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Photoperiod affects distribution of dynorphin A in the brain of Siberian hamster

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Abstract. Dynorphin $A_{1–17}$ (DYN $A_{1–17}$) acting in the CNS is known to affect thermoregulation, water and energy balance in the short time scale. In this study a long-term alteration of these functions induced by changes of day length in the highly photoperiodic species, the Siberian hamster (*Phodopus sungorus*) was studied using immunohistochemistry for DYN $A_{1–17}$. We found that in the long day (LD, L:D 16 h:8 h) more brain areas express DYN $A_{1–17}$ peptide than in the short day (SD, L:D 8 h:16 h) conditions. Structures of the hypothalamo-pituitary axis as well as cells of the ependyma, subcomissural organ and choroid plexus of the lateral and third brain ventricles are immunoreactive to anti-dynorphin IgG only in the LD. This might indicate a seasonal regulatory role of DYN $A_{1–17}$ in physiological adaptations to severe climate changes.

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INTRODUCTION

Siberian hamster (*Phodopus sungorus*) is a highly photoperiodic species showing a wide range of adaptations to extreme climate changes (Hoffman 1973, Lynch and Puchalski 1986). Under short day (SD), winter conditions it undergoes involution of the gonads leading to attenuation of reproductive functions (Hoffman 1973, Niklowitz and Hoffman 1988, Pawlak et al. 2005). It also exhibits a molt to thick, white winter pelage (Lynch and Puchalski 1986), weight loss (Lynch and Puchalski 1986, McElroy et al. 1986) with late onset of hypophagia and episodes of torpor lasting for about 4–8 h, during which body temperature decreases from 37°C to about 12–15°C (Dark et al. 1999, Elliot et al. 1987, Lynch and Puchalski 1986). All these adaptations can be induced by manipulating day length (Elliot et al. 1987, Lynch and Puchalski 1986).

Preprodynorphin (ppDYN) mRNA and particular peptides derived from ppDYN are widely distributed throughout the rodent brain and pituitary (DePaoli et al. 1994, Khachaturian et al. 1982, Neal and Newman 1989). Many of these structures are also target areas of melatonin (Sliwowska et al. 2004). Since the duration of the nocturnal elevation of melatonin level has been shown to be the most relevant biological signal for seasonal changes in the Siberian hamster (Lynch and Puchalski 1986, Klante and Steintechner 1994) it may imply that the dynorphinergic system is somehow involved in the seasonal changes in CNS.

It was suggested, that dynorphin $A_{1–17}$ (DYN $A_{1–17}$) has a significant regulatory role in thermoregulation (Cavicchini et al. 1989) most probably acting at the level of thermosensitive hypothalamic neurons (Yakimova et al. 1998). DYN $A_{1–17}$ also affects water balance through an inhibitory action (*via* kappa-opioid receptors) on vasopressin release from the paraventricular nuclei (Shuster et al. 2000) and through pituicytes in the pars nervosa of pituitary (Boersma and Van Leeuwen 1994). DYN $A_{1–17}$ is also thought to regulate reproduction (Boersma and Van Leeuwen 1994) as it co-localizes with follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Khachaturian et al. 1986) in the gonadotrophs in pars distalis of the pituitary. Moreover DYN $A_{1–17}$ is co-released with FSH and LH (Knepel et al. 1985) and this release is inhibited or decreased by the same agents that inhibit LH/FSH release (Schwaninger et al. 1987).

It is suggested that the binding affinity of DYN $A_{1–17}$ may be altered by changing photoperiod, as the short day (SD) conditions render golden hamsters less sensitive to a replacement testosterone therapy, which in LD diminishes binding affinity of H^3naloxone induced by castration (Tubbiola and Bittman 1994). If, therefore, change of day length affects expression of dynorphin in the brain, it is important to examine whether the physiological adaptations to certain photoperiods are accompanied by changes in the dynorphinergic system.

The aim of our study is to investigate whether photoperiod alters DYN $A_{1–17}$ distribution in the brain regions controlling physiological functions involved in winter adaptations in the Siberian hamster.

