeding behavior. Endocrinology 144, 4729-4733. http://dx.doi.org/10.1210/en.2003-0536

Nakahara, K., Akagi, A., Shimizu, S., Tateno, S., Qattali, A.W., Mori, K., Miyazato, M., Kangawa, K., Murakami, N., 2016. Involvement of endogenous neuromedin U and neuromedin S in thermoregulation. Biochem. Biophys. Res. Commun. http://dx.doi.org/10.1016/j.bbrc.2016.01.155

Nakazato, M., Hanada, R., Murakami, N., Date, Y., Mondal, M.S., Kojima, M., Yoshimatsu, H., Kangawa, K., Matsukura, S., 2000. Central effects of neuromedin U in the regulation of energy homeostasis. Biochem. Biophys. Res. Commun. 277, 191-194. http://dx.doi.org/10.1006/bbrc.2000.3669

Niimi, M., 2006. Centrally administered neuropeptide S activates orexin-containing neurons in the hypothalamus and stimulates feeding in rats. Endocrine 30, 75-79. http://dx.doi.org/10.1385/ENDO:30:1:75

Oishi, M., Kushikata, T., Niwa, H., Yakoshi, C., Ogasawara, C., Calo, G., Guerrini, R., Hirota, K., 2014. Endogenous neuropeptide S tone influences sleep-wake rhythm in rats. Neurosci. Lett. 581, 94-97. http://dx.doi.org/10.1016/j.neulet.2014.08.031

Okamura, N., Garau, C., Duangdao, D.M., Clark, S.D., Jüngling, K., Pape, H.C., Reinscheid, R.K., 2011. Neuropeptide S enhances memory during the consolidation phase and interacts with noradrenergic systems in the brain. Neuropsychopharmacology 36, 744-752. http://dx.doi.org/10.1038/npp.2010.207

Pañeda, C., Huitron-Resendiz, S., Frago, L.M., Chowen, J.A., Picetti, R., de Lecea, L., Roberts, A.J., 2009. Neuropeptide S reinstates cocaine-seeking behavior and increases locomotor activity through corticotropin-releasing factor receptor 1 in mice. J. Neurosci. 29, 4155-4161. http://dx.doi.org/10.1523/JNEUROSCI.5256-08.2009

Raiteri, L., Luccini, E., Romei, C., Salvadori, S., Calò, G., 2009. Neuropeptide S selectively inhibits the release of 5-HT and noradrenaline from mouse frontal cortex nerve endings. Br. J. Pharmacol. 157, 474-481. http://dx.doi.org/10.1111/j.1476-5381.2009.00163.x

Reinscheid, R.K., 2007. Phylogenetic appearance of neuropeptide S precursor proteins in tetrapods. Peptides 28, 830-837. http://dx.doi.org/10.1016/j.peptides.2007.01.008

Riley, C.L., Dao, C., Kenaston, M.A., Muto, L., Kohno, S., Nowinski, S.M., Solmonson, A.D., Pfeiffer, M., Sack, M.N., Lu, Z., Fiermonte, G., Sprague, J.E., Mills, E.M., 2016. The complementary and divergent roles of uncoupling proteins 1 and 3 in thermoregulation. J. Physiol. 594, 7455-7464. http://dx.doi.org/10.1113/JP272971

Rizzi, A., Vergura, R., Marzola, G., Ruzza, C., Guerrini, R., Salvadori, S., Regoli, D., Calo, G., 2008. Neuropeptide S is a stimulatory anxiolytic agent: a behavioural study in mice. Br. J. Pharmacol. 154, 471-479. http://dx.doi.org/10.1038/bjp.2008.96

Roth, A.L., Marzola, E., Rizzi, A., Arduin, M., Trapella, C., Corti, C., Vergura, R., Martinelli, P., Salvadori, S., Regoli, D., Corsi, M., Cavanni, P., Caló, G., Guerrini, R., 2006. Structure-activity studies on neuropeptide S: identification of the amino acid residues crucial for receptor activation. J. Biol. Chem. 281, 20809-20816. http://dx.doi.org/10.1074/jbc.M601846200

