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Cold exposure induces tyrosine phosphorylation of BIT through NMDA receptors in the rat hypothalamus

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Abstract

The hypothalamus has a central role in maintaining homeostases of physiological conditions including body temperature and energy balance. To examine molecular responses to cold exposure in the hypothalamus, we examined changes in protein tyrosine phosphorylation in the suprachiasmatic nucleus of the hypothalamus after acute cold exposure in rats. It was found that brain immunoglobulin-like molecule with tyrosine-based inhibitory motifs (BIT, also called SHPS-1, SIRPα or p84), a transmembrane glycoprotein with two ITIM motifs, showed enhanced tyrosine phosphorylation after cold exposure. Its tyrosine phosphorylation induced by cold exposure was also found in other hypothalamic nuclei including the paraventricular nucleus, lateral hypothalamic area, ventromedial hypothalamus, and arcuate nucleus. This phosphorylation was blocked by AP-5, an NMDA receptor antagonist, indicating that it was mediated by NMDA receptors. These results suggest that BIT is involved in the mechanism of neuronal responses to cold exposure in the hypothalamus.

Keywords: BIT; SHPS-1; SIRPα; p84; Cold exposure; Hypothalamus; Tyrosine phosphorylation

The hypothalamus has been implicated in maintaining body temperature at nearly constant levels under different external conditions. Upon cold exposure, acute vasoconstriction, piloerection, shivering thermogenesis, and nonshivering thermogenesis are triggered by the action of the hypothalamo-sympathetic nerve axis. Thermogenesis is also enhanced under the cold environment through thyroid hormones, whose secretions are regulated by the hypothalamo-hypophysial axis.

Hypothalamic control of homeostasis is achieved by cooperative functions of various hypothalamic nuclei. The suprachiasmatic nucleus of the hypothalamus (SCN) is a master circadian oscillator and generates a daily rhythm in body temperature [1–3]. We have been shown that the SCN is also involved in the control of glucose metabolism through autonomic regulations of insulin, glucagon, and adrenaline secretions [4,5]. In addition, these were suggested to be mediated by SCN neurons that send autonomic neural inputs to the pancreas and liver [6,7]. Other hypothalamic nuclei including the ventromedial hypothalamus (VMH), lateral hypothalamic area (LHA), paraventricular nucleus (PVH), dorsomedial nucleus, and arcuate nucleus (ARH) are also involved in the mechanism of thermogenesis which is related to the controls of energy metabolism and food intake [8].

Brain immunoglobulin-like molecule with tyrosine-based inhibitory motifs (BIT) is a transmembrane glycoprotein also referred to as SHPS-1, SIRP-a, and p84 [9,10]. It has been implicated in intracellular signal transduction mechanisms of receptor tyrosine kinases and integrins [11–14]. We have previously shown that tyrosine phosphorylation of BIT was induced in the suprachiasmatic nucleus of the hypothalamus and in the retina in response to light stimulation [15,16]. In

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addition, administration of anti-BIT monoclonal antibody into the third ventricle of a rat caused a phase shift of a circadian locomotor activity [17]. These results indicate that BIT is involved in neuronal transmission from the retina to the SCN to regulate master circadian clock located in the SCN.

To investigate the molecular responses of hypothalamic neurons to cold exposure, we examined protein tyrosine phosphorylation in various regions of the rat hypothalamus. We found a protein that was tyrosine-phosphorylated in response to cold exposure.

Materials and methods

Animals and cold exposure. Eight-week-old male Wistar strain rats were used in all experiments. They were housed under a 12-h light and 12-h dark cycle (lights on 07:00–19:00) at 24 ± 1 °C for at least 2 weeks before experiments. Food (type MF, Oriental Yeast, Tokyo) and water were freely available. Cold exposure was carried out at 4 °C and animals were sacrificed by decapitation.

