

Caffeine treatment regulates neuropeptide S system expression in the rat brain

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Abstract

Caffeine has marked effects on sleep, arousal and food intake. However, the neuronal mechanisms underlying these actions are not fully understood. Neuropeptide S (NPS) is a recently discovered neuropeptide regulating both sleep and feeding. Here, we examined the effect of acute and chronic caffeine treatment on the expression of neuropeptide S and its receptor (NPS-R) in the hypothalamus and brainstem of rats by using real-time PCR. Our results showed that acute caffeine treatment induces a marked decrease in the mRNA levels of NPS in the brainstem, whilst the expression levels NPS-R are increased in both hypothalamus and brainstem after caffeine treatment. The timing of both processes differs, with acute treatment affecting brainstem NPS-R expression and chronic treatment affecting hypothalamic NPS-R expression. Overall, these data suggest a possible role for the NPS system in mediating some of the behavioral effects of caffeine.

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Caffeine is the most commonly used stimulant to combat inappropriately timed sleepiness. Acute delivery of caffeine previous to sleep extends sleep latency, decreases total sleep time, and increases sleep fragmentation in humans and rodents [8,19,23,25,31]. The specific effects of caffeine administration are dose-dependent [10,33]. In addition to its wakefulness-promoting effects, caffeine administration to rats markedly decreases food intake and body weight [4,6]. However, the molecular mechanisms mediating caffeine-induced arousal and anorexia are still not fully understood. Two mechanisms have been proposed for the stimulant effects of caffeine. One is via the dopamine–adenosine interaction involving adenosine A_{2A} receptors in the striatum [7,11]. The second mechanism is through antagonism at the adenosine A₁ receptor present on wake-promoting basal forebrain neurons [9,22,27,28]. These mechanisms could explain the effects of caffeine on sleep but not its potent action on feeding. Thus, the possibility of direct actions at other wake-promoting neurons in other brain areas remains open.

Neuropeptide S (NPS) is a recently discovered 20-amino-acid neuropeptide [32]. NPS binds and activates a G-protein coupled receptor called Neuropeptide S receptor (NPS-R) [2,24,32]. In the rat, NPS expression is located almost exclusively in a brainstem area close to the locus coeruleus (LC) and Barrington's nucleus. Weak NPS expression has also been described in the dorsomedial nucleus of the hypothalamus (DMN) and the amygdala [32]. Conversely, NPS-R is widely expressed in the brain [32]. The specific anatomical distribution of NPS in LC area initially suggested that NPS could be involved in the regulation of the sleep–wake cycle and in arousal. This was further demonstrated by central administration of NPS producing an increase in arousal and locomotor activity, as well as reductions of all sleep stages [32]. These results suggest that NPS/NPS-R system induces and/or maintains the awake state. Interestingly, additional evidence has also demonstrated a role for NPS in the regulation of feeding. It has been recently reported that lateral ventricle administration of NPS causes a marked decrease in food intake [26]. Overall, these data indicate that NPS system has important effects on many of the same behavioral phenomena as those regulated by caffeine administration.

To check the possibility of an interaction between caffeine action and the NPS system, we investigated whether there is activation of NPS system expression following the administra-

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tion of feeding-reducing and sleep-reducing doses of caffeine [1,3,5,18] by using real-time semiquantitative RT-PCR for NPS and NPS-R.

Adult male Sprague–Dawley rats (250–300 g) were housed in a temperature-controlled room (normal housing temperature, 22 °C) with a 12 h light/dark cycle (light: 8:00–20:00). Food and water were available ad libitum prior to the initiation of the experiment. All experiments were conducted in accordance with the Ethics Committee of the University of Santiago de Compostela. The experiments were performed in agreement with the Rules of Laboratory Animal Care and International Law on Animal Experimentation.

We studied the effect of both acute and chronic caffeine treatments. For the acute administration rats were killed 2 h after single intraperitoneal caffeine (Sigma; Poole, UK) injection (100 mg/kg, dissolved in saline) [1,3,5,18]. For the chronic treatment, rats were injected during 2 days with two intraperitoneal caffeine injections (100 mg/kg dissolved in saline) every 24 h [1,3,5,18]. Control animals were treated with the same intraperitoneal volume of vehicle. We used the same control group for the two treatments, so for this reason the animals treated with caffeine for 2 h were treated with vehicle at the same times that the 48 h caffeine-treated group were treated with caffeine, with the exception of the last treatment, two hours before they were killed, when they were treated with caffeine (2 h caffeine treatment). Sixteen animals per experimental group were used and the experiments were repeated twice. To confirm the efficiency of the treatments, body weight and food intake were measured. All animals were killed two hours after the last treatment (vehicle for the controls and 48 h treated group and caffeine for the 2 h treated group). The selected dose of caffeine has been reported to exert substantial effects on both arousal and sleep [1,3,5,18].