Smith, K.L., Patterson, M., Dhillo, W.S., Patel, S.R., Semjonous, N.M., Gardiner, J.V., Ghatei, M.A., Bloom, S.R., 2006. Neuropeptide S stimulates the hypothalamo-pituitary-adrenal axis and inhibits food intake. Endocrinology 147, 3510-3518. http://dx.doi.org/10.1210/en.2005-1280

Whittle, A.J., Vidal-Puig, A., 2012. NPs -- heart hormones that regulate brown fat? J. Clin. Invest. 122, 804-807. http://dx.doi.org/10.1172/JCI62595

Xu, Y.L., Gall, C.M., Jackson, V.R., Civelli, O., Reinscheid, R.K., 2007. Distribution of neuropeptide S receptor mRNA and neurochemical characteristics of neuropeptide S-expressing neurons in the rat brain. J. Comp. Neurol. 500, 84-102. http://dx.doi.org/10.1002/cne.21159

Xu, Y.L., Reinscheid, R.K., Huitron-Resendiz, S., Clark, S.D., Wang, Z., Lin, S.H., Brucher, F.A., Zeng, J., Ly, N.K., Henriksen, S.J., de Lecea, L., Civelli, O., 2004. Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. Neuron 43, 487-497. http://dx.doi.org/10.1016/j.neuron.2004.08.005

Zhao, P., Shao, Y.F., Zhang, M., Fan, K., Kong, X.P., Wang, R., Hou, Y.P., 2012. Neuropeptide S promotes wakefulness through activation of the posterior hypothalamic histaminergic and orexinergic neurons. Neuroscience 207, 218-226. http://dx.doi.org/10.1016/j.neuroscience.2012.01.022

Zhao, Z., Huang, L., Wu, H., Li, Y., Zhang, L., Yin, Y., Xiang, Z., 2010. Neuropeptide S

mitigates spatial memory impairment induced by rapid eye movement sleep deprivation in rats. Neuroreport 21, 623-628. http://dx.doi.org/10.1097/WNR.0b013e328339b5f9

Figure legends

Figure 1

Icv injection of NPS increased the body temperature. Effect of icv injection of saline and NPS (A, B) (baseline temperature: Saline: 37.07±0.187, NPS 0.01 nmol: 37.06±0.363, NPS 0.1 nmol: 37.05±0.084, NPS 0.5 nmol: 36.74±0.128, NPS 1 nmol: 37.29±0.211, NPS 5 nmol: 37.28±0.076). The difference from baseline temperature is displayed every minute (A) (saline vs 1 nmol: $F=4.735$ $P<0.00001$) The area under the curve is displayed in (B) (*saline vs 0.1 nmol: $t=2.528$ $P=0.0281$, vs 0.5 nmol: $t=2.850$ $P=0.016$, vs 1.0 nmol: $t=3.757$ $P=0.003$, vs 5.0 nml: $t=4.260$ $P=0.001$) on back surface temperature. Effect of icv injection of saline and NPS on rectal temperature (C, D) (baseline temperature: Saline: 37.85±0.139, NPS 0.5 nmol: 37.86±0.181, NPS 1 nmol: 37.98±0.097). The difference from baseline temperature is displayed every 15 minute (A) (saline vs 1 nmol: $F=2.748$ $P=0.0414$). The area under the curve is displayed in (B) (*saline vs 1.0 nmol $t=3.875$ $P=0.003$). Each bar graph on the right side represents the area under the curve (AUC) of temperature from the baseline for 60 min after injection. Each symbol or bar and vertical line represents the mean ± SEM (n=6). Asterisks indicate significant differences from the saline group (*$P<0.05$).

Figure 2

Effect of icv injection of saline or 1 nmol NPS on locomotor activity (n=5) (A). Bar graph represents the AUC of locomotor activity counts for 60 min after icv injection of saline, 0.5 nmol or 1 nmol NPS (B). Effect of icv injection of saline, 0.5 nmol or 1 nmol NPS on calorie consumption. Each bar graph represents the calorie consumption in 60 min after icv injection of saline or NPS (n=7) (C). Bar graph represents the average