METHODS

Experimental animals

Siberian hamsters (*Phodopus sungorus*) from our own breeding colony were housed from their birth in long-day conditions (LD, Light:Darkness 16 h:8 h). At weaning they were divided into two groups, one (3 females and 3 males) was transferred to the short-day conditions (SD, L:D 8 h:16 h) while the other, consisting of the same number of animals, remained in the LD conditions. They were housed in groups of 3 in standard conditions with constant access to food and water. After 10 weeks in the experimental conditions animals were weighed and – in case of the SD animals – evaluated by two independent observers for changes in fur color. All SD animals achieved points 3 and 4 on 4-point scale of fur change (where 1 is no change, 2 – small change of the fur color on the animal's head but no change on sides, 3 – substantial changes of color on the head and sides, and 4 – white fur). At mid-light phase (approx. 2:00 P.M.) animals were sacrificed by intraperitoneal injection of sodium pentobarbital solution (Vetbutal, Biovet Puławy, Poland; 6 mg/kg of body weight) and perfused transcardially with phosphate buffer saline (0.01M PBS, pH = 7.4–7.6) and fixative (4% paraformaldehyde and 15% picric acid diluted in PBS). The brains were postfixed for at least 48 h before embedding in paraplast (Paraplast Regular, Sigma, St. Louis) and then cut into 8 μm coronal slices.

All experiments were conducted in accordance with the Polish Law on Animal Protection and the guidelines established by the Declaration of Helsinki concerning Care and Use of Animal in Research.

Procedures

Indirect immunohistochemistry staining (IHC) was performed using goat polyclonal antibody against human Dynorphin $A_{1–17}$ diluted 1:2500. Clustal W (1.82) (Higgins et al. 1996) alignment showed that human, rat and Guinea pig dynorphin $A_{1–17}$ peptides have identical protein sequence. Afterwards, the slides were incubated with a biotinylated porcine monoclonal anti-rabbit, anti--mouse and anti-goat IgG (DAKO, LSAB KO690).A porcine serum (5%, Normal DAKO X0901) was used for blocking the nonspecific binding of the secondary antibody. Antibody binding and serum blocking was performed at +4°C in high humidity conditions. The staining was developed with the streptavidin-horseradish peroxidase complex (DAKO, KIT Streptavidin Peroxidase Conjugated KO690) and diaminobenzidine (DAB, 3,3'Diaminobenzidine, Tablet Set, Sigma Fast). TRIS buffer (TRIS/HCl pH 7.4, T-8404, Sigma) was used for attenuation of the reaction. All slides were mounted with DPX (DPX Mountant, No 360299 BDH Chemicals Ltd.). Sections were assessed with Nikon Eclipse E600 microscope combined with digital camera (Panasonic GP-KR222E). Identification of the labeled structures was done by comparison with the Stereotaxic Atlas of the Golden Hamster Brain (Morin and Wood 2001).

Specificity of the primary antibody was checked by 24 h preincubation with the synthetic human DYN $A_{1–17}$ peptide (5 nM/ml, Sigma) that was used for immunization of the goat. Then IHC staining on brain tissue was performed using the saturated antibody. No DYN $A_{1–17}$ immunoreactivity (DYN-ir) was observed. Additionally, one section on each coverslip was used as a control. On these sections the first antibody (IgG anti-DYN $A_{1–17}$) was replaced with PBS. No staining was observed on these sections.

RESULTS

A set of physiological differences in experimental animals subjected to 10 weeks of exposure to either long day (L:D 16 h:8 h) or short-day (L:D 8 h:16 h) photoperiod was observed. Under SD conditions the animals of both sexes showed the same changes of fur color, turning from dark-grey to almost white. The average weight of animals housed in SD conditions was lower than the weight of animals housed in the LD regime (females 32.5 ± 4.8 g *versus* 35.9 ± 2.1 g and males 38.1 ± 2.7 g *versus* 44.4 ± 2.7 g, respectively, $P>0.05$).

Dynorphin $A_{1–17}$ immunoreactivity was present in several brain structures in Siberian hamsters kept in both SD and LD conditions. No sex-related differences in distribution of DYN-ir material were found.

Most of the structures in the brains of Siberian hamsters showed positive immunoreactivity to anti-DYN $A_{1–17}$ antibodies in both photoperiods. Nevertheless, a distinct pattern of DYN-ir structures characteristic only for long-day (LD, L:D 16 h:8 h) or short-day (SD, L:D 8 h:16 h) conditions was observed.

Under LD conditions DYN-ir cells and fibers were found in the median eminence (Fig. 1A) and pars tuberalis of the pituitary gland. No such staining was observed in the brains of SD animals (Fig. 1B).