Both the hypothalamus and the brainstem were dissected as previously reported [12–15] and frozen immediately in dry ice. Total RNA was extracted from the tissues using Trizol (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

Two micrograms of total RNA were used for each RT reaction as previously described [12,20,21]. Real-time reverse-transcription polymerase chain reaction (RT-PCR) analyses were performed in a fluorescent temperature cyler (LightCycler, Roche Molecular Biochemicals; Mannheim, Germany). The 20 μ l amplification mixture contained 2 μ l of RT reaction products plus 3 mM MgCl₂, 0.5 μ M of each primer, and 1X LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals). After initial denaturation at 95 °C for 20 s, reactions were cycled 40 times using the following parameters for NPS and NPS-R detection: denaturation at 95 °C for 2 s, annealing at 60 °C for 15 s, and extension at 72 °C for 15 s. Hypoxanthine guanine phosphoribo transferase (HPRT) cDNA was used as housekeeping gene and amplified under the same conditions. We used the following primer sequences (5'-3'): NPS-forward: TGT CGC TGT CCA CAA TGC AT; NPS-reverse: AAT CAG ATT TTC CAG ACA CCT TAG AAG; NPS-R-forward: TGC AGG GAG CAA AGA TCA CA; NPS-R-reverse: AAT CTG CAT CTC ATG CCT CTC A; HPRT-forward CAG TCC CAG

CGT CGT GAT TA; HPRT-reverse AGC AAG TCT TTC AGT CCT GTC [20,21]. To check the specificity of our primers, PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining with UV light using a GelDoc 1000 Documentation System (Bio-Rad Laboratories Inc., Richmond, CA). The amount of PCR products formed in each cycle was evaluated on the basis of SYBR Green I fluorescence. For mathematical analysis, the crossing points (CP) values were used for each transcript. CP is defined as the point at which fluorescence rises appreciably above the background fluorescence. Cycle-to-cycle fluorescence emission readings were monitored and quantified using the second derivative maximum method of the LightCycler software package 3.3 (Roche Molecular Biochemicals; Roche Molecular Biochemicals, Mannheim, Germany). This method determines the crossing points of individual samples by an algorithm that identifies the first turning point of the fluorescence curve. This turning point corresponds to the first maximum of the second derivative curve and correlates inversely with the log of the initial template concentration. It should be noted that a lower CP means a higher expression rate and that a difference of one cycle number means a two-fold difference in mRNA levels, when PCR amplification efficiencies are 2 (100%). PCR efficiencies were calculated from the given slopes in LightCycler Software 3.3; the obtained values were: 1.99 for HPRT, 1.86 for NPS and 1.82 for NPS-R. NPS and NPS-R mRNA levels were normalized with respect to of HPRT level in each sample. Data were normalized by using the delta-delta Ct method.

Data were expressed as mean \pm S.E.M. and analyzed by using StatView 4.57 (Abacus Concepts). Statistically significance was determined by ANOVA and post-hoc Bonferroni test. $P < 0.05$ was considered significant. mRNA levels were presented as percentage change in relation to control group (vehicle-treated).

Real-time semiquantitative RT-PCR analysis showed a strong NPS mRNA signal in the brainstem. However, contrary to the reported data [32], no NPS mRNA expression was detected in any of the hypothalamic samples assayed (16 hypothalamic samples from 16 different rats) (Fig. 1A). On the other hand, NPS-R mRNA expression was strongly detected, at similar levels, in both hypothalamus and brainstem (Fig. 1B).

Chronic intraperitoneal administration of caffeine to rats markedly decreased food intake over the 48 h treatment period (Fig. 2A). In addition, administration of caffeine induced a significant reduction on the body weight gain of the animals after 48 h of treatment (Fig. 2B).

Intraperitoneal administration of caffeine for 2 h induced a marked decrease on NPS mRNA content in the brainstem (Fig. 3A). Furthermore, acute caffeine treatment induced a clear increase in the mRNA levels of NPS-R in the brainstem (Fig. 3B) but not in the hypothalamus (Fig. 3C).

Intraperitoneal administration of caffeine for 48 h did not induce any change in the mRNA content of NPS or NPS-R in the brainstem (Fig. 4A and B). On the other hand, chronic caffeine treatment induced a clear increase in the mRNA levels of NPS-R in the hypothalamus (Fig. 4C).