calorie consumption from 10 minutes to 60 minutes after administration of saline or NPS (D). Effect of icv injection of 1 nmol NPS on plasma adrenalin (E) and corticosterone (F). Samples of blood were collected 15 min after injection of saline or NPS. Each symbol or bar and vertical line represents the mean ± SEM. Asterisks indicate significant differences vs saline-treated group (*: $P<0.05$). (A saline vs 1 nmol: $F=4.697$ $P<0.00001$, B saline vs 0.5 nmol: $t=3.119$ $P=0.0097$, saline vs 1 nmol: $t=6.296$ $P<0.001$, C saline vs 1nmol: $F=2.452$ $P=0.0185$,D saline vs 1 nmol: $t=3.469$ $P=0.005$, E saline vs NPS: $t=3.061$ $P=0.01$, F saline vs NPS: $t=2.457$ $P=0.031$)

Figure 3

Effect of pretreatment with timolol on the increase in back surface temperature induced by NPS (A, B). Timolol was administered 30 min before icv injection of NPS. A: (clear circles): pretreated with saline followed by saline (solid circles): pretreated with saline followed by NPS (clear squares): pretreated with timolol followed by saline (solid squares): pretreated with timolol followed by NPS. Baseline temperature: Saline+Saline: 37.54±0.179, Saline+NPS: 37.40±0.208, Timolol+Saline: 37.59±0.159, Timolol+NPS: 37.45±0.207. Bar graph on the right side represents the AUC of temperature from the baseline for 60 min after icv injection of saline or NPS in rats pretreated with saline or timolol. Each symbol or bar and vertical line represents the mean ± SEM (n=6). Asterisks indicate significant differences (*: $P<0.05$). (A saline+saline vs saline+NPS: $F=2.946$ $P<0.0001$, B saline+saline vs saline+NPS:$t=3.232$ $P= 0.008$)

Figure 4

Diurnal variation of NPS mRNA expression in the brainstem (A). Samples were collected at 4-hour intervals starting from 07:30 h. Expression of NPSR mRNA in the central nervous system. Each of the samples were collected at 10:00 h (light period) and 22:00 h (dark period) (C). Diurnal variation of NPSR mRNA expression in the hypothalamus (B). Samples were collected at 4-hour intervals starting from 07:30 h. Gray area indicates the dark period. Each symbol or bar and vertical line represents the mean ± SEM (n=4). Asterisks indicate significant differences (*: P=0.037 t=2.569). Diurnal variation of NPS mRNA expression in the brainstem was considered to be a biological rhythm on the basis of cosinor analysis. Diurnal variation of NPSR mRNA expression in the hypothalamus was considered to be a biological rhythm on the basis of one-way ANOVA.

Figure 5

Effect of SHA68 on back surface temperature in the dark phase. Each bar and vertical line represents the mean ± SEM (n=4). Asterisks indicate significant differences from the saline group (*P <0.05, vehicle vs SHA68 t=3.278 P=0.013).

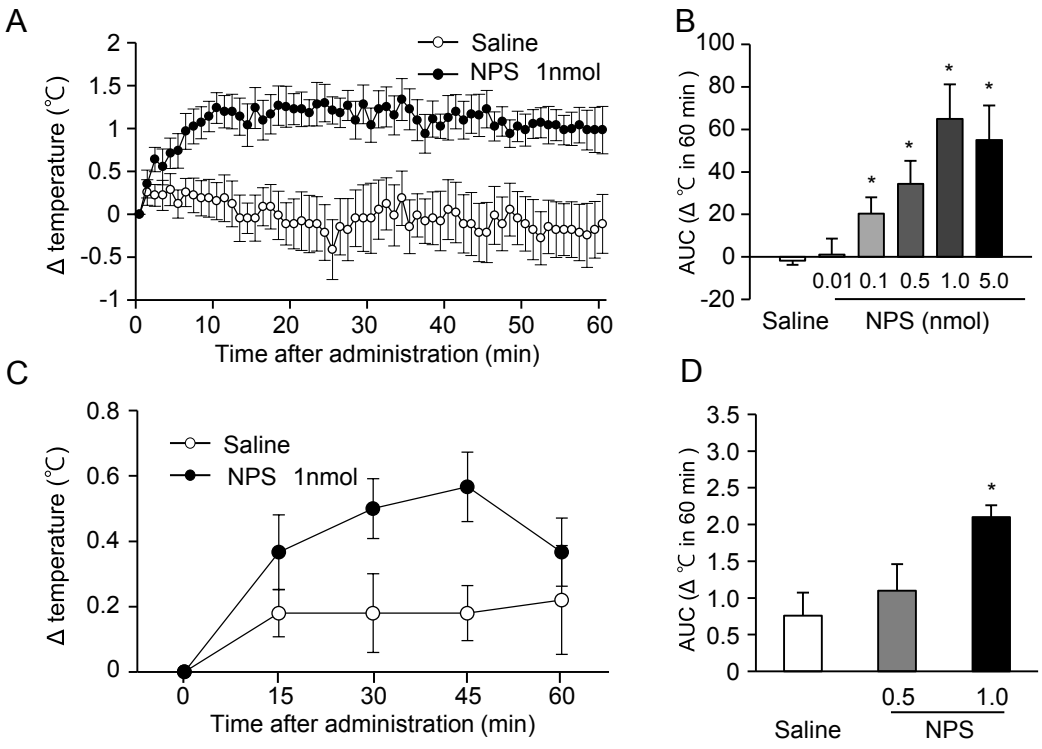
A
Saline
NPS 1nmol
Δ temperature (℃)
Time after administration (min)
B
AUC (Δ ℃ in 60 min)
0.01 0.1 0.5 1.0 5.0
Saline
NPS (nmol)
C
Saline
NPS 1nmol
Δ temperature (℃)
Time after administration (min)
D
AUC (Δ ℃ in 60 min)
0.5 1.0
Saline
NPS

