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IN THE RAT PINEAL GLAND, BUT NOT IN THE SUPRACHIASMATIC NUCLEUS, THE AMOUNT OF CONSTITUTIVE NEURONAL NITRIC OXIDE SYNTHASE IS REGULATED BY ENVIRONMENTAL LIGHTING CONDITIONS

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To investigate whether the expression of neuronal isoform of nitric oxide synthase is photoneurally regulated, we examined the amount of the enzyme by means of Western Blot analysis under different environmental lighting conditions in two photoneuronally regulated tissues, the pineal gland and the suprachiasmatic nucleus of the rat. In the pineal gland nitric oxide synthase immunoreactivity was strikingly decreased when rats had been exposed for 8 days to light:dark 20:4 conditions or to constant light. The decline in nitric oxide synthase immunoreactivity following LL reversed after 4 days under light:dark 12:12. We conclude that in the rat pineal the amount of neuronal nitric oxide synthase is controlled by environmental lighting conditions. That it is not justified to extrapolate from the pineal to other photoneuronally regulated centers is illustrated by the present finding that the suprachiasmatic nucleus did not reveal changes in the amount of neuronal nitric oxide synthase.

Nitric oxide (NO) is formed from L-arginine by the enzyme nitric oxide synthase (NOS). To date, one inducible and two constitutive isoforms of the enzyme have been identified (1). Inducible NOS (type II, 130 kDa) is expressed in macrophages upon treatment with cytokines. This isoform of NOS is tightly associated with calmodulin to keep the enzyme in its tonically active state. On the other hand, endothelial (type III, 130 kDa) and neuronal (type I, 150 kDa) isoforms of NOS are believed to be constitutively present. They exhibit strong Ca^{2+}/calmodulin requirement and are regulated by receptor-stimulated Ca^{2+} influx.

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Abbreviations: NO: nitric oxide; NOS: nitric oxide synthase; SCN: suprachiasmatic nucleus; NE: norepinephrine; LL: constant light; LD: light:dark; NOS-IR: NOS-immunoreactivity.

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The neuronal isoform of NOS is abundant in the rat pineal gland (2) where it mediates cGMP transduction. NOS activity appears to be regulated through concurrent stimulation of α_1- and ß-adrenoceptors by the primary transmitter norepinephrine (NE; 2,3), released from sympathetic nerve endings innervating the organ (4). The neural circuits regulating NE release include the hypothalamic suprachiasmatic nucleus (SCN; 5) and the superior cervical ganglion (SCG; 6). An endogenous clock in the SCN, which is entrained by light acting through the retina, produces a rhythm of NE release. In rats, NE release is low during the light phase and increases during the dark phase (7). The nocturnal induction of NE release can be abolished by stimulus deprivation, e.g. housing the rats in constant light (LL) (8).

It has recently been found that chronic elongation of the light phases decreases pineal NOS activity (9). This raises the question of whether the drop in NOS activity is due to NOS inactivation, e.g. (de)phoshorylaton (10,11), or to a decline in the amount of NOS. With this in mind, we investigated the amount of neuronal NOS in the rat pineal gland following exposure of the animals to different environmental lighting conditions.

MATERIALS AND METHODS

Animals.

Male Sprague-Dawley rats (mean body weight 200-250 g) were obtained from a commercial breeder (Interfauna, Tuttlingen). They were kept under standard laboratory conditions (LD 12:12; lights on at 6.00 h; illumination with fluorescent strip lights, 200 lux at cage level; 20 ± 1°C; water and food ad libitum) two weeks prior to experimentation. Then animals were exposed to the following lighting conditions for 8 days: LD 12:12, LL or LD 20:4 (lights on at 6.00 h). Subsequently one group of LL rats was returned to LD 12:12 for 4 days (LL/LD rats). All animals were killed at 10:00-11:00 h. For the study of differences throughout the 24 h cycle, four groups of rats were housed under LD 12:12 cycles with time of lights on shifted 6 h for each group. In this experiment, rats were killed at 11.30-12.00 h under light or dim red light. Pineal glands, SCN and olfactory bulbs were removed after decapitation under light ether anesthesia. All investigations were conducted on groups of four rats each.

Materials.

Chemicals and antibodies were obtained from the following sources: monoclonal antibody to NOS raised against a 22.3 kDa protein fragment (amino acids 1095-1289) of human neuronal NOS from Affiniti (Nottingham, UK); alkaline phosphatase-conjugated goat anti-mouse antibody from Dianova (Hamburg, FRG); equipment for electrophoresis and electrotransfer was from Bio-Rad Laboratories (Munich, FRG).