Caffeine has a profound effect on both sleep and food intake in humans and rodents [4,6,8,19,23,31]. In spite of this evidence,

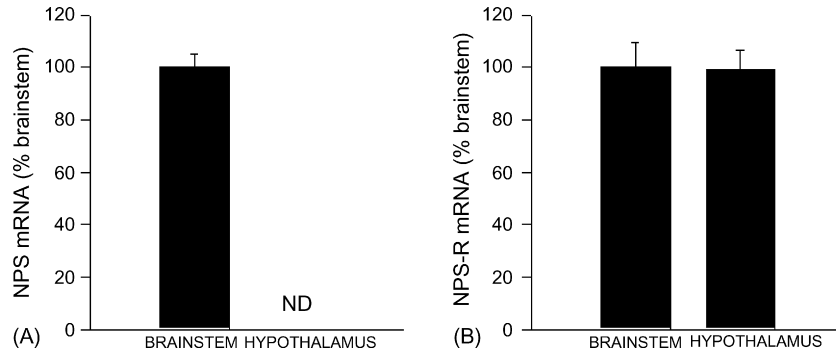


Fig. 1. NPS (A) and NPS-R (B) mRNA levels in the brainstem and the hypothalamus. Data are expressed as percentage of brainstem mRNA expression values. ND: non-detected.

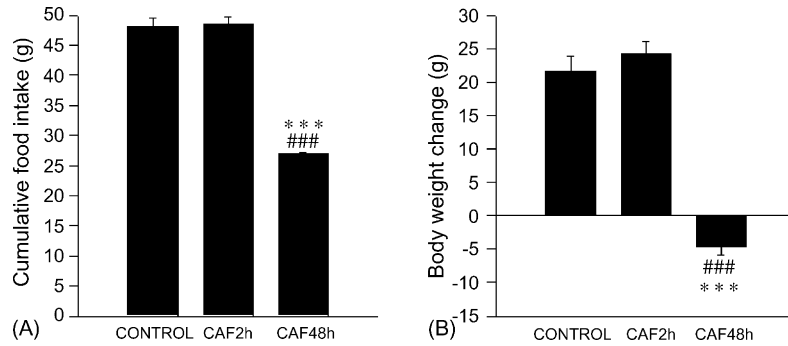


Fig. 2. Cumulative food intake (A) and body weight change (B) of control rats (vehicle-treated) and caffeine-treated rats for 2 h and 48 h. *** $P < 0.001$ vs. control; ### $P < 0.001$ caffeine 2 h vs. caffeine 48 h.

the molecular mechanisms underlying caffeine induced wakefulness and anorexia are not perfectly understood.

The hypothalamus and the brainstem play an important role in the regulation of both sleep–wake cycle and feeding [29,30]. The discovery of the hypocretin/orexin system and its impli-

cation on food intake and arousal/sleep control, established a new hypothalamic neuropeptide linking both physiological processes [16,29,30]. It has been further demonstrated since, that caffeine activates hypothalamic orexin neurons [18]. This effect might explain caffeine’s stimulatory actions but not its

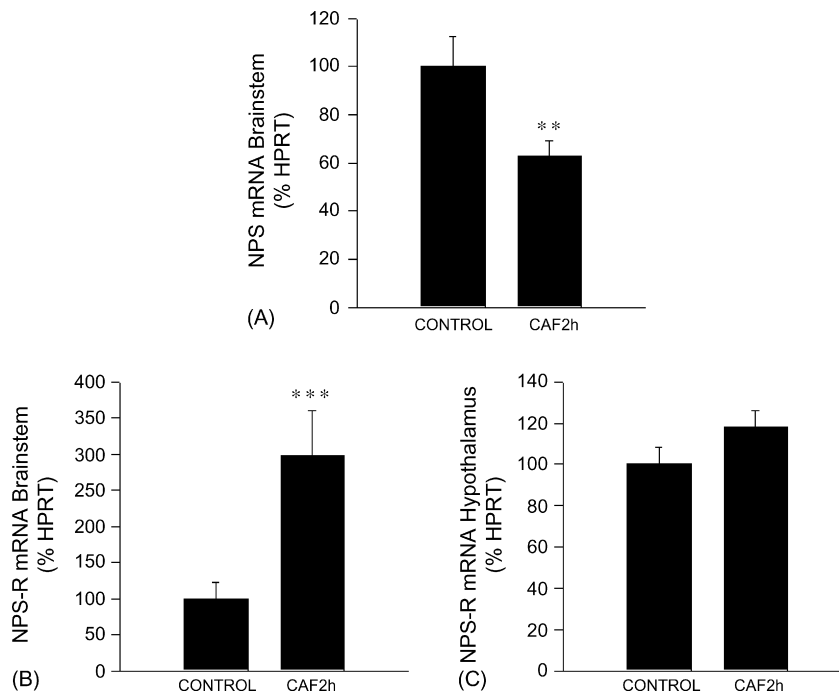


Fig. 3. NPS mRNA levels in the brainstem (A), NPS-R mRNA levels in the brainstem (B) and NPS-R mRNA levels in the hypothalamus (C) of control (vehicle-treated) and caffeine-treated rats for 2 h. ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control.