Fig. 1

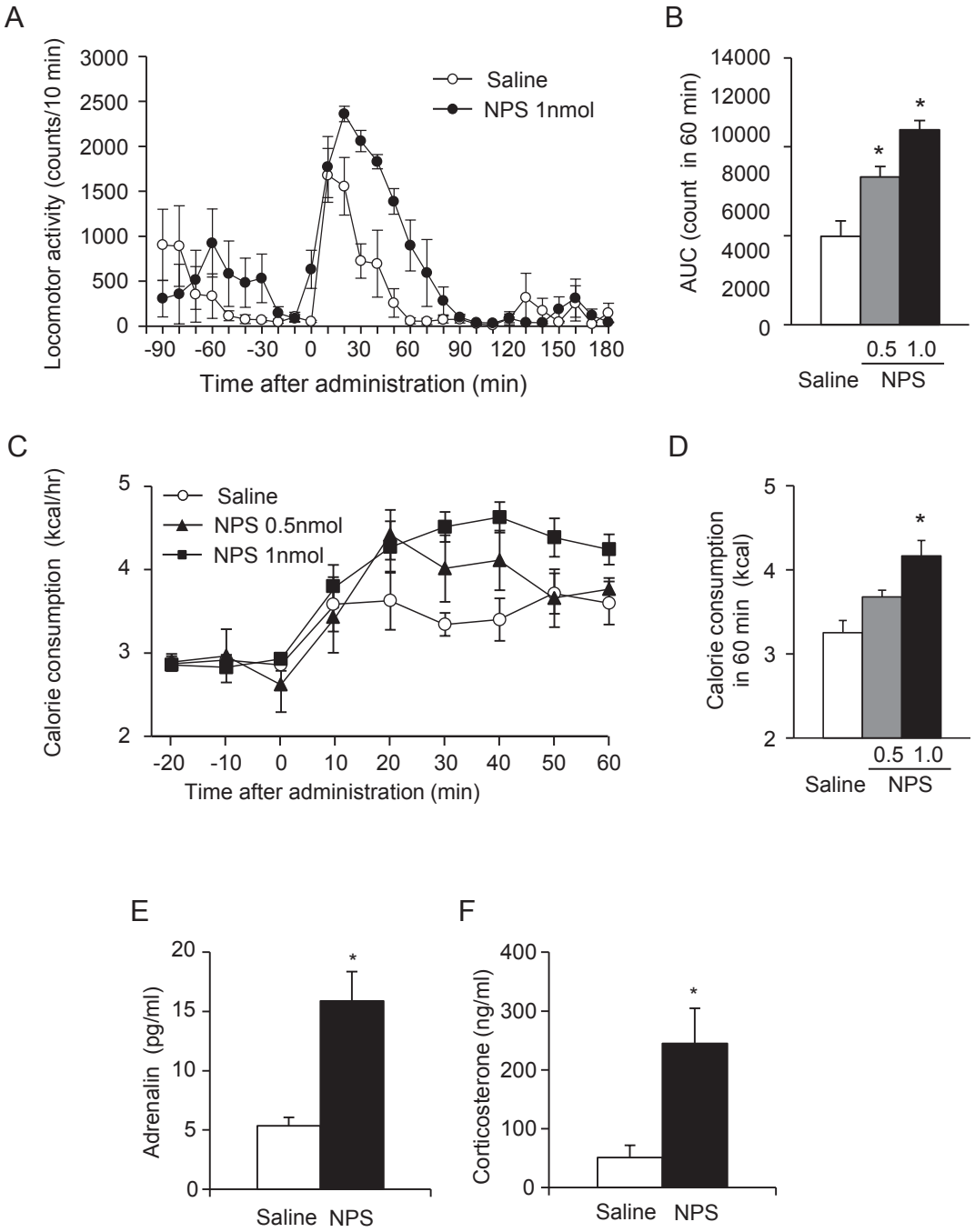
A
Locomotor activity (counts/10 min)
3000
2500
2000
1500
1000
500
0
Saline
NPS 1nmol
-90 -60 -30 0 30 60 90 120 150 180
Time after administration (min)
B
AUC (count in 60 min)
14000
12000
10000
8000
6000
4000
2000
0
*
*
0.5 1.0
Saline NPS
C
Calorie consumption (kcal/hr)
5
4
3
2
Saline
NPS 0.5nmol
NPS 1nmol
-20 -10 0 10 20 30 40 50 60
Time after administration (min)
D
Calorie consumption in 60 min (kcal)
5
4
3
2
*
0.5 1.0
Saline NPS
E
Adrenalin (pg/ml)
20
15
10
5
0
*
Saline NPS
F
Corticosterone (ng/ml)
400
300
200
100
0
*
Saline NPS

