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ORIGINAL ARTICLE

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Cholinergic input to the supraoptic nucleus increases Fos expression and body temperature in rats

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Abstract To examine the role played by cholinergic input and processes in the supraoptic nucleus (SON) in the control of body temperature and water intake in rats, we used microdialysis to stimulate and analyze SON without disturbing the behavior of unanesthetized rats. After microdialysis, we also investigated immunoreactivity for c-Fos protein in the brain as an index of neuronal activation. Stimulation with neostigmine, an acetylcholine esterase inhibitor, through the microdialysis probe increased the extracellular concentration of acetylcholine in the SON. This cholinergic stimulation dose-dependently increased body temperature but did not significantly change the water intake. The stimulation markedly increased c-Fos-like immunoreactivity (Fos-IR) in the SON and certain hypothalamic areas, including the paraventricular nucleus (PVN) and median preoptic nucleus (MnPO). Fos-IR was also evident in certain regions of the pons and brainstem, including the locus ceruleus (LC), area postrema (AP), and nucleus of the solitary tract (NTS). Addition of atropine, a muscarinic receptor antagonist, to the dialysis medium containing neostigmine attenuated the increase of Fos-IR and suppressed the neostigmine-induced responses in body temperature. These results suggest that cholinergic input and activation of the muscarinic cholinoceptive neurons in the SON contribute to the regulation of body temperature. Activation of noradrenergic pathways in the brainstem including LC and NTS may be involved in the thermoregulation mechanism.

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Introduction

The primary physiological function of the supraoptic nucleus (SON) is believed to be secretion of osmoregulatory hormones in response to various stimuli related to osmotic changes and fluid balance. SON and the paraventricular nucleus (PVN) form a major neuroendocrine effector system. However, the neurochemical aspects of functional regulation and those of the relationship between the effector system and osmosensory systems remain unclear. It has been suggested that the cholinergic system plays a significant role in this regulation, and that activation of hypothalamic muscarinic cholinergic processes induces water intake [8, 11, 16]. Studies indicate that a number of choline-acetyltransferase-positive cells are located in a region dorsolateral to SON and that they send fibers directly to SON [2, 19, 22]. Cholinomimetics have been shown to excite a portion of the cells in SON [6, 10]. The neuronal networks of osmoregulatory systems including SON overlap with the body temperature regulation mechanism [1]. It has been reported that electrical stimulation of SON [13] and glutamate microinjection into SON [1] increase body temperature. On the other hand, it has also been reported that hypothalamic injection of cholinomimetics induces hypothermia [14, 17, 31]. Recently we showed that cholinergic stimulation of the anterior hypothalamus and preoptic area is accompanied by a fall in body temperature and increased water intake [42].

In this study, to examine the role of cholinergic input to the SON and that of cholinergic processes in the regulation of body temperature in rats, we used microdialysis for cholinergic stimulation and analysis of endogenous extracellular acetylcholine (ACh) in SON. We added neostigmine, a cholinesterase inhibitor, to the microdialysis perfusion medium for cholinergic stimulation. First,

we monitored the peritoneal temperature during cholinergic stimulation using a telemetric system. Following that, we measured rectal temperature at the completion of microdialysis, and examined the relationship between the activation of cholinergic processes in SON, c-Fos expression in the specific nuclei and rectal temperature as well as water intake in the same animals. c-Fos expression has often been used as a marker of neuronal activity at the cellular level in the mammalian nervous system. Osmotic changes [21, 35] and heat exposure [29] induce c-Fos-like immunoreactivity (Fos-IR) in SON and PVN. Application of microdialysis enables us to predetermine and attenuate the effects of acute injury, which unavoidably occurs during microinjection [32, 33], and also makes the stimulation and simultaneous analysis of neuronal activity possible [41].

Materials and methods

Animals and surgical procedure

All experimental procedures involved in this study were approved by the Committee for Animal Experimentation of Gunma University, School of Medicine, and meet the guidelines of the Japanese Association for Laboratory Animal Science.

Male Wistar rats weighing 210–240 g was used. They were kept at 24°C temperature with a 12-h light, 12-h dark cycle (lights on from 07:00 hours). The animals were fed standard laboratory food and water ad libitum. One week before the experiment, rats were stereotaxically implanted with a guide cannula in the upper end of the right SON (coordinates: anterior–posterior 1.4, lateral 2.0, depth from dura 8.5 mm: according to the atlas of Pellegrino et al. [30]) under pentobarbital anesthesia (55 mg/kg i.p.) for in vivo brain microdialysis. The guide cannula was anchored firmly to the skull in each rat by dental adhesive and acrylic resin. In addition, in other group of rats, a miniature transmitter equipped with a thermister (Model 10T-T, Star Medical, Tokyo Japan, an implantable transmitter sealed with epoxy resin, size: 15.5 mm× 9.0 mm×4.7 mm) was implanted into the peritoneal cavity to measure the peritoneal temperature using telemetry.