Antibodies. A monoclonal anti-BIT antibody, 1D4, was previously described [18]. Rabbit polyclonal anti-BIT antibody was raised against a recombinant protein corresponding to amino acids 401–509 of rat BIT, which was expressed in *Escherichia coli* as a GST-fusion protein. Monoclonal anti-phosphotyrosine antibody, 4G10, was purchased from Upstate Biotechnology. Anti-mouse IgG and anti-rabbit IgG conjugated with horseradish peroxidase were from Cell Signaling Technology.

Preparation of tissue extracts. Rat brains were quickly removed after decapitation and frozen on dry ice. Using a cryostat, 600-μm thick coronal brain sections were obtained. Then, the SCN, PVH, ARH, VMH, LHA, and cerebral cortex were punched out from the sections using stainless steel pipes. The samples were homogenized in TNE buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 50 mM NaF, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 5 mM 2-mercaptoethanol), and the homogenates were centrifuged at 15,000*g* for 10 min. The supernatants were collected and the protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard.

Intracranial injection of AP-5. A cannula made of polyethylene tubing (PE-10; Clay Adams) was inserted into the right lateral cerebral ventricle (LCV) as previously described [17]. After recovering from surgery for 3 days, AP-5 (Sigma, 40 nmol/20 μl), an antagonist of NMDA receptor, or vehicle (aCSF; artificial cerebrospinal fluid) was administered into the LCV through the cannula with a rate of 20 μl/2 min. Ten minutes after injection, rats were subjected to cold exposure.

Gel electrophoresis and immunoblot analysis. Protein samples were separated by SDS–PAGE on 8% or 10% polyacrylamide gels. For immunoblot analysis, proteins were transferred to nitrocellulose membranes using a semi-dry electroblotting apparatus. After blocking with 0.1% Tween 20 in Tris-buffered saline (Tween/TBS, pH 7.4), the membranes were incubated with a primary antibody for 1 h, followed by a secondary antibody for 1 h and washed with Tween/TBS. Immunoreactive bands were detected with chemiluminescence reagents (Perkin–Elmer, Boston) and the illumination was detected with X-ray films (Kodak).

Immunoprecipitation and Concanavalin A–Sepharose precipitation. For immunoprecipitation, brain lysates were incubated for 6 h at 4 °C with anti-BIT monoclonal antibody (1D4) bound to protein G–Sepharose (Amersham). For Concanavalin A (Con A)–Sepharose precipitation, lysates were incubated overnight at 4 °C with 20 μl Con A–Sepharose (Amersham). The beads were washed four times with the lysis buffer, resuspended in the SDS sample buffer (0.125 M Tris–HCl, pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue), boiled for 3 min, and applied on a SDS–polyacrylamide gel.

Results

Effect of cold exposure on protein tyrosine phosphorylation in the SCN

In order to investigate the molecular mechanism underling the hypothalamic response to cold exposure, we searched for proteins that were phosphorylated at their tyrosine residues after cold exposure at 4 °C. The cold exposure was started at 10:00 and animals were sacrificed at indicated times, and protein tyrosine phosphorylation in the SCN was examined by immunoblotting using an anti-phosphotyrosine antibody (Fig. 1A). A marked increase in tyrosine phosphorylation of a 90 kDa protein was observed 1–12 h after cold exposure. We also examined serine- and threonine phosphorylations of proteins using anti-phosphoserine and anti-phosphothreonine antibodies, but no significant difference was detected (data not shown).

Since protein tyrosine phosphorylation in the SCN showed a daily change [15], it is possible that the change in the phosphorylation of the 90 kDa protein (Fig. 1A) might be due to its daily rhythm. To determine whether this is the case, a group of rats was exposed to 4 °C from 10:00 to 16:00, while a control group was kept at 25 °C. SCN samples were then prepared at 16:00 from these two groups. At 16:00, tyrosine phosphorylation of the 90 kDa protein was higher in cold-exposed rats than in control rats as seen in Fig. 1B, confirming that the increase in the phosphorylation is dependent on the change in the environmental temperature. Similar results were obtained at 14:00, 18:00, and 22:00 (data not shown).