Fig. 2

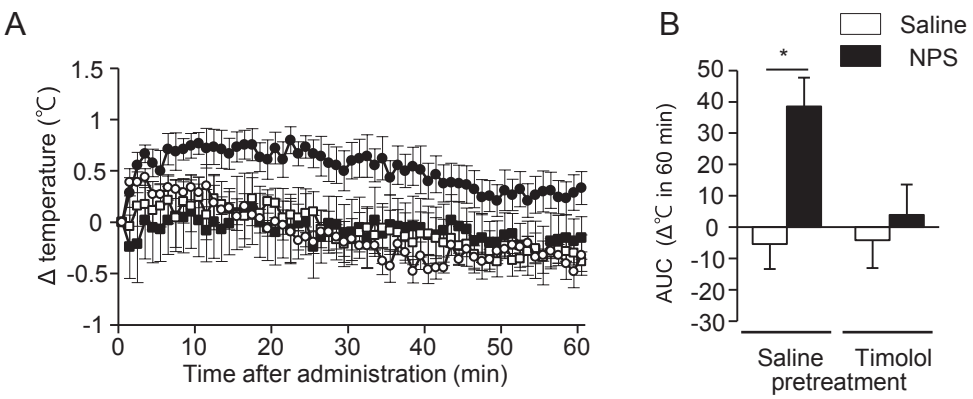
A
Δ temperature (℃)
1.5
1
0.5
0
-0.5
-1
0
10
20
30
40
50
60
Time after administration (min)
B
Saline
NPS
*
AUC (Δ℃ in 60 min)
50
40
30
20
10
0
-10
-20
-30
Saline
Timolol
pretreatment