Microdialysis, water intake, and body temperature

One week later, a microdialysis probe with dialysis membrane, 1 mm in length and 0.5 mm outer diameter (CMA 10, Carnegie Medicine, Stockholm Sweden), was inserted into the right SON through the guide cannula. The pointed end of the probes reached a depth of 9.5 mm from the dura. The brain microdialysis was performed without disturbing the behavior of unanesthetized rats. All the microdialysis experiments were started between 09:00 and 10:00 hours. Animals were deprived of food throughout the microdialysis. The probe was perfused with control Ringer medium (in mM: Na 147, K 4.0, $CaCl_2$ 3.0) containing 10 µM eserine at 2 µl/min using a microinfusion pump. Generally, acetylcholine (ACh) in microdialysis dialysate is not detectable without the use of an ACh esterase inhibitor [20]. After an equilibration period of 3 h, four baseline fractions were collected every 30 min. The basal concentration of the dialysate ACh in the three fractions was measured, the fourth baseline fraction was collected, and then the perfusion medium was changed to the medium containing 1, 2 and 5 mM neostigmine for 2 h. In the control group, the perfusion with control Ringer medium was continued throughout the microdialysis. In another group of rats, to examine the effects of a muscarinic antagonist, 2 mM atropine sulfate was added to the perfusion medium containing 2 mM neostigmine, after an equilibration period of 3 h and following collection of baseline fractions. Concentrations of ACh in the dialysate were analyzed using an HPLC system equipped with electrochemical detectors as previously reported [39, 40]. The recovery rate of ACh with the microdialysis probe in vitro was 7.5±0.2% (n=10). The recovery was tested by placing the probe in Ringer solution containing 1 pmol/µl ACh, and then perfusing the probe at a flow rate of 2 µl/min at 37.5°C. The position of the microdialysis probe was verified under microscopy after microdialysis and preparation of brain sections. In the rats used successfully, the microdialysis probe membrane penetrated the right-side SON.

The peritoneal temperature of rats with an implanted miniature transmitter was monitored during microdialysis using a telemetric system. Measurement of the peritoneal temperature was taken once per second throughout the experiment, using a telemetric system receiver (IMT-10RT, Star Medical, Tokyo Japan) interfaced to an on-line computer. Five rats were successfully used for the control and neostigmine groups, respectively. In the other group of rats, rectal temperature was measured only once at the end of microdialysis, to avoid the stress associated with this procedure. Five rats were successfully used in each group. The temperature was measured to the nearest 0.1°C using a quick-response type of thermister probe inserted into the rectum of each animal at the completion of microdialysis. During microdialysis, water intake was measured by weighing the bottle. All values are presented as means ±SE. The data were analyzed by ANOVA with Bonferroni's post hoc analysis.

Tissue preparation and immunohistochemistry

After microdialysis, rats were anesthetized with sodium pentobarbital (70 mg/kg i.p.) and perfused transcardially, first with heparinized saline, and then with a 4% paraformaldehyde solution prepared with 0.2 M phosphate buffer (pH 7.4). Brains were postfixed in paraformaldehyde solution and cryoprotected in a solution of 30% sucrose in 1/15 M phosphate buffer saline (PBS, pH 7.4) for 2 days, then sectioned in the coronal plane (25 µm thick) on a frozen microtome for c-Fos immunohistochemistry. Free-floating sections were rinsed in PBS and incubated in 3% normal goat serum in PBS containing 0.3% Triton X-100 for 2 h. Sections were then transferred into a polyclonal primary antibody which was raised from rabbit against a peptide derived from a conserved region of human c-Fos protein (Calbiochem PC38). Sections were incubated in the primary antibody at 4°C for 18 h. The antigen–antibody complex was visualized using avidin–biotin–peroxidase immunohistochemistry (Vectastain ABC kit, Vector, Burlingame, Calif., USA). Brain sections were mounted on slides, dried and coverslipped with Eukitt (O. Kindler, Germany). For staining control, the primary antibody was omitted from the staining procedures. No staining could be seen on these control sections.

Hypothalamic and extrahypothalamic brain nuclei or structures, identified according to the stereotaxic atlas [30], were photographed using a Nikon microscope at a 100-fold magnification. c-Fos-IR was quantified in each brain nuclei or structure by counting the number of c-Fos-positive cells in typical photographs of those regions in each of the five animals. From each brain sample, serial coronal tissue sections were made, and adjacent sections were stained with Cresyl violet for exact neuroanatomical determination of the nuclear boundaries. No attempt was made to quantify the intensity of staining within positive cells.