In our previous study, we showed that a light stimulation during the dark period enhanced tyrosine phosphorylation of BIT, a membrane glycoprotein with a high affinity for Concanavalin A (Con A) [15]. The apparent molecular weight of BIT was close to that of the protein that underwent tyrosine phosphorylation on cold exposure. A precipitation of SCN lysates from cold-exposed rats with Con A–Sepharose beads showed enhanced tyrosine phosphorylation levels of a 90 kDa Con A-binding protein by a cold exposure (Fig. 2A). These indicated that the 90 kDa phosphotyrosil protein might be identical to BIT.

To confirm this, BIT was immunoprecipitated with a specific antibody against BIT from SCN extracts and analyzed by immunoblotting with an anti-phosphotyrosine antibody. The immunoprecipitated materials with the anti-BIT antibody showed enhanced tyrosine

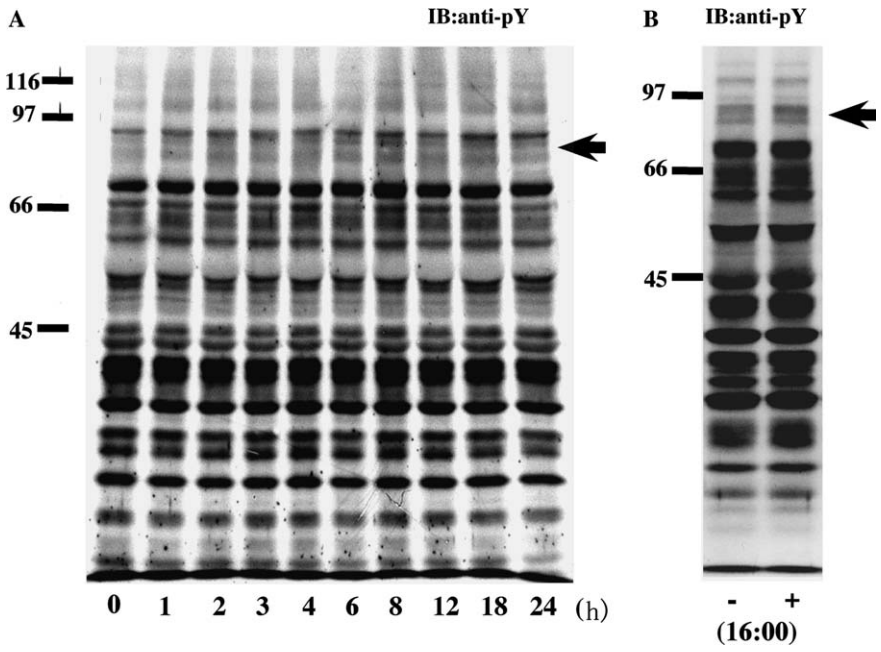

Fig. 1. Changes in protein tyrosine phosphorylation in the SCN on cold exposure. (A) Rats were exposed to cold (4 °C) from 10:00 for the indicated time periods. Brains were frozen, sliced, and SCNs were cut out. The tissue samples were then homogenized and analyzed by immunoblotting with anti-phosphotyrosine antibody (4G10). Each lane of the blot represents the SCN of a rat. (B) A rat was exposed to cold (4 °C) from 10:00 to 16:00, while a control rat was maintained under a room temperature (24 °C). Then, brains of rats were dissected at 16:00 and SCN extracts were prepared. Tyrosine phosphorylation was analyzed as described above.

phosphorylation after the cold exposure (Fig. 2B). In addition, the molecular weight of this protein was the same as that of the Con A-precipitated phosphotyrosil protein (Fig. 2A). These results suggest that cold exposure elicits tyrosine phosphorylation of BIT in the SCN.