Fig. 3

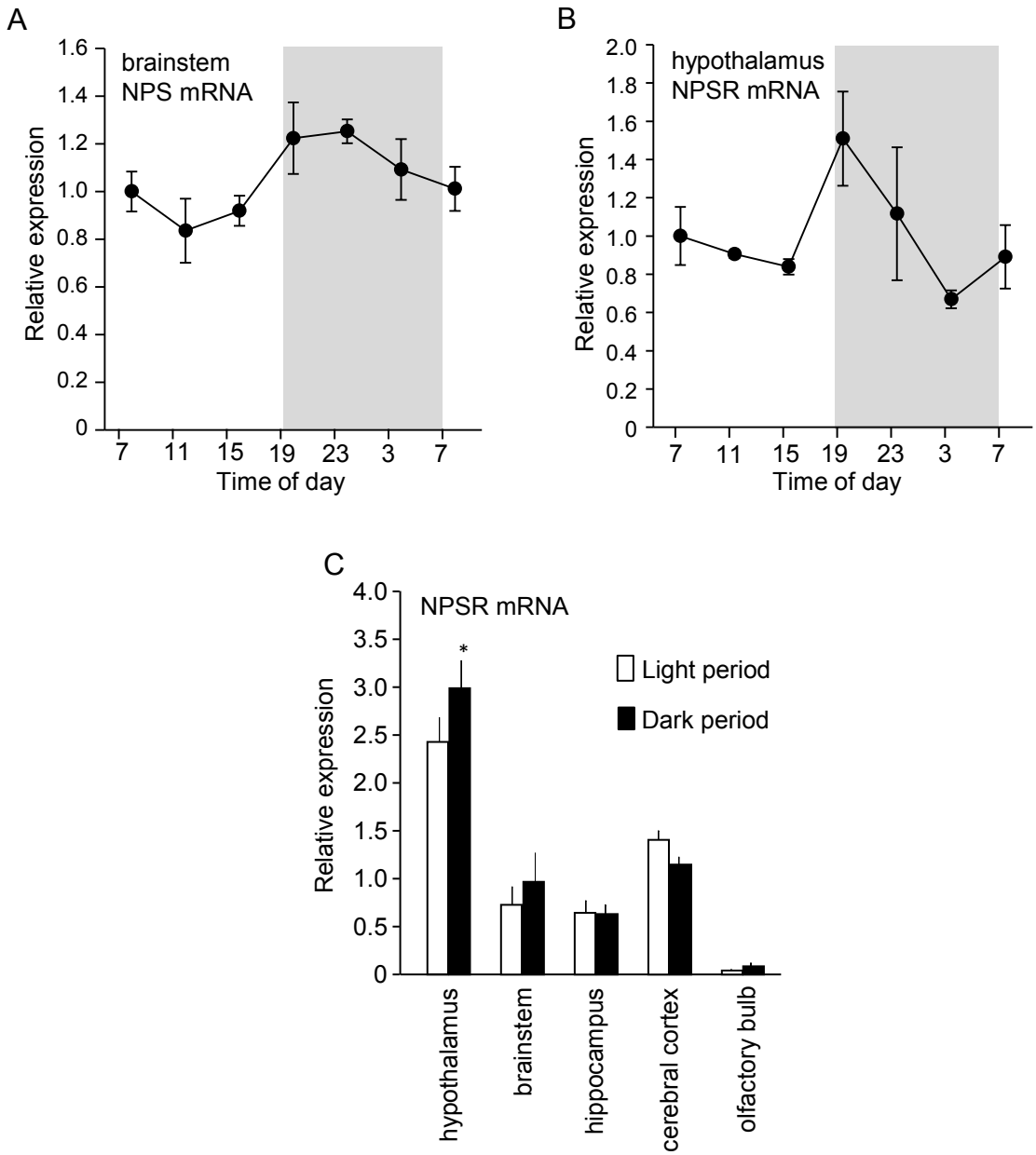
A
brainstem
NPS mRNA
Relative expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
7
11
15
19
23
3
7
Time of day
B
hypothalamus
NPSR mRNA
Relative expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
7
11
15
19
23
3
7
Time of day
C
NPSR mRNA
Relative expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
Light period
Dark period
hypothalamus
brainstem
hippocampus
cerebral cortex
olfactory bulb

Fig. 4

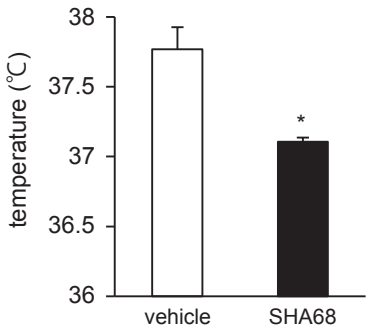

Fig. 5