Partial purification of NOS.

NOS was partially purified as described by Bredt and Snyder (12) with slight modifications. All procedures were carried out at 4°C.

After decapitation, pineal glands/SCN/olfactory bulbs were immediately homogenized by sonication in 30/30/60 µl of 50 mM Tris-HCl, pH 7.4, containing antipain (10 µg/ml), chymostatin (10 µg/ml), dithiothreitol (1 mM), EDTA (0.1 mM), EGTA (0.1 mM), leupeptin (3 µM), mercaptoethanol (12 mM), pepstatin A (1 µM), trypsin-inhibitor (10 µg/ml), phenylmethylsulfonyl fluoride (1 mM). The homogenates were centrifuged for 1 h (100.000 x g) and the supernatant fractions (and as indicated the pellets) were used for SDS electrophoresis.

Protein determination.

Protein concentrations of the supernatant samples were analysed according to the method described by Lowry et al. (13).

Gel electrophoresis.

For Western blot analysis of rat pineal/SCN/olfactory bulb supernatant 60/150/300 µg protein were used per lane. Supernatants (and as indicated pellets) were dissolved in up to 100 µl sample buffer (0.15 M Tris-HCl, pH 6.8, containing 4% SDS, 22% glycerol and 0.05% bromophenol blue) and prepared by adding 10 µl mercaptoethanol and heating for 10 min at 90°C. SDS gel electrophoresis was performed using 7.5% acrylamide/bisacrylamide according to the method of Laemmli (14).

Western Blot analysis.

Electroblotting onto nitrocellulose (0.45 µm) was carried out in a semi-dry blot system applying a 3 mA current per cm^2 for 1 h. All subsequent incubations were followed by washing in four changes of phosphate-buffered saline (10 mM PBS, pH 7.4), the second and third wash containing 0.1% Triton-X100. The membranes were blocked in 1% BSA in PBS for 30 min and incubated in primary antibody (1:250 in PBS containing 1% BSA) for 2 h. The following immunostaining was performed using an alkaline phosphatase-conjugated secondary antibody (1:50.000) for 1 h incubation. Control experiments were performed by omitting the first and/or the second antibody.

RESULTS

To find out whether there are circadian changes in the amounts of NOS, we compared NOS-immunoreactivity (NOS-IR) at various time-points during LD 12:12. Irrespective of the tissue, we were unable to detect clear differences in the intensities of NOS-IR (data not shown). We, further, investigated the effect of LL exposure on NOS-IR. In comparison to LD 12:12 treatment, NOS-IR of the pineal supernatant fraction was markedly reduced (Fig. 1). To find out whether the decline in NOS-IR is reversible, rats were moved from LL to LD 12:12 for 4 days (Fig. 1). In comparison to animals continuously kept under LL, pineal NOS-IR was clearly increased. To investigate the possibility that LL-treatment induces a translocation of pineal NOS from the soluble to the particulate fraction, NOS-IR of the pellets was also considered. In this fraction we did not detect NOS-IR following either LD 12:12 or LL treatment (Fig. 2). Contrary to the pineal gland, NOS-IR of SCN and olfactory bulb did not exhibit any detectable changes

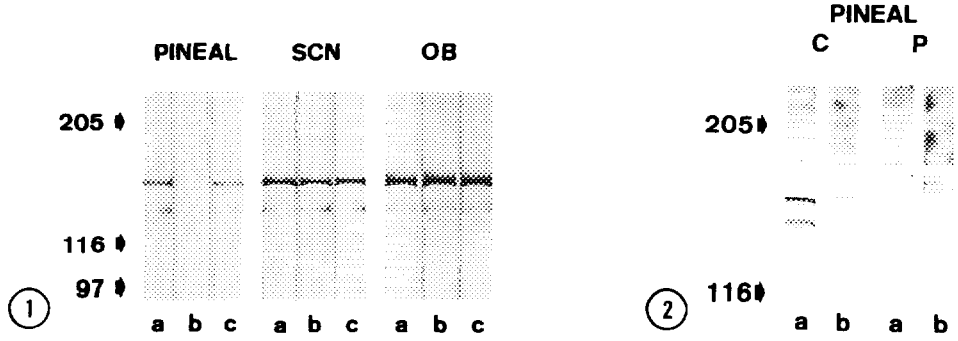

Fig. 1. Western blot analysis of neuronal NOS after exposure to constant light. Supernatant fractions of rat pineal gland, nucleus suprachiasmaticus (SCN) and olfactory bulb (OB) were separated on 7,5 % SDS-PAGE. Immunoreaction was conducted with monoclonal antibodies raised against a protein fragment of human neuronal NOS. Tissues were taken from rats killed 5 h after lights on. Animals were exposed to light:dark 12:12 (lanes a), constant light (lanes b) and or moved back from constant light treatment to light:dark 12:12 for 4 days (lanes c). Position of molecular weight markers are shown on the left (kDa).