Substantial photoperiod-dependent, qualitative differences in immunostaining was observed in the cells of the subcomissural organ, ependyma (Fig. 1C) of the III ventricle and the choroid plexus (Fig. 1E) of the third and lateral ventricles as well as in the smooth muscle cell layer of meningeal vessels. In the LD conditions the staining was heavy, while it was absent in the SD conditions (Fig. 1D, F). A photoperiod-related difference in labeling of the fibers was also found. In the LD conditions staining was mainly located in the preoptic area and arcuate nuclei of the hypothalamus, while in SD conditions staining was present in the substantia nigra pars compacta, dorsal raphe nucleus and nuclei of the solitary tract (data not shown).

The density and localization of DYN-ir cells in the pituitary gland was different in LD and SD animals despite the presence of DYN-ir in pars nervosa (fibers) and pars distalis (cells) in both photoperiods. In the LD conditions DYN-ir in both parts of the pituitary tended to concentrate around blood vessels, while diffuse staining was observed in SD conditions.

DISCUSSION

A widespread distribution of DYN-immunoreactive cells and nerve fibers was observed in the brain of the Siberian hamster (*Phodopus sungorus*). Staining in some of these structures occurred in photoperiod-dependent manner. Since all animals were housed at room temperature (20 ± 2°C) and given food and water *ad libitum*, photoperiod seems to be the only differentiating factor in this study. However, we can not exclude some secondary changes, e.g. change in feeding activated by change in photoperiod (Lynch and Puchalski 1986, McElroy et al. 1986). We found no sex-dependent differences in the density and distribution of DYN-ir staining.