Results

Effects of SON perfusion with neostigmine on body temperature and water intake

Perfusion of neostigmine, an ACh esterase inhibitor, into the right SON through the microdialysis probe increased body temperature. Figure 1 illustrates the change of peri-

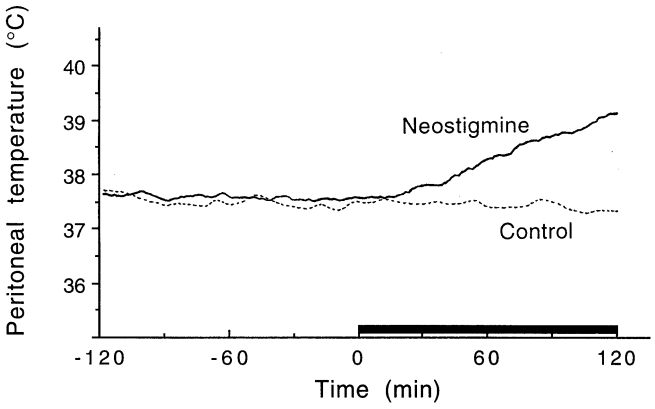

Fig. 1 Changes of peritoneal temperature during perfusion of 2 mM neostigmine into the supraoptic nucleus (*SON*). An example of the effect of neostigmine perfusion into the SON on peritoneal temperature. Peritoneal temperature was monitored during microdialysis using a telemetry system. After an equilibration period of perfusion with control Ringer medium through the microdialysis probe for 3 h, temperature measurement began, and basal peritoneal temperature was monitored for 2 h, after which the perfusion medium was changed to one containing 2 mM neostigmine. The perfusion with neostigmine medium continued for 2 h. The *dotted line* indicates an example of data from a rat perfused with control Ringer medium throughout the microdialysis. The *solid bar* on the *abscissa* indicates the duration of perfusion with neostigmine. Five rats were used in both 2 mM neostigmine and control Ringer medium groups

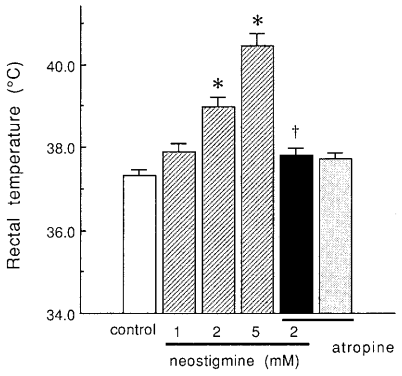

Fig. 2 Dose-dependent changes in rectal temperature after a 2-h perfusion of neostigmine into SON and the effect of atropine. After an equilibration period of 3 h, four basal fractions were collected for 2 h, and then the perfusion medium was changed to the medium containing 1, 2 and 5 mM neostigmine, except control. To examine the effects of muscarinic antagonist, after an equilibration period of 3 h and the collection of basal fractions, the perfusion medium was changed to one containing 2 mM atropine or 2 mM atropine + 2 mM neostigmine. The perfusion with these media continued for 2 h. Data are mean ±SE values for five rats. *Significantly different from the respective control values obtained after a 2-h perfusion with the control Ringer medium at $P<0.05$. †Significant difference observed between the values obtained during the perfusion of 2 mM neostigmine and of 2 mM atropine + 2 mM neostigmine at $P<0.05$

toneal temperature during perfusion with 2 mM neostigmine. The peritoneal temperature gradually increased following a latency period of about 10–20 min. The temperature of rats perfused with control Ringer medium did not change markedly during the microdialysis (Fig. 1). The peritoneal temperature at 0, 60 and 120 min during perfusion with 2 mM neostigmine was 37.3±0.14, 38.0±0.21 and 39.2±0.34°C, respectively (SE, n=5). The temperature at 0, 60 and 120 min during perfusion with control Ringer medium was 37.4±0.13, 37.3±0.15 and 37.2±0.18°C, respectively (SE, n=5). Perfusion with neostigmine significantly increased peritoneal temperature at 120 min compared to the temperature measured before perfusion of neostigmine and that taken following the perfusion of control Ringer medium ($P<0.05$).

In the other groups of rats, rectal temperature was measured at the end of microdialysis. Figure 2 shows the dose-dependent effects of perfusion with 1, 2 and 5 mM neostigmine on the rectal temperature at 2 h. In the neostigmine-induced hyperthermic rats, saliva spreading was observed at the end of perfusion with 2 and 5 mM neostigmine. Figure 2 also shows the effects of atropine, a muscarinic antagonist, on the neostigmine-induced increase in rectal temperature. Addition of 2 mM atropine to the perfusion medium containing 2 mM neostigmine suppressed neostigmine-induced hyperthermia. However, perfusion of neostigmine into SON had no significant effect on water intake (Fig. 3). Perfusion of 2 mM atropine Ringer medium had a tendency to increase rectal temperature and decrease water intake; however, the change was not significant.