To analyze the phosphorylation levels of BIT, immunoprecipitation is expected to be more specific than Con A–Sepharose precipitation, but it requires more than 10 rats for each lane of an immunoblot. In addition, the analysis of tyrosine phosphorylation by Con A–Sepharose precipitation was shown to give basically the same results as that using immunoprecipitation. Thus, we used Con A–Sepharose precipitation for further analyses.

Tyrosine phosphorylation of BIT in other hypothalamic nuclei

Next we examined whether tyrosine phosphorylation of BIT was also increased in other hypothalamic nuclei on cold exposure. Cold exposure was done from 10:00 to 16:00, and the hypothalamic paraventricular nucleus (PVH), arcuate nucleus (ARH), lateral hypothalamic area (LHA), ventromedial hypothalamus (VMH), and cerebral cortex in addition to the SCN were dissected. BIT was precipitated with Con A–Sepharose from the brain extracts and the phosphorylation levels were determined with the anti-phosphotyrosine antibody.

As shown in Fig. 3A, tyrosine phosphorylation of BIT on cold exposure was observed in all hypothalamic nuclei examined, with highest increase in the VMH. In contrast, its tyrosine phosphorylation was not significantly enhanced in the cerebral cortex. Time courses of its tyrosine phosphorylation in each hypothalamic nucleus (Figs. 3B and C) showed that it started within 1 h after the beginning of cold exposure and then gradually increased during the following 1–6 h. In the VMH and PVH, a second peak was found at 8 h after the start of the cold exposure.

We have previously shown that tyrosine phosphorylation of BIT was induced by glutamate in primary cultures of cortical neurons [17]. It was thus possible that cold-induced tyrosine phosphorylation was triggered by glutamate. To test this possibility, we injected AP-5 (D-2-amino-5-phosphonovalerate), a competitive NMDA receptor antagonist, into the lateral ventricle of a rat brain prior to the cold exposure. As shown in Fig. 4, tyrosine phosphorylation of BIT induced by cold exposure was blocked by the pretreatment with AP-5, suggesting that this phosphorylation was dependent on NMDA receptors.

Discussion

The purpose of this study was to investigate the molecular responses to cold environment in the

