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An Immunohistochemical Study of Temperature-Related Changes in Galanin and Nitric Oxide Synthase Immunoreactivity in the Hypothalamus of the Toad

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Galanin (GAL) and nitric oxide synthase (NOS) have been implicated in the control of thermogenesis in mammals. An experimental protocol was designed to determine whether or not the expression of these molecules in the hypothalamus of the toad could be related to environmental temperature changes. Exposure of the animals to low temperature increased the number and intensity of NOS-positive neurons in the magnocellular hypothalamic region, in contrast to a weak immunoreactivity observed in control animals kept in a natural environment at a spring-summer temperature (23–27°C). Also a significantly higher number of GAL-immunoreactive (-IR) cells was observed in the preoptic area as compared to that observed in controls, while no difference in the intensity of GAL immunostaining intensity was detected. These results show a temperature-related expression of GAL and NOS in the hypothalamus and preoptic area of the toad. The results suggest a possible role of GAL and NOS in the regulation of hibernation in these animals.

Most species exhibit neurochemical, anatomical, and behavioral changes designed to optimize reproduction and survival in response to seasonal alterations in climate and food availability. Annual endocrine changes in species that migrate or hibernate are well characterized because of the essential behavioral extremes that seasonal changes elicit.

Environmental temperature is a significant external factor affecting physiological responses. Seasonal or circannual rhythms in the hypothalamic content of corticotropin-releasing factor, thyrotrophin-releasing hormone, neurotensin, and neuromedin N have been observed in rats (Bissette *et al.*, 1995). Within temperate climates, many anuran species hibernate underwater; at least five species of frogs in northern regions hibernate on land and endure freezing temperatures (Storey, 1990). Furthermore, it is agreed that most frogs do not bury themselves in the mud, but simply rest on the surface (Cunjak, 1986; Penny, 1987). This allows tegumentary gas exchange, the mode of winter "breathing" (Merrel, 1977). Artificially induced hibernation decreases the density of luteinizing hormone-releasing hormone-like immunoreactivity (LHRH-LI) in the median eminence (ME) of the toad, *Bufo japonicus.* These seasonal changes in LHRH-LI seem to coincide with seasonal reproductive activity in this species (Jokura and Urano, 1985).

Previous reports have shown the sequence of galanin (GAL) in frogs (Chartrel *et al.*, 1995) as well as the complete distribution of GAL (González Nicolini *et al.*, 1995) and nitric oxide synthase (NOS) (Suburo *et al.*, 1998) in hibernating animals and both molecules have been implicated in the regulation of thermogenesis in the rat (Menéndez *et al.*, 1992; De Luca *et al.*, 1995). In the present study a possible effect of the environmental

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temperature on GAL in the preoptic area and on NOS in the magnocellular hypothalamic system of the toad (*Bufo arenarum*) has been examined. The avidin-biotin complex (ABC) method (Hsu *et al.*, 1981) was employed for immunohistochemical staining. Nomenclature usage in this paper is that used in the brain atlas of *Rana pipiens* (Wada *et al.*, 1980) and by Neary and Northcutt (1983) and González Nicolini *et al.* (1995).

MATERIALS AND METHODS

Experimental Procedures

The effect of hibernation on GAL- and NOS-LIs was examined in 18 male toads captured in spring (October-November). They were divided into two groups: 9 hibernating animals and 9 control animals. Hibernation was artificially induced by maintaining the animals at 6–8°C in constant darkness for 2 weeks (Jokura and Urano, 1985), while the controls were kept in a natural environment with free access to water and food. The thermal shift corresponded to the temperature in the annual cycle when wild toads begin to hibernate. During hibernation, toads were not fed. Another control group (SHAM, $n = 9$) was kept at room temperature (23–27°C) in constant darkness and not fed for 2 weeks.