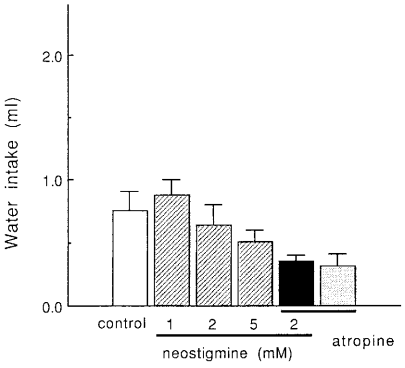

Fig. 3 Water intake during a 2-h perfusion of neostigmine into SON and the effect of atropine. Water intake was measured from the same rats as in Fig. 2. Data are mean ±SE values for five rats

Figure 4 schematically shows the position of the tips of the microdialysis probe in hemilateral (right-side) SON-perfused rats successfully perfused with control Ringer medium and medium containing 2 mM neostigmine for 2 h. The probe membrane penetrated the right SON. In the other groups of rats, the probe membrane also penetrated SON (not shown).

Effects of SON perfusion with neostigmine on the extracellular concentration of ACh

ACh concentrations in the dialysate of SON are shown in Fig. 5. ACh concentrations in the dialysate increased dose-dependently with the perfusion of 1 and 2 mM neo-

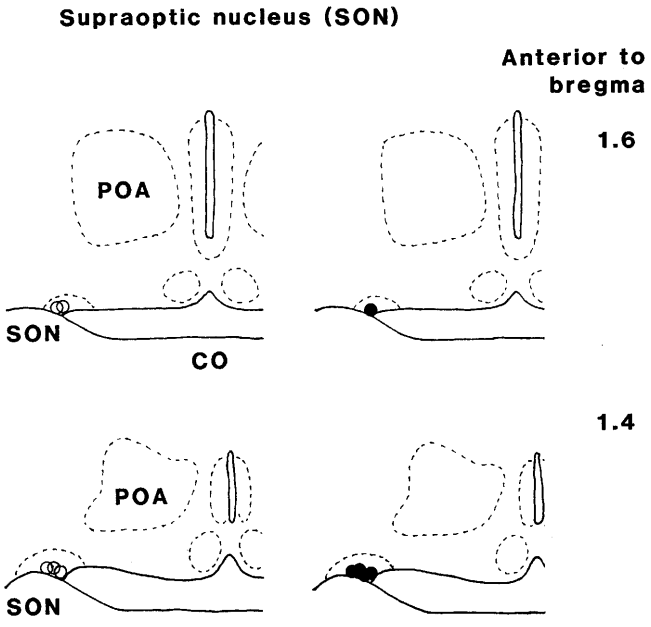

Fig. 4 Location of the tips of microdialysis probes. The *open* and *filled circles* indicate the locations of the probe tips in rats perfused with control Ringer medium and 2 mM neostigmine, respectively (n=5), into SON. Frontal sections of the brain are schematically illustrated at the level of SON (1.6 and 1.4 mm anterior to bregma). (*CO* Optic chiasma, *POA* preoptic area, *SON* supraoptic nucleus)

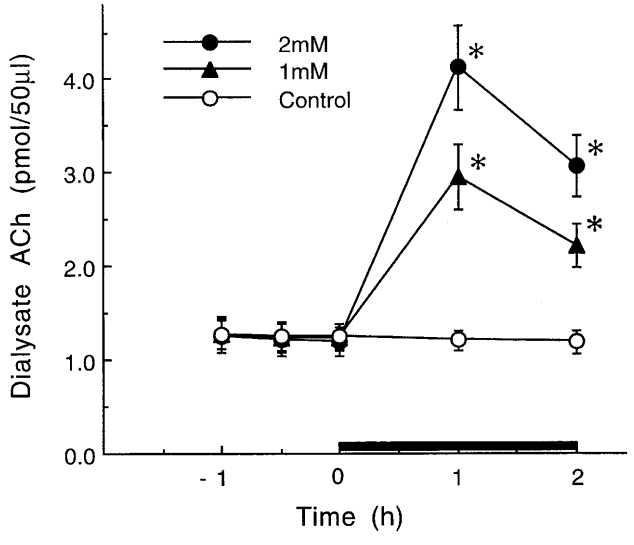

Fig. 5 Changes of extracellular acetylcholine concentrations in SON after perfusion of control Ringer medium and neostigmine. After a 3-h equilibration period of perfusion with control Ringer medium, the basal fractions were collected and the concentrations of dialysate acetylcholine (*ACh*) were measured. The perfusion medium was then changed to the medium containing 1 or 2 mM neostigmine except for the SON control group. The perfusion with neostigmine medium continued for 2 h. Five rats were used for control, 1 and 2 mM neostigmine groups, respectively. Data are mean ±SE values. *Solid bar* on *abscissa* indicates the duration of perfusion with neostigmine. *Significantly different from the values following the perfusion of control Ringer medium and the basal values before perfusion with neostigmine at $P<0.05$

stigmine. The basal concentrations of ACh in the dialysate perfused with 1 and 2 mM neostigmine were 1.24±0.14 pmol/50 µl and 1.20±0.15 pmol/50 µl, respectively, without correction for recovery across the probe membrane. The ACh concentrations had increased to about 2.4 and 3.3 times the respective basal level 1 h after the beginning of neostigmine perfusion. The basal concentration of ACh in the control group did not change significantly during the microdialysis. Perfusion of neostigmine through the microdialysis probe locally elevated the endogenous extracellular ACh in SON.