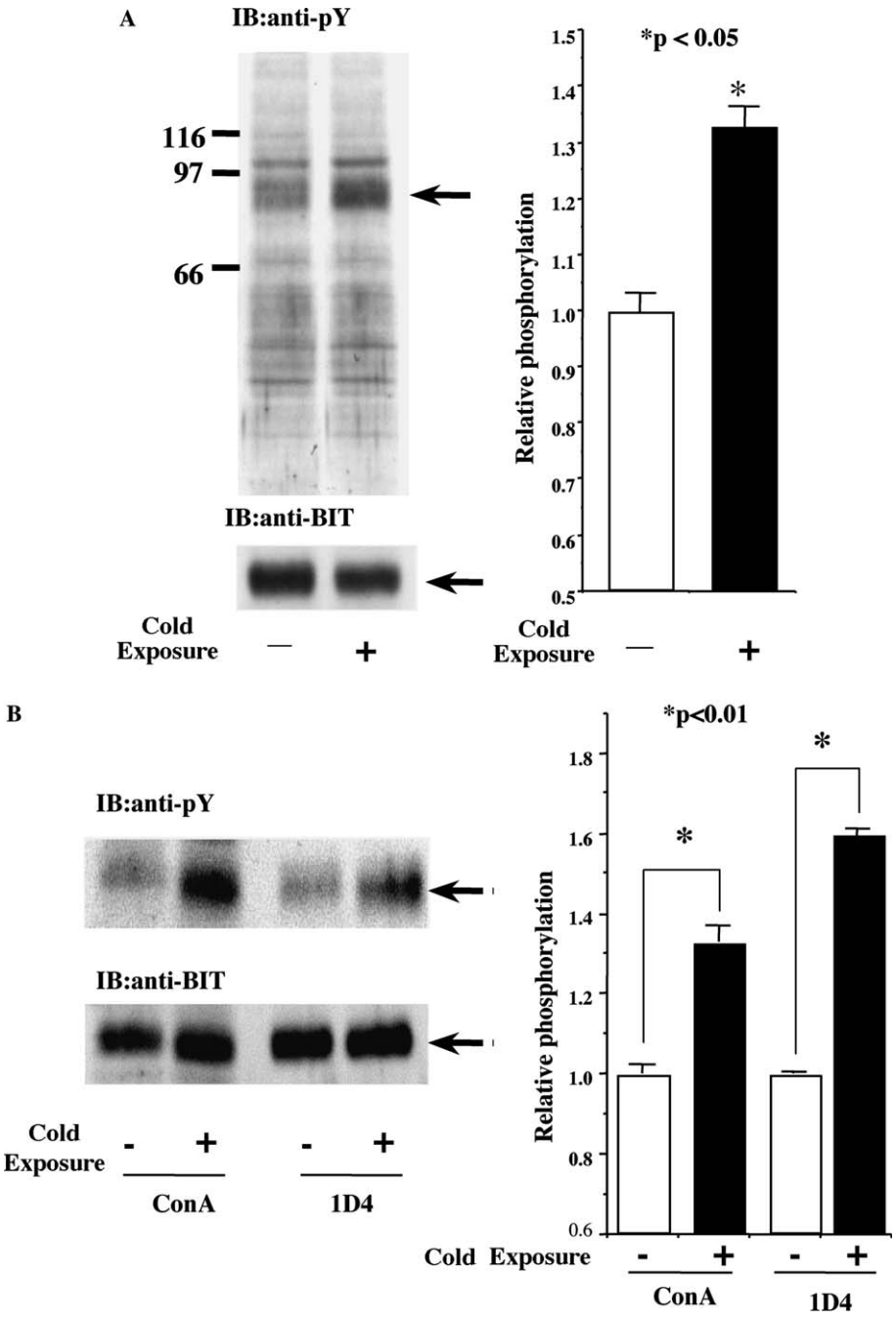

Fig. 2. Characterization of a 90 kDa phosphotyrosil protein in the SCN. SCN lysates were prepared from control rats (−) or rats exposed to cold at 4 °C for 6 h (+). (A) The lysates were incubated with Con A–Sepharose beads, and bound proteins were analyzed by immunoblotting with anti-phosphotyrosine (4G10) and anti-BIT antibodies (left). Tyrosine phosphorylation levels were quantified, normalized with the anti-BIT immunoreactivities (right). (B) SCN extracts from control and cold-exposed rats were immunoprecipitated with anti-BIT antibody or Con A–Sepharose and analyzed by immunoblotting with the anti-phosphotyrosine and anti-BIT antibodies. Data represent means ± SEMs from 3 groups, each of which was consisted of four independent rats for Con A precipitation and from each 3 groups which consisted of 10 rats in the case of immunoprecipitation using anti-BIT antibody.

hypothalamus. Our previous studies have suggested that the SCN functions to control the autonomic nervous system [4–7,19] which is important for physiological responses to cold environment. In this study, we first focused on cold-induced changes in proteins in the SCN and found that tyrosine phosphorylation of BIT, a 90 kDa protein with a high affinity for Con A, was increased on cold exposure.

BIT, also referred to as SHPS-1, SIRP-α, and p84, is a transmembrane glycoprotein with three Ig domains in its extracellular region [16,17,23]. Its cytoplasmic domain is tyrosine phosphorylated on a variety of stimuli