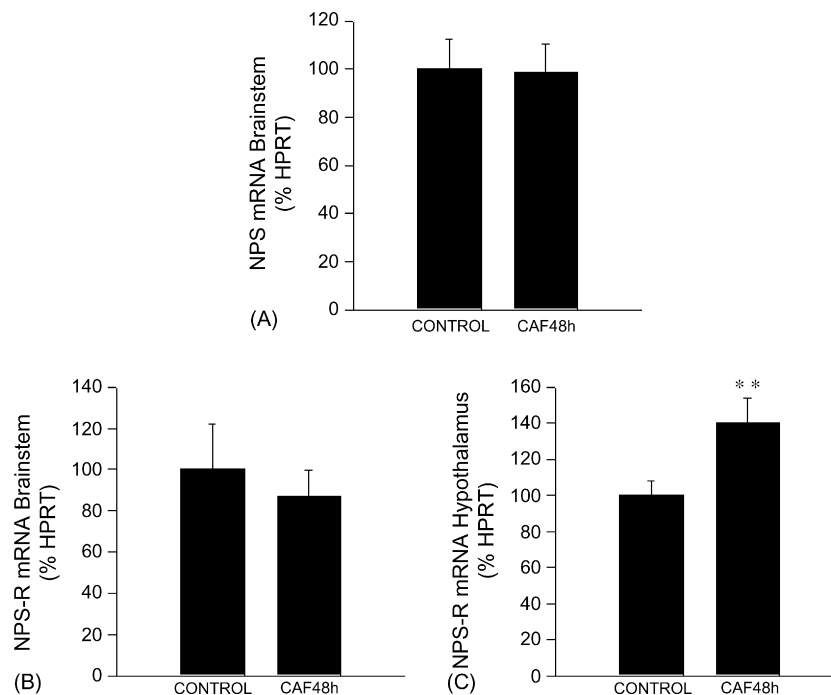


Fig. 4. NPS mRNA levels in the brainstem (A), NPS-R mRNA levels in the brainstem (B) and NPS-R mRNA levels in the hypothalamus (C) of control (vehicle-treated) and caffeine-treated rats for 48 h. ** $P < 0.01$ vs. control.

anorexigenic effects, given the appetite-promoting effect of hypocretin/orexins [16,29,30].

Current evidence indicates that neuropeptide S (NPS)-containing neurons play a critical role in the regulation of sleep–wake cycle and food intake [26,32]. On the basis of this evidence, we hypothesized that caffeine's action might be mediated through NPS system in the brain. Firstly, we examined the mRNA expression of NPS in the rat brain. Our data showed a strong NPS mRNA signal in the brainstem. Nevertheless, contrary to the reported data [32], no NPS mRNA expression was detected in any of the hypothalamic samples assayed. The reason of this discrepancy is not clear but it may be related to the experimental approach, real-time PCR versus *in situ* hybridization [32]. NPS-R mRNA expression was strongly detected, at similar levels, in both hypothalamus and brainstem.

We investigated the effect of peripheral caffeine administration on NPS system mRNA expression in the rat brain. We detected that NPS mRNA levels were markedly decreased in the brainstem of caffeine-treated rats. Interestingly this effect was just detected after acute, but not chronic, treatment, suggesting a time-dependent effect of caffeine on NPS mRNA expression. Given the inhibitory actions of caffeine on both sleep and feeding, an increase in NPS mRNA expression after caffeine administration would be more expected. To investigate this paradoxical effect and to further check the caffeine actions on NPS system in the rat brain, we examined NPS-R mRNA expression in the brainstem and hypothalamus of caffeine-treated rats. Our results showed that acute caffeine treatment induced a marked increase of NPS-R mRNA signal in the brainstem, without changes in the hypothalamus. On the other hand, chronic caffeine treatment induced an increase in NPS-R mRNA specifically in the

hypothalamus. These results suggest the existence of a time-dependent effect of caffeine on NPS-R mRNA expression and that the inhibitory effect of caffeine on sleep and feeding might be due to increased NPS signaling in the brain. These data might also explain the paradoxical decrease of NPS mRNA content in the brainstem after caffeine treatment. Considering that caffeine treatment strongly increased NPS-R mRNA levels in the brainstem, the decrease in NPS mRNA expression may be a compensation mechanism. Similar compensatory mechanism have been also described for several feeding-regulating neuropeptides [12,15,17].

In summary, NPS system is likely playing a role in acute and chronic responses to caffeine. The functions and anatomy of the NPS system may allow it to coordinate physiological and behavioral responses to caffeine and several other psychostimulant drugs.

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