Effects of SON stimulation with neostigmine on Fos-IR

Cholinergic stimulation of hemilateral SON with neostigmine through the microdialysis probe for 2 h markedly increased Fos-IR on both sides of SON, as well as in other hypothalamic areas including PVN and the median preoptic nucleus (MnPO). Figure 6 shows Fos-IR in the hypothalamic area following the perfusion with control Ringer medium and medium containing 2 mM neostigmine. Low numbers of cell nuclei exhibiting Fos-IR were observed in the rats perfused with control Ringer medium (Fig. 6A, D, G). Neostigmine-perfused rats had many Fos-IR cells in SON, MnPO, and PVN (Fig. 6B, E, H). The addition of 2 mM atropine to the perfusion medium containing 2 mM neostigmine reduced the neostigmine-induced Fos-IR expression (Fig. 6C, F, I). At the level of the pons, there were many positive-staining cells on both sides of the locus ceruleus (LC) in the rats whose SON was perfused with neostigmine but not in control rats (Fig. 7A, B). In the medulla, immunostaining was evident in certain regions, including the area postrema (AP) and the nucleus of the solitary tract (NTS) (Fig. 7E). Also, the increase of Fos-IR in these regions of the brain was suppressed by the addition of atropine to the perfusion medium containing neostigmine (Fig. 7C, F). Fos-IR in the same brain nuclei was also found (not shown) in rats perfused with neostigmine for 1 h. Quantitative analysis of c-Fos-positive cells in the brain regions from all five rats confirmed this result (Fig. 8). Co-administration of atropine significantly reduced the neostigmine-induced distinct expression of Fos-IR in the following brain nuclei and structures: SON, MnPO, PVN, LC, NTS, and AP.

Discussion

Our present data show that cholinergic input to SON and activation of muscarinic cholinergic processes in SON correspond closely to increased body temperature. Cholinoceptive neurons and muscarinic receptors exist in SON [19, 22, 36] and cholinomimetics excite a portion of the cells [6, 10]. In the present work, we used neostigmine to stimulate cholinoceptive neurons in SON by elevating the extracellular concentration of ACh, which was spontaneously released from the cholinergic terminals.