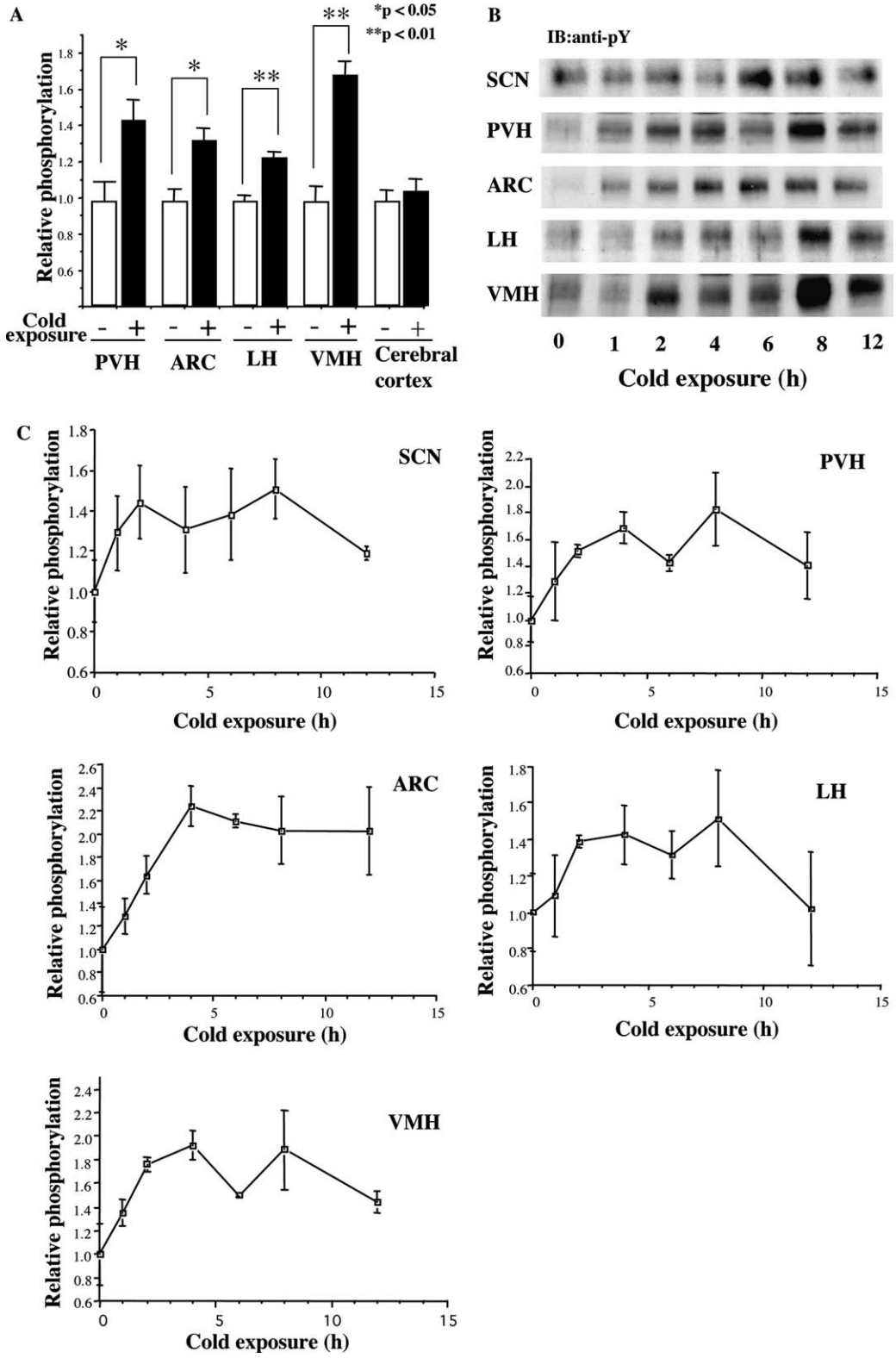

Fig. 3. Tyrosine phosphorylation of BIT in various hypothalamic nuclei. (A) Rats were exposed to cold for 6 h and several hypothalamic nuclei as well as the cerebral cortex were dissected. Then tissue extracts were prepared, precipitated with Con A–Sepharose and analyzed by immunoblotting with anti-phosphotyrosine and anti-BIT antibodies. Tyrosine phosphorylation levels were quantified and normalized with immunoreactivities of anti-BIT antibody. Data represent means ± SEMs. (B) Rats were exposed to cold from 10:00 for the indicated times, and hypothalamic nuclei were dissected from frozen brain slices. Tyrosine phosphorylation levels of BIT were examined as described above. (C) Phosphorylation of