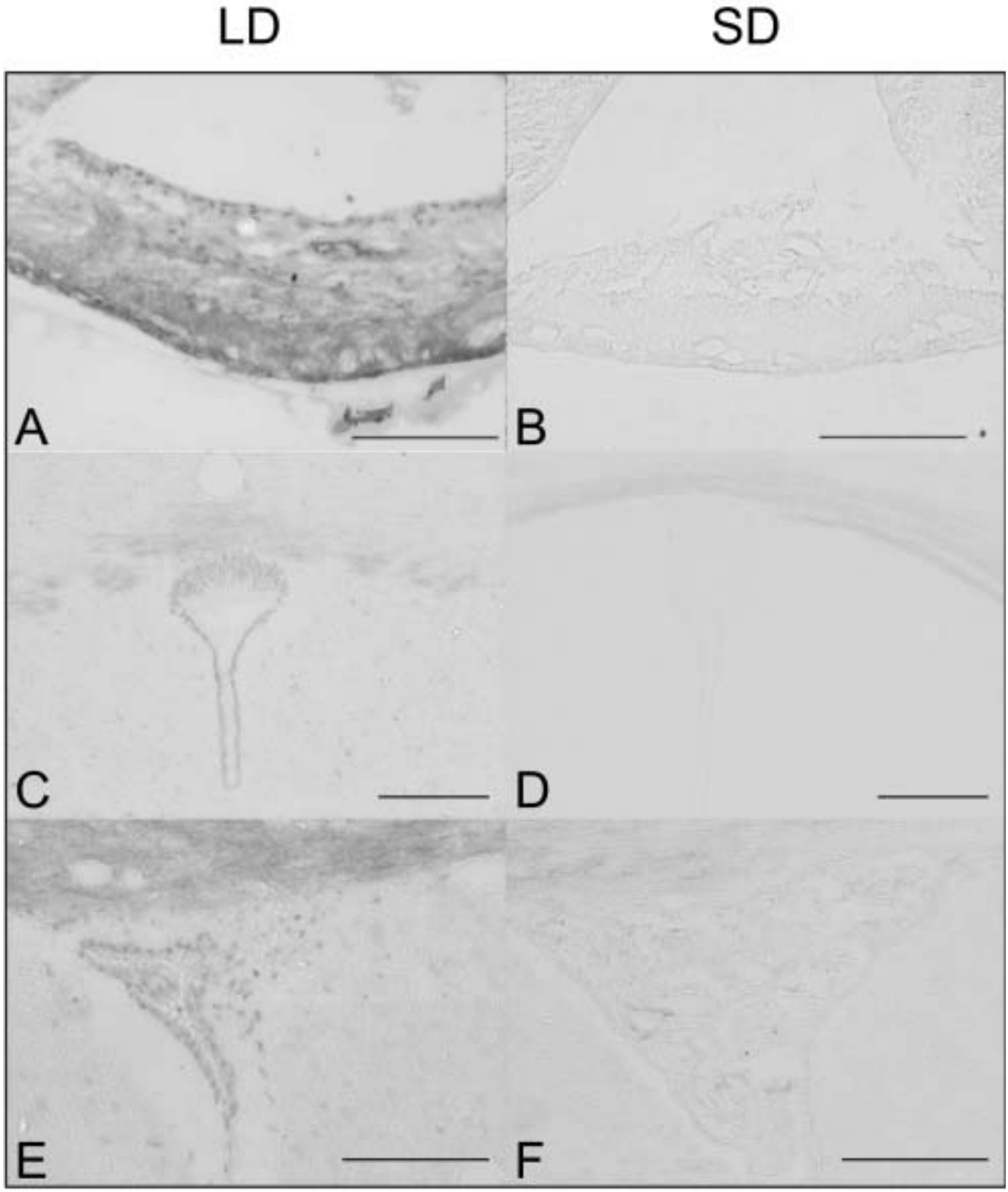

Fig. 1. Regional expression of DYN A_{1-17} protein in Siberian hamster brain after housing in LD (L:D 16:8 h) and SD (L:D 8:16 h) conditions: (A/B) median eminence; (C/D) subcomissural organ; (E/F) the choroid plexus of the lateral ventricle (LD/SD). Scale bar is 100 μm.

To the best of our knowledge this is the first description of photoperiodic changes in dynorphin $A_{1–17}$ distribution in the brain and pituitary gland of a seasonal species of rodent. Our study shows that the DYN $A_{1–17}$ localization in the Siberian hamster is similar to that described previously in the Guinea pig, rat and Syrian hamster (Khatchaturian et al. 1982, Neal and Newman 1989, Shuster et al. 2000) at the same time covering broader material including the pituitary gland. In contrast to previous publications we observed fewer DYN-ir cells and more DYN-ir fibers. This difference might be due to the species and/or methodological differences, as unlike in the studies on the rat and Syrian hamster (Neal and Newman 1989), we did not use colchicine to stop axonal transport of DYN $A_{1–17}$ along microtubules. A qualitative difference was nevertheless found in the paraventricular nuclei of the hypothalamus. Contrary to results obtained in the rat and Syrian hamster in our study we did not find any DYN-ir cells or fibers in this region. This might be due to the fact that in the rats the level of DYN $A_{1–17}$ undergoes circadian fluctuations, reaching its peak in the hypothalamus at night (Przewlocki et al. 1983). Siberian hamsters in our study were sacrificed at midday, when the level of dynorphin in the hypothalamus, especially in structures responsible for water balance control is low. On the other hand Shuster and co-workers (2000) suggest that DYN B is the peptide regulating water balance acting on vasopressin neurons in the PVN of Guinea pigs. As DYN B has not yet been studied in Siberian hamsters this remains to be clarified.

In the majority of structures investigated in this study DYN-ir was observed under both photoperiodic conditions. However, more structures were seen in the LD (L:D 16 h:8 h) than in SD (L:D 8 h:16 h) regime. The most striking difference was the status of the dynorphinergic system in the hypothalamo-pituitary axis. Fibers in the hypothalamic structures such as preoptic (POA) and arcuate nuclei (Arc), involved in rats in control of thermoregulation (Cavicchini et al. 1989, Yakimova et al. 1998) and reproduction (Khatchaturian et al. 1986, Knepel et al 1985, Schwaninger et al. 1987) were DYN-ir only in the LD regime. This, accompanied by immunoreactivity in the median eminence (ME) together with pars tuberalis (p.t.) and pars distalis (p.d.) of the pituitary gland that are involved on the hormonal control of molting and lactation may indicate possible role of dynorphin in photoperiod-dependent changes of Siberian hamster physiology.

As mentioned before, the POA and Arc nuclei are involved in thermoregulation. In our study, stable temperature in the cages and food provided *ad libitum* might have prevented the early onset of thermoregulatory torpor bouts. In hamsters normally these episodes start 10–13 weeks after the switch to SD conditions (Dark et al. 1999, Lynch and Puchalski 1986). In our case, the animals were sacrificed 10 weeks after switching to the SD regime, which could have been too early for the torpor behavior to show. This could explain the absence of DYN-ir in structures responsible for thermoregulation in the SD conditions. On the other hand seasonal changes of DYN-ir in the smooth muscle cell layer of meningeal and pituitary vessels might suggest that Siberian hamsters were potentially able to fall into torpor, as dynorphin is suggested to have direct impact on the blood flow (Mikhailova et al. 2003). We also found DYN-ir in SD conditions in the nuclei of the solitary tract, a structure mediating control of bradycardia by dynorphin (Mikhailova et al. 2003), as well as in the substantia nigra pars compacta and the dorsal raphe nucleus, structures controlling voluntary movement, mood and sleep/wake cycle. Presence of DYN-ir in SD conditions in these structures might be responsible for rendering Siberian hamsters potentially able to fall into torpor.