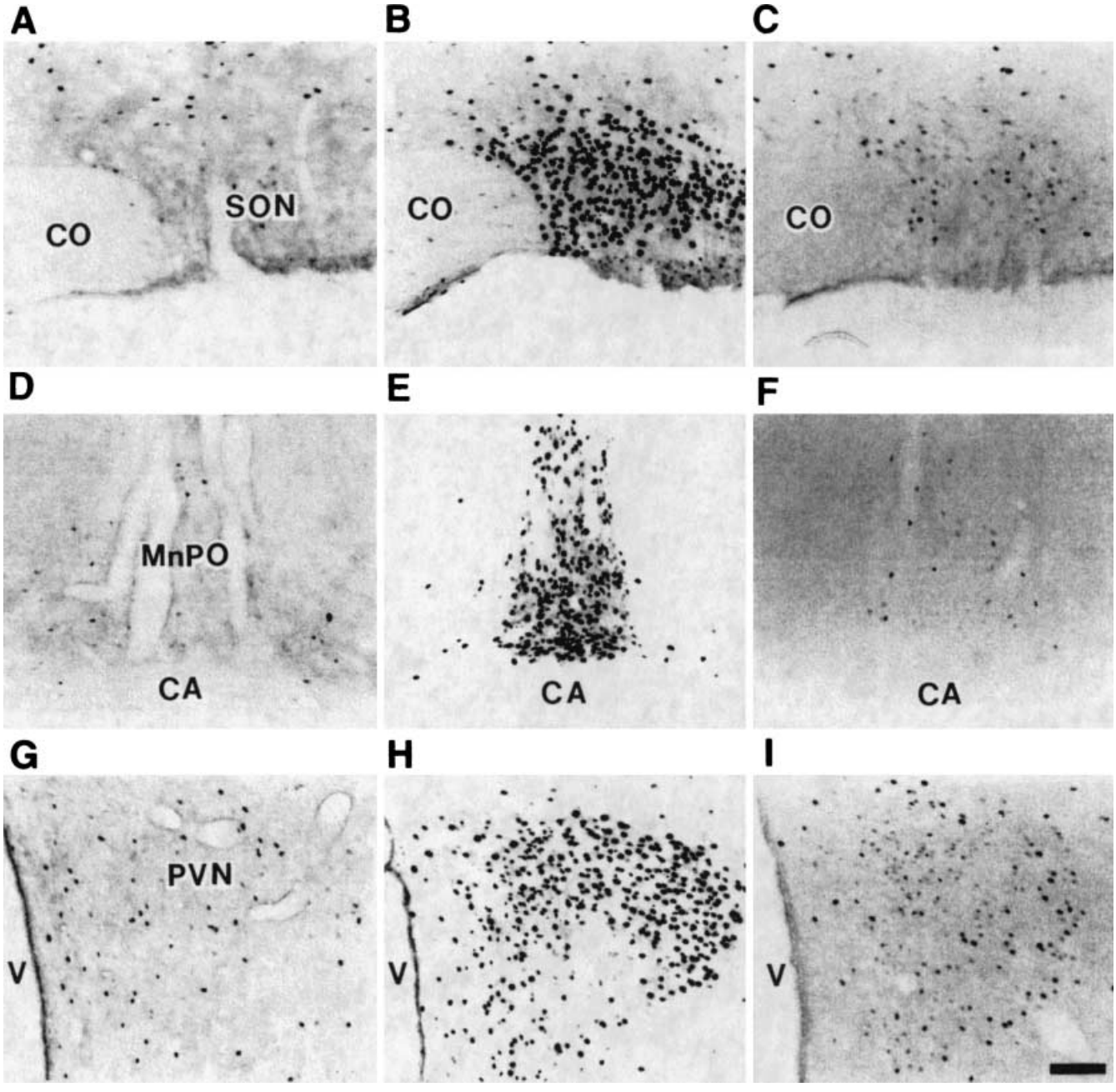

Fig. 6A–I Distribution of c-Fos-immunoreactive cell nuclei in coronal sections at the level of the hypothalamus. After microdialysis with control Ringer medium, neostigmine-containing or neostigmine- and atropine-containing medium into SON as in Fig. 2, rats were killed and used for analysis of c-Fos expression. Representative sections for SON (**A–C**), median preoptic nucleus (*MnPO*) (**D–F**) and paraventricular nucleus (*PVN*) (**G–I**): control (**A**, **D**, **G**), 2 mM neostigmine (**B**, **E**, **H**) and 2 mM neostigmine and atropine (**C**, **F**, **I**). *Bar* represents 50 µm. (*CA* Anterior commissure, *CO* optic chiasma, *V* third ventricle)

Perfusion with neostigmine through the microdialysis probe dose-dependently elevated the endogenous extracellular ACh concentration at the perfusion sites (Fig. 5). This indicates endogenous stimulation of the cholinoceptive neurons. The neostigmine-induced increase in rectal temperature and increase of Fos-IR were suppressed or attenuated by the addition of atropine, a muscarinic receptor antagonist, to the dialysis medium containing neostigmine (Figs. 2, 6 and 7). Thus, cholinergic input to SON and activation of the muscarinic cholinergic processes increase c-Fos expression in SON and in certain brain regions including PVN, MnPO, LC, AP, and NTS (Figs. 6, 7), and are probably involved in body temperature regulation (Figs. 1, 2).

The primary function of SON is secretion of osmoregulatory hormones: SON neurons are sensitive to osmotic stimuli [18] and respond to such stimuli by secreting the antidiuretic vasopressin and oxytocin [15, 44, 45]. It is well known that thirst drive is partly regulated by cholinergic action, and that cholinergic stimulation of