BIT was analyzed as shown in (B) and Western blots from three independent groups of rats were quantified. Data represent means ± SEMs. PVH, hypothalamic paraventricular nucleus; ARH, hypothalamic arcuate nucleus; LHA, lateral hypothalamic area; and VMH, ventromedial hypothalamus.

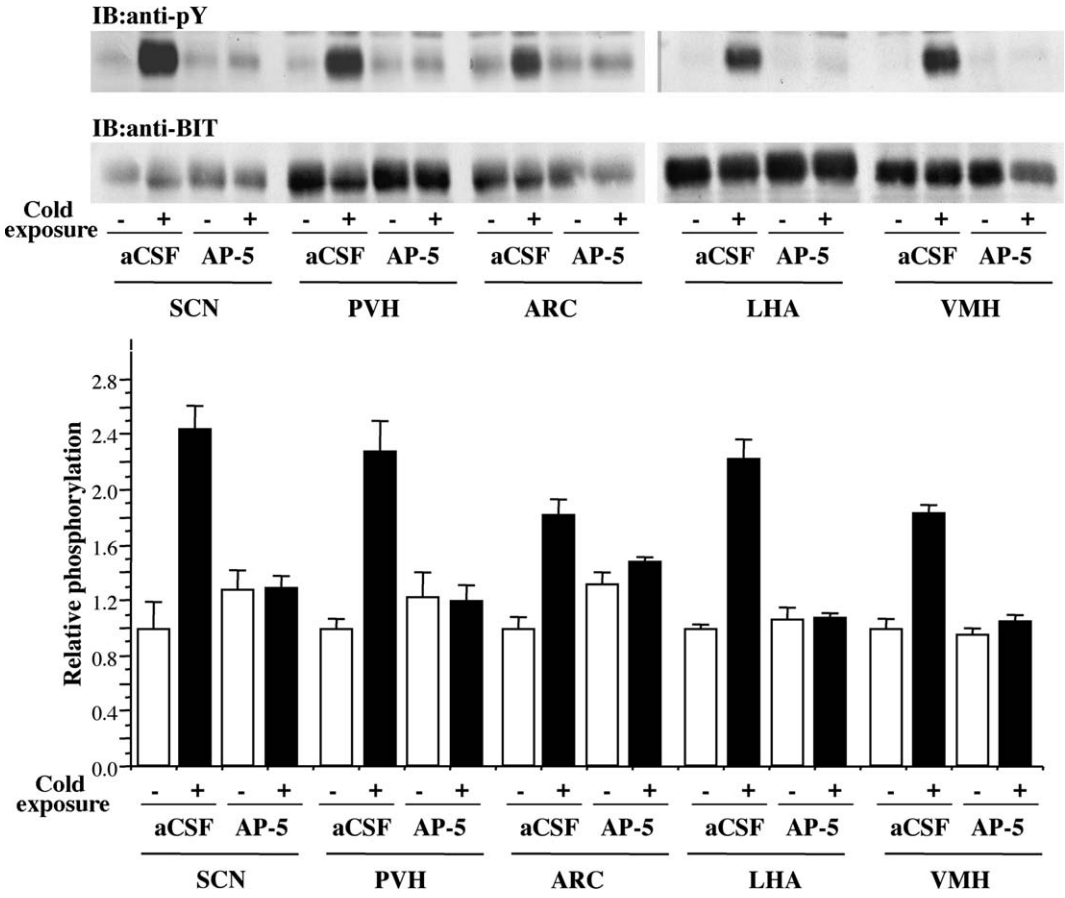

Fig. 4. Effect of an NMDA-R blocker, AP-5, on cold-induced tyrosine phosphorylation of BIT. Prior to cold exposure, AP-5 was injected into the lateral cerebral ventricle of the rat brain. After 10 min, the rats were exposed to cold for 4 h and extracts of hypothalamic nuclei (the SCN, PVH, ARC, LHA, and VMH) were analyzed by immunoblotting with the anti-phosphotyrosine and anti-BIT antibodies. Data represent means ± SEMs.