After anesthesia with 3-aminobenzoic acid ethyl ester methanesulfonate (MS 222, Sandoz Ltd., Basel, Switzerland) control and experimental animals were perfused through the ascending aorta with 50 ml (28°C) of 0.6% NaCl followed by 300 ml (4°C) of a mixture of formalin and picric acid (4% paraformaldehyde and 0.4% picric acid in 0.16 M sodium phosphate buffer (PBS), pH 6.9; Zamboni and De Martino, 1967). The brains were dissected, immersed in the same fixative overnight, and then transferred to PBS (pH 7.4) containing 10% sucrose, 0.02% Bacitracin (Sigma, St. Louis, MO), and 0.01% sodium azide (Merck, Darmstadt, Germany) for at least 48 h. A complete series of parallel diencephalic frontal sections (20 μm thickness) was cut in a cryostat (Microm, Zeiss, Germany) and processed for the avidin-biotin complex (ABC) technique (Hsu *et al.*, 1981) to minimize technical variations.

ABC Method

The sections were mounted onto chrome alum-gelatin precoated glass slides, allowed to dry for at least 1 h, and rinsed twice in PBS. After this, they were incubated for 18–48 h in a humid chamber at 4°C with GAL rabbit antiserum (against bovine and rat GAL, Peninsula Laboratories, Belmont, CA), diluted 1:2000, and with NOS rabbit antiserum (against rat NOS, Wellcome Research Labs., Kent, England) diluted 1:2000 in PBS containing 0.2% (w/v) bovine serum albumin, 0.03% Triton X-100, and 0.1% (w/v) sodium azide. The slides were rinsed twice in PBS and incubated at room temperature for 30 min in biotinylated goat anti-rabbit secondary antibodies (1:200, Vector Laboratories, Burlingame, USA), rinsed twice in PBS, and incubated in an ABC (Elite Kit, Vector Laboratories) for 1 h at room temperature. Peroxidase activity was demonstrated by reaction with 3, 3′-diaminobenzidine using glucose oxidase and nickel salts for enhancement of the reaction product (Shu *et al.*, 1988). After dehydration the sections were coverslipped with Permount medium.

Quantification

The numbers of cell profiles immunostained in the preoptic area and the magnocellular hypothalamic region were counted in nine controls and nine hibernating animals. The boundaries of each area were established using parallel series of sections stained with the Nissl technique (see Neary and Northcutt, 1983; González Nicolini *et al.*, 1995). The numbers of GAL- and NOS-immunopositive neurons in serial sections of each area were counted, and the mean expressed as a single value for the final statistical analysis. A double-blind analysis of sections was followed by two independent researchers. To establish the staining intensity, the counting system consisted of a superfine pitch trinitron video acquisition board (Sony) attached to a 486 personal computer (frame grabber OCULUS-T CX, CORECO Inc., Canada) with an OPTIMAS 4.10 computerized image analysis system. Video images were obtained by a high performance CCD (COHU) camera attached to a Zeiss microscope equipped with a ×16 objective and darkfield condenser. A one-way ANOVA followed by Student's t test (two-tailed unpaired) assessed differences in the number of cell profiles and

the average labeling intensity across the groups. Data are given as mean ± SEM. The criterion level for significance was set at $P < 0.001$.

Controls

For control purposes, parallel incubations of sections with GAL and NOS antiserum (dilutions as above) preabsorbed with synthetic pig GAL peptide (Peninsula Laboratories) and NOS peptide (peptide 49, amino acids 251–270; Bredt *et al.,* 1991) (10^{-6} M) were performed. Also, sections were incubated with only primary or secondary antibodies and processed for ABC.

All sections were studied in a Nikon Microphot FXA microscope. Agfapan APX 25 (Agfa Gevaert AG, Leverkusen, Germany) was used for bright-field photography.

RESULTS

There were seasonal variations in the immunoreactivity of both GAL and NOS and on no occasion were there GAL- and NOS-IR structures in the immunoabsortion control experiments. Also, animals kept at room temperature, in constant darkness and not fed (SHAM) exhibited GAL- and NOS-LIs similar to those of controls.

Galanin

In control toads GAL-IR cell bodies occurred along the entire rostrocaudal extension of the medial preoptic nucleus. They occupied the periventricular region and were aligned parallel to the walls of the third ventricle (Fig. 1A). Hibernating animals showed an increase in the number of GAL-IR neurons in the preoptic area ($P < 0.001$) (Fig. 1B). After 2 weeks of hibernation there was a twofold increase in the number of GAL-IR cells (Fig. 2A). However, there were no changes in the GAL-IR intensity within cells (Fig. 2B).