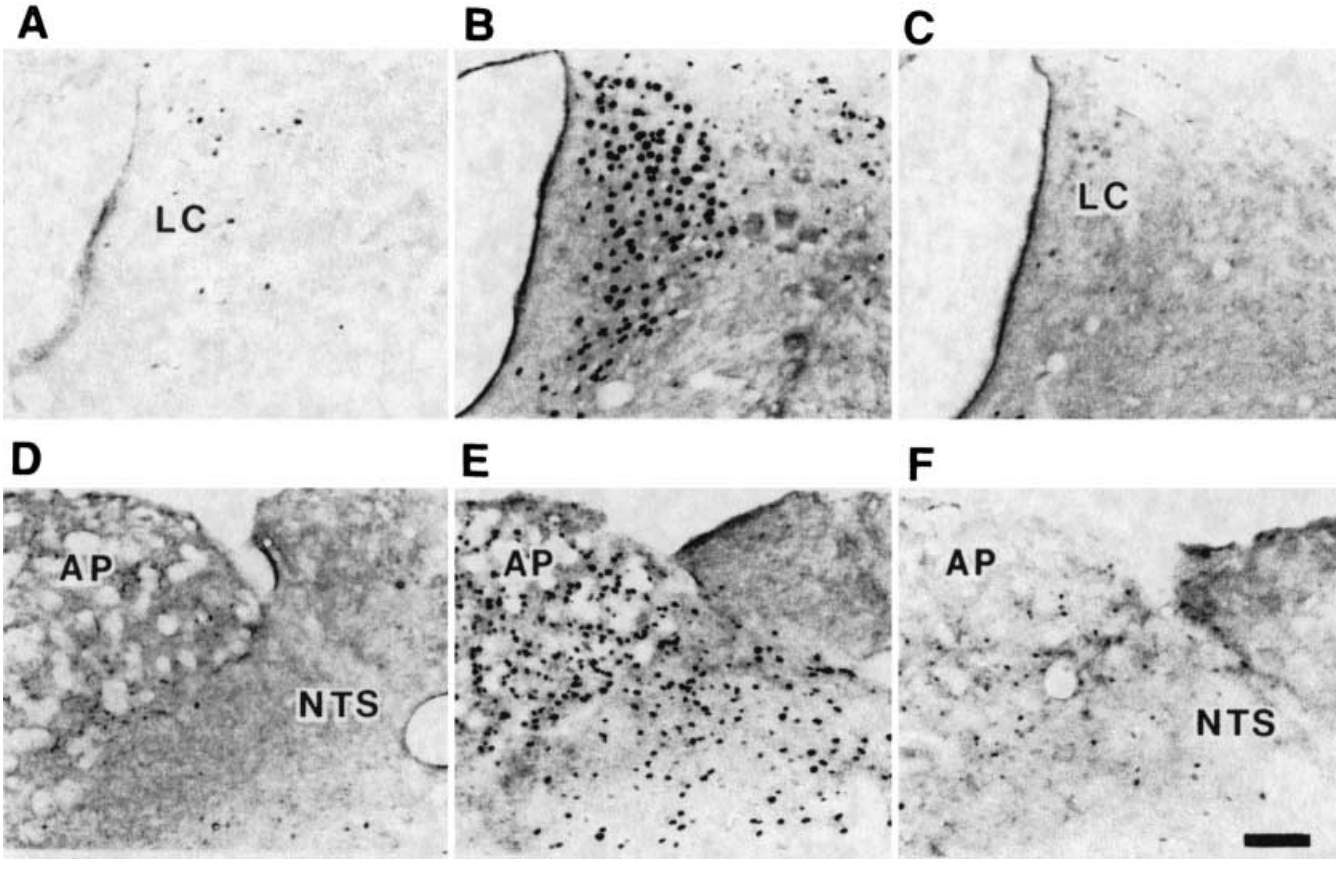

Fig. 7A–F Distribution of c-Fos-immunoreactive cell nuclei in coronal sections at the level of the pons and medulla. Representative sections of the locus ceruleus (*LC*) (**A–C**) and nucleus of the solitary tract (*NTS*) and area postrema (*AP*) (**D–F**): control (**A**, **D**), 2 mM neostigmine (**B**, **E**) and 2 mM neostigmine and atropine (**C**, **F**). *Bar* represents 50 μm

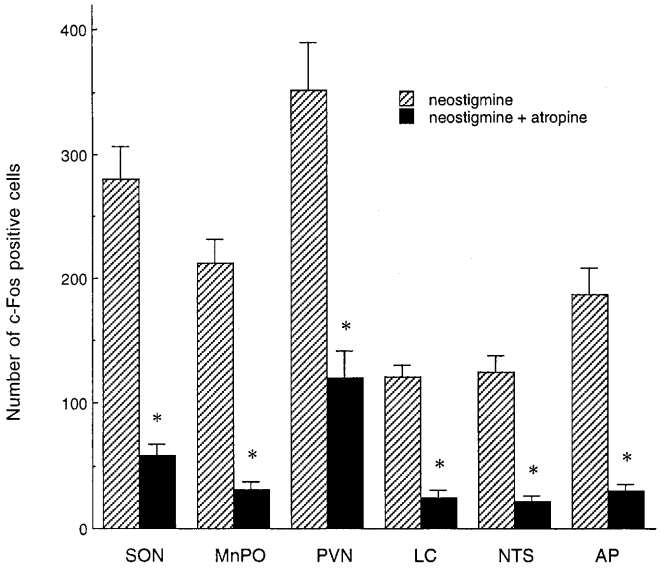

Fig. 8 Quantitative evaluation of the numbers of c-Fos-immunoreactive cells in the brain nuclei and structures. The number of c-Fos-positive cells was calculated in SON, MnPO, PVN, LC, NTS, and AP. Data represent means ±SE of cell counts per brain nuclei or structure in a typical photograph of those regions for each of the five rats per group, 2 mM neostigmine, and 2 mM neostigmine + 2 mM atropine. *In all the brain regions, SON, MnPO, PVN, LC, NTS, and AP, the number of c-Fos-positive cells during the co-administration of atropine is significantly different from the corresponding numbers in the presence of neostigmine at $P<0.05$

the lateral hypothalamus [11] and perifornical regions [8, 16] induces drinking. In our data, no significant increase in water intake was observed during the cholinergic stimulation of SON (Fig. 3). It seems unlikely that the cholinergic input to SON and the activation of muscarinic processes involve sensory signals causing thirst and the onset of drinking behavior, although the activation of cholinergic input to SON may increase antidiuretic vasopressin release [23, 28] and save water consumption. On the other hand, activation of cholinergic input and cholinergic processes in the lateral hypothalamus [11] and perifornical region [8, 16] may involve sensory signals of thirst and the onset of drinking. This suggests that there are different or independent regulatory mechanisms in thirst or drinking and in the secretion of antidiuretic hormones.