The Arc and POA nuclei of hypothalamus together with the pars distalis of the pituitary are also involved in control of reproductive functions. Experiments on rats suggest the positive feedback role of dynorphin in these structures (Neal and Newman 1989). Previous studies in our laboratory showed that the diameters of testes in males from our breeding colony kept under SD conditions decreased markedly by the 10th week of SD (Pawlak et al. 2005). Therefore, we suggest that the absence of DYN immunoreactivity in Arc and POA might be correlated with the involution of gonads of Siberian hamsters.

Another seasonal regulatory action of dynorphin could be that exerted upon release of prolactin (PRL) from lactotrophs situated in the pars distalis (Badura and Goldman 1992, Hazlerigg 2001) of the pituitary gland. In our study we found a distinct pattern of DYN-ir distribution within the pars distalis of LD and SD animals. Dynorphin is known to stimulate the release of PRL by inhibition of dopaminergic (DA) input on the cells in the pars tuberalis that directly activate cells in the pituitary pars distalis in the turkey (Youngren et al. 1999). If this is true for other species and both at the levels of hypothalamus and pars tuberalis, dynorphin might prove to

be the unknown factor suggested by Hazlerigg (2001) in studies on melatonin regulation of PRL release. Pinealectomy renders Siberian hamsters insensitive to DA agonists and releases PRL from DA inhibition resulting in prevention of molt into winter pelage (Badura and Goldman 1992). This might mean that melatonin is required for the release of the inhibitory action of DA on the pituitary lactotrophs from dynorphin suppression. If therefore the long melatonin signal of SD conditions would prevent dynorphin synthesis in the pars tuberalis of the pituitary, the absence of DYN-ir in medial eminence and pars tuberalis observed in our study in SD conditions would allow DA neurons to suppress PRL release, which would result in molt to winter pelage. This is consistent with the fact that all the animals in SD conditions exhibited profound changes in fur color. As this was the early stage of SD-induced changes, melatonin should act as a potent inhibitor of dynorphin synthesis with no sign of refractoriness (Schwaninger et al. 1987).

To the best of our knowledge our results are the first evidence of distinct photoperiod-dependent changes in DYN-ir in ependyma, subcommissural organ and choroid plexus of the third (and in the case of choroid plexus also lateral) ventricle as well as in the smooth muscle cell layer of meningeal vessels in a seasonal species of rodent.

DYN $A_{1–17}$ is known to either diffuse or be actively transported through the ependymal layer, as it potently and dynamically affects thermoregulation after intraventricular infusion (Cavicchini et al. 1989). In our study, dynorphin peptide was found in animals kept in LD conditions in the ependyma, and the choroid plexus formation. It is well known that DYN $A_{1–8}$ and DYN $A_{1–13}$ peptides can be transported through the blood-brain barrier (Turner et al. 1998). Also proteolitic enzymes allowing digestion of ppDYN peptide into smaller, functional proteins were found in the choroid plexus (Nyberg et al. 1991). This might suggest that dynorphin synthesized and/or cleaved at this location in a photoperiod-dependent manner might then be transported *via* blood and cerebrospinal fluid to a structure where it elicits its seasonal action.

Dynorphin was also found in the subcomissural organ under the LD regime. This structure is believed to be functionally interconnected with the adrenal glands (Gul'iants and Siziakina 1980). Finding DYN-ir cells in this region might therefore suggest that dynorphin not only acts at the hypothalamic and pituitary levels, but also affects the HPA axis at the adrenal level.

CONCLUSIONS

In the present study a relationship between the regional expression of DYN $A_{1–17}$ protein and photoperiod was observed. LD conditions were associated with intense staining in the structures of the hypothalamo-pituitary axis and structures controlling release of substances through blood-brain barrier and into the cerebrospinal fluid. In contrast, SD conditions were associated with staining in structures responsible for bradycadia, control of voluntary movement and mood and sleep/wake cycle. These findings suggest that dynorphin A may play a significant role in transfer of seasonal-melatonin signal onto the neuroendocrine regulatory effector system. The qualitative character of our results allows us to draw only preliminary conclusions and therefore further investigation on the role of dynorphin in seasonal adaptations to winter conditions is needed.

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