Nitric Oxide Synthase

A remarkable seasonal variation was found in NOS-LI. There were barely discernable NOS-IR neurons in the magnocellular region of the hypothalamus of control animals and only occasional lightly immunostained cells were observed (Fig. 1C). Neuronal perikarya containing NOS-LI scattered in the magnocellular region were large and pale. In contrast, in hibernation-induced animals NOS-LI significantly increased in both intensity and number of immunostained cells (Figs. 1D, 2C, 2D).

Thus, artificially induced hibernation, or exposure to a chill and constant dark environment, increased immunoreactivity to NOS and GAL in the hypothalamus of the toad.

DISCUSSION

Galanin

The 29 amino acid peptide GAL is widely distributed in the central nervous system (Melander *et al.*, 1986) and the primary structure of GAL has been identified with only two amino acid substitutions (Chartrel *et al.*, 1995). The paraventricular nucleus of the hypothalamus (PVN) of the rat (Melander *et al.*, 1986) and the toad (González Nicolini *et al.*, 1995) is particularly rich in GAL-positive neurons. Akabayashi *et al.* (1994) showed in rats the presence of two distinct diurnal rhythms for GAL in the dorsal region of the medial hypothalamus. Acute injections of GAL into the PVN produce a shift of the rat's metabolism toward a state of energy conservation characterized by reduced thermogenesis, so that GAL may be a PVN anabolic neurotransmitter, together with norepinephrine (Siviy *et al.*, 1989) and neuropeptide Y (Menéndez *et al.*, 1990). These agents can be further subdivided into antithermogenic agents (GAL and norepinephrine) and inducers of fat deposition (neuropeptide Y) (Menéndez *et al.*, 1992). The increased number of GAL-IR neurons in hibernating animals may reflect a sensitivity to environmental changes. Nevertheless, the possibility exists that the exposure to low temperature could be stressful for the animals and that hormonal changes induced by stress could be responsible for the modifications of the number of GAL-IR neurons. In fact, it has been found recently that GAL inhibits corticosteroid secretion in amphibians (Gasman *et al.*, 1996). The present results suggest that GAL