including insulin, brain derived neurotrophic factor, and integrins in cultured cells [22,23]. Tyrosine phosphorylation of BIT results in association with SHP-2, a protein tyrosine phosphatase with two SH2 domains, to positively regulate the MAP kinase cascade [13,21]. We have previously shown that tyrosine phosphorylation of BIT was induced in the SCN of the hypothalamus in response to photic stimulation on the retina [11]. In addition, administration of anti-BIT monoclonal antibody into the third cerebral ventricle of a rat caused a phase shift of a circadian locomotor activity in rats [12]. These results indicate that BIT is involved in neuronal transmission from the retina to the SCN to regulate master circadian clock located in the SCN.

Although cold exposure and light can induce tyrosine phosphorylation of BIT, time courses of its phosphorylation were different between these two cases. When the rat retina was exposed to light in the dark period, the phosphorylation level of BIT in the SCN was rapidly increased, peaking at 15 min after the light exposure [11]. This rapid phosphorylation induced by light exposure could be due to a rapid activation of glutamatergic inputs from the retina to the SCN. On the other hand, cold exposure at 4 °C gradually increased the tyrosine phosphorylation of BIT in the SCN and the phosphorylation peak was observed a few hours after the start of the cold exposure. This might indicate that activation of some neurons or release of some neurotransmitters took place with a slow time course on cold exposure. In this connection, it was reported that c-Fos expression was induced in different populations of hypothalamic neurons 3 h, 24 h, and 14 days after the start of cold exposure [7].

Tyrosine phosphorylation of BIT induced by cold exposure was found not only in the SCN but also in other hypothalamic nuclei including the PVH, VMH, LH, and ARH. This indicates that neurons in all these areas might be stimulated on cold exposure. This is consistent with the finding that [^{14}C]deoxyglucose incorporation was increased in a wide range of hypothalamic nuclei after cold exposure [8]. On the other hand, induction of c-Fos protein was found in more restricted neurons [20]. These discrepancies might be because c-Fos is induced in subpopulations of activated neurons.

It has been shown that light-induced phase shifts of circadian rhythms were mediated by glutamatergic neurons from the retina to the SCN [18]. Our previous studies indicated that light-induced tyrosine phosphorylation

of BIT seemed to be mediated by glutamate [11]. An immunohistochemical examination showed that BIT is present not only in the hypothalamus, but also in many regions of the rat brain. In this study, we found that AP-5, an antagonist of NMDA receptors, suppressed the tyrosine phosphorylation of BIT in hypothalamic nuclei after cold exposure (Fig. 4). In addition, neurons containing NMDA receptors are observed in various brain areas. Taken together, it is possible that tyrosine phosphorylation of BIT is involved not only in circadian phase resetting and cold adaptation, but also in other brain functions mediated by NMDA receptors. Thus, it must be determined whether tyrosine phosphorylation of BIT could take place in association with other neuronal activities.

These present results indicate that BIT is involved not only in photic entrainment of the circadian rhythms, but also in hypothalamic control of cold adaptation. This raised a possibility that tyrosine phosphorylation of BIT is involved in a wide variety of neuronal functions mediated by NMDA receptors. For further analysis, it would be needed to prepare antibodies that specifically recognize the phosphorylated forms of BIT to localize the region of tyrosine phosphorylation in the hypothalamus. It must also be required to search ligands for the extracellular region of BIT to clarify the functions of BIT in the nervous system.

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