Fig. 2. Western blot analysis of pineal cytosolic (C) and particulate (P) neuronal NOS after exposure to light:dark 12:12 conditions (lanes a) and constant light (lanes b). Crude homogenate was separated by centrifugation and proteins were subjected to 7,5 % SDS-PAGE. Immunoreaction was conducted with monoclonal antibodies raised against a protein fragment of human neuronal NOS. Positions of molecular weight markers are shown on the left (kDa).

following LL or LL/LD exposure (Fig. 1). Further, we examined how different photoperiodic information affects the amount of NOS. In pineal glands of rats kept under LD 20:4 for 8 days the extended light period led to a clear decrease in NOS-IR compared to LD 12:12 animals (Fig. 3). In SCN and olfactory bulb NOS-IR did not differ between LD 20:4 and LD 12:12.

DISCUSSION

We show in this study that in the rat pineal the amount of neuronal NOS varies considerably under different lighting conditions. Two aspects illustrate this notion: (i) Exposure of rats to longer periods of light for a number of days leads to a down-regulation of the amount of NOS and (ii) the down-regulation is reversed when the animals were moved back to LD 12:12 for several days. Our findings, together with previous observations that NOS expression changes after different experimental treatments (15,16,17), show that the current concept of the

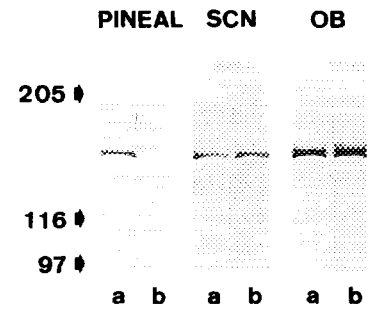

Fig. 3. Western blot analysis of neuronal NOS after exposure to different photoperiods. Supernatant fractions of rat pineal gland, nucleus suprachiasmaticus (SCN) and olfactory bulb (OB) were separated on 7,5 % SDS-PAGE. Immunoreaction was carried out with monoclonal antibodies raised against a protein fragment of human neuronal NOS. Tissues were taken from animals killed 5 h after lights on. Rats were exposed to light:dark 12:12 (lanes a) or 20:4 (lanes b). Arrowheads indicate molecular weight markers (kDa).

neuronal isoform of NOS being constitutively expressed does not appear to be generally valid. Interestingly, day/night fluctuations of the NOS could not be detected in the present study. This observation together with the finding that chronic treatment is required to demonstrate changes in NOS shows that the NOS response is rather slow.

Our findings are in perfect harmony with those of Schaad et al. (9). These authors have shown that NOS *activity* decreases with increasing light exposure during the 24-h cycle and vice versa. Because of the good agreement of our and their results we suspect that the changes in NOS activity (9) are due to changes in the amount of NOS. Since NOS amount and hence activity respond slowly to differences in photoperiod, the enzyme could be important for *monitoring chronic changes in photoperiod*.

Our findings are also of interest with regard to the regulation of pineal cGMP synthesis. Considering that NO formation is an important step in pineal adrenergic cGMP transduction (2,3,18) the non-availability of NOS may explain the decline of the cGMP response to adrenergic stimulation following LL exposure (19). This suggests that changes in the amount of NOS appear to play a major role in the control of adrenergic cGMP response.

That it is not justified to conclude from the pineal to other photoneuronally regulated centers is illustrated by the present observation that in the SCN the amount of neuronal NOS did not show any signs of up- or down-regulation. The failure of changes

in the enzyme's amount is remarkable with regard to the previous finding that the NOS of the SCN (20) plays a major role in resetting of the endogenous clock (21,22,23). Since resetting is restricted to the night (23,24) one might expect that the amount of NOS is increased during this period. However, this is not the case. Therefore, the nocturnal resetting is apparently not due to an increase in the availability of neuronal NOS.

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