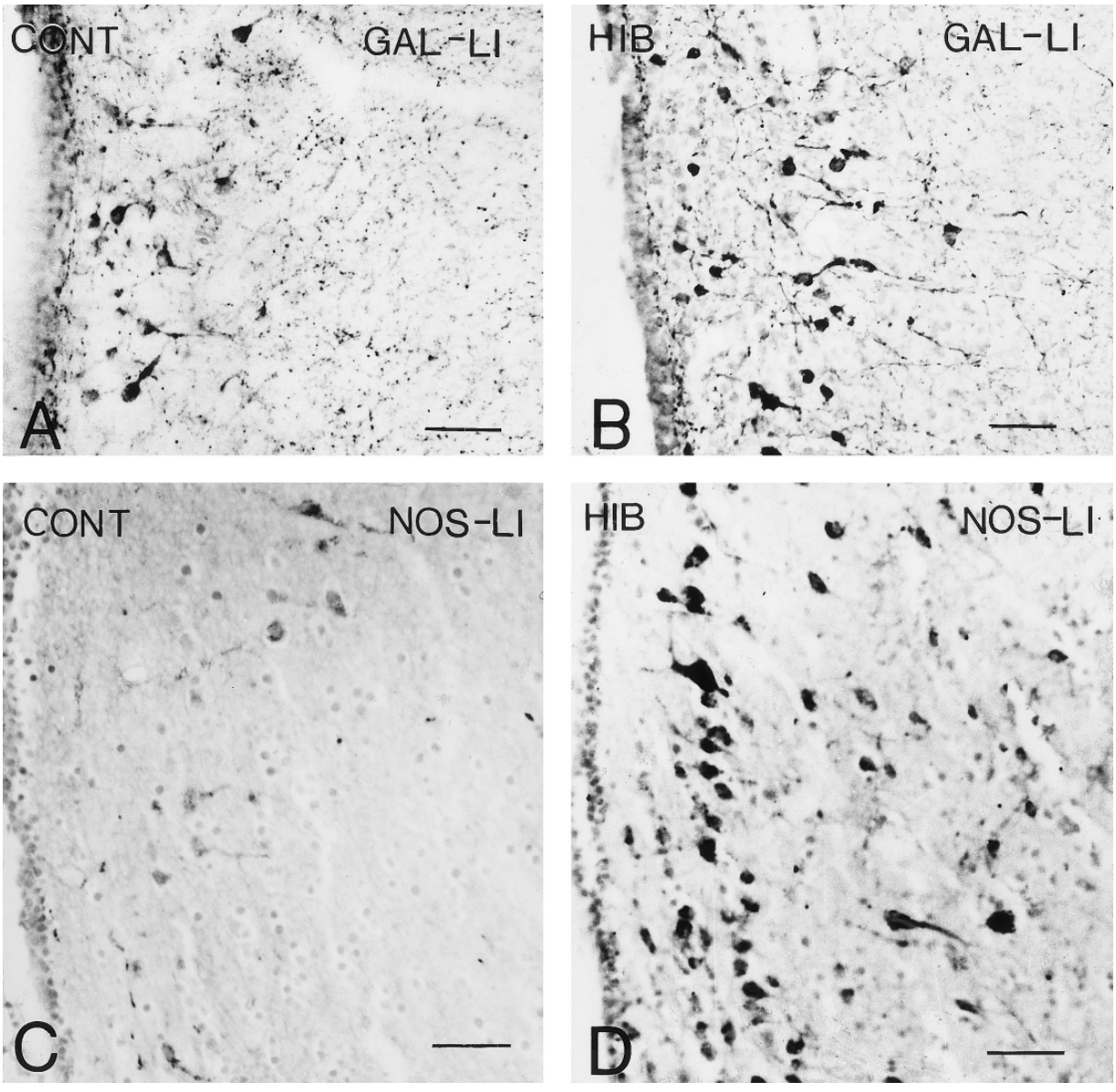

FIG. 1. Photomicrographs showing immunostained sections with GAL antiserum in the preoptic area of control (A) and hibernating (B) toads, and NOS antiserum in the magnocellular region of control (C) and hibernating (D) toads. Note that whereas in the controls (A, C) few neurons are present, in the hibernated-induced animals several GAL-IR (B) and NOS-IR (D) neurons can be observed. Bars = 100 μm.

is concerned with the control of energy expenditure in toads, as in mammals. Alternatively, an endocrine relation between growth hormone (GH) and GAL was shown in preoptic cells of the hypothalamus of the toad (González Nicolini *et al.*, 1997). The increases in GAL-LI observed in the present study could result from increased plasma GH levels known to be higher during the winter months in amphibia (Mosconi *et al.*, 1994).

Nitric Oxide Synthase

An increased NOS-LI in the magnocellular region of hibernating animals is described. The presence of NOS in a nucleus, important for several neuroendocrine functions, suggests that nitric oxide (NO) may control pituitary hormone secretion as in mammals (Brann, 1995). The effect of NO might be related to its well-established vasodilatory action, possibly exerted near