The osmoregulatory mechanism and the thermoregulatory system overlap, and a recent study suggests that the MnPO is one site of integration [29]. It is possible that cholinergic input and the subsequent neural activation of some SON cells also serve to transduce the signals involved in thermoregulation. The preoptic area (POA) and anterior hypothalamus (AH), major central

thermosensitive sites, contain both warm-sensitive and cold-sensitive neurons and play an important role in the regulation of body temperature [5, 46]. It is believed that warm-sensitive and cold-sensitive neurons contribute to heat loss and heat production, respectively. Warm-sensitive neurons send efferent signals: excitatory signals for heat loss and inhibitory signals for heat production. Conversely, the removal of tonic inhibition from the warm-sensitive neurons of POA and AH leads to an increase in rectal temperature [5]. We suppose that muscarinic cholinergic input to SON successively inhibits the function of warm-sensitive neurons in the POA and AH, and consequently, contributes to the increase of body temperature.

Activation of SON cells and increased c-Fos expression occur in the central administration of prostaglandins [43] and heat exposure [29] and also when the injection of hypertonic saline or dehydration induce osmotic changes [25, 29, 35]. SON may be part of the hypothalamic mechanism controlling heat production and loss. Perhaps activation of the sympathetic nervous system contributes to an increase of heat generation and a decrease of heat loss. The control of thermoregulatory muscle tone, shivering, skin blood flow and sweating may be factors. In addition, it has been reported that neurons in SON can signal the sympathetic system to increase heat production in brown adipose tissue (BAT). The hypothalamic regulation of BAT thermogenesis through the sympathetic nervous system is well known [24, 34]. It has been reported that BAT thermogenesis is activated by a coronal transection just caudal to the POA [5]. This also shows that BAT thermogenesis is controlled by an inhibitory signal from POA.

Cholinergic stimulation of SON enhances c-Fos expression in LC, AP, and NTS (Fig. 7). Enhanced c-Fos expression in those nuclei may imply the activation of brainstem noradrenergic pathways. The principal brain noradrenaline system consisting almost entirely of noradrenergic neurons originates in the LC [3, 12]. Although the specificity and relationship between LC activity and the activity of peripheral sympathetic nerves remains to be elucidated, some functional connection has been suggested [9]. Activation of peripheral sympathetic nerves is an important factor in increasing body temperature. On the other hand, it is unclear whether c-Fos induction in the LC and NTS, or activation of the brainstem noradrenergic system affects the hyperthermia induced by the SON cholinoceptive process. It has been reported that LC and NTS neurons exert an inhibitory influence rather than a stimulatory effect on the sympathetic nerves [26, 37]. However, some researchers have suggested heterogeneity in the spatial organization of the brainstem circuitry underlying parasympathetic and sympathetic activities. Microinjection of glutamate into the medial and lateral NTS region rostral to the obex elicited decreases in arterial pressure and heart rate, while glutamate injections into a site caudal to the obex, in the commissural region of the NTS, produced elevations in heart rate and arterial pressure sympathetically [27]. In the LC, glutamate-induced depressor points are located dorsal to the sites eliciting the pressor response [4]. Some physiological studies have suggested that LC neurons respond reciprocally to peripherally induced changes in arterial pressure or sympathetic activity [7, 38]. In our study, it remains unclear whether the induction of c-Fos in LC and NTS supports activation of the sympathetic nervous system, or whether the c-Fos induction in these nuclei is a reciprocal response against increased sympathetic activity and hyperthermia induced by cholinergic stimulation of SON.

In this study, we used microdialysis to examine the role of cholinergic input to SON in water intake and body temperature regulation, and we showed that cholinergic stimulation of SON increases the extracellular concentration of ACh and Fos-IR in SON, with an accompanying increase in rectal temperature. The presence of Fos-IR was also evident in certain brain regions including PVN, MnPO, LC, AP, and NTS. Co-administration of atropine from the microdialysis probe suppressed or attenuated the increase in rectal temperature and the increase of Fos-IR. Our data suggest that cholinergic input and activation of the muscarinic processes in SON contribute to the regulation of body temperature through modulation of the autonomic nervous system. There is little information available regarding the relationships between neuroactivity in a specific nuclei and endogenous hyperthermia. Further research in this area may advance our understanding of hypothalamic thermoregulation.

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