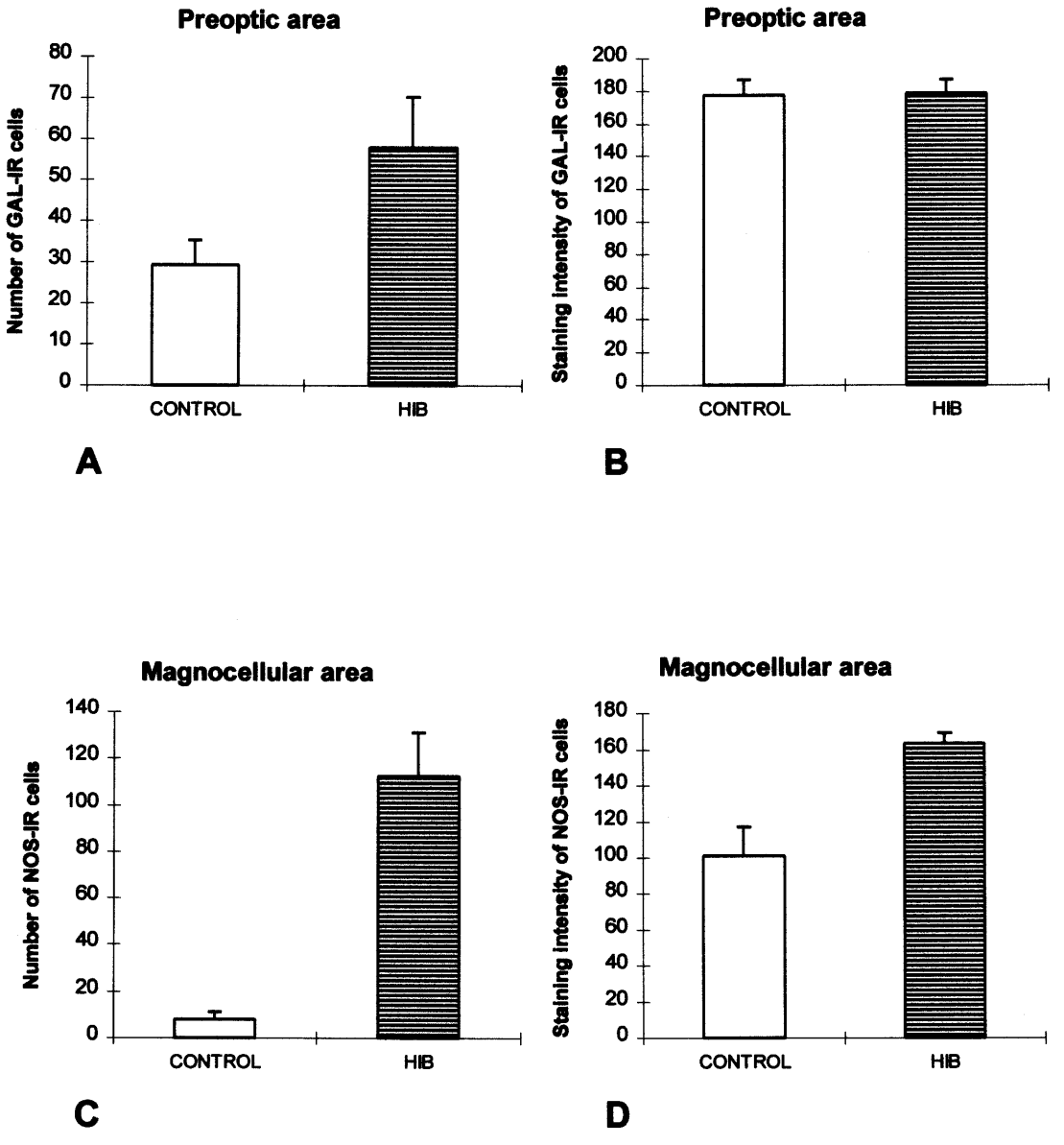

FIG. 2. Effects of hibernation (shadowed columns) on the expression of GAL-IR neurons and immunostaining intensity in the preoptic area (A, B) and NOS-IR neurons and immunostaining intensity in the magnocellular region (C, D) in the toad as compared to controls (white columns). The survival time in the experiment was 2 weeks. The number of immunostained neurons and the immunostaining intensity are expressed as the means ± S.E.M. Statistical analysis was done by one way ANOVA followed by Student's *t* test. $^{**}P < 0.001$, $n = 9$.

the portal blood vessels in the ME or in the posterior pituitary. In this way, the amount of hormone released into the ME to reach the anterior pituitary may be modulated. Another possibility is that NO may influence local blood circulation in the magnocellular nucleus. The hypothesis that NO regulates hypothalamic-hypophyseal blood flow is supported by the known vasodilatory action of NO (Bredt and Snyder, 1992) and the presence of a dense NOS innervation around blood vessels in the ME of the rat (Ceccatelli *et al.*, 1992) and the toad (Suburo *et al.*, 1998). Moreover, hibernation is not the only physiological stimulus that increases NOS since it has been shown that lactation (Ceccatelli and Eriksson, 1993), dehydration (Villar *et al.*, 1994), and also stress (Calzá *et al.*, 1993) upregulate NOS mRNA in the PVN of the rat. The increased

NOS-LI may thus be part of a general response to stimuli that change magnocellular region activities. Whether the mechanism is directly related to hormone or blood flow regulation is unclear. Alternatively, it has been suggested that NO may be a signaling molecule for the regulation of thermogenesis during cold acclimation both centrally and peripherally in rats (De Luca *et al.*, 1995).

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