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Invited Review

Nitric oxide synthase in the pineal gland

M.O. López-Figueroa and M. Møller
Institute of Medical Anatomy, University of Copenhagen, Denmark

Summary. The recent discovery of nitric oxide (NO) as a biological messenger molecule with unique characteristics has opened a new field in pineal research. This free radical gas is synthesized by the enzyme nitric oxide synthase (NOS) from L-arginine. The activation of adrenoreceptors in the membrane of the pinealocytes mediates the increase in NO through a mechanism that involves G proteins. In the pinealocyte, NO stimulates guanylyl cyclase resulting in an increased intracellular content of cGMP. The role of cGMP in pineal metabolism, however, is still enigmatic

Using enzyme histochemistry and immunohistochemistry, the presence of NOS has been confirmed in the pineal gland of some species. In the rat and especially in the sheep, NOS is located in nerve fibres innervating the gland. These nerve fibres also contain the neuropeptides vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI), and are probably of parasympathetic origin. In cell cultures and tissue sections NOS immunoreactivity has been shown to be present in pinealocytes of the rat and bovine but not in the sheep. Finally, NOS is also present in the endothelial cells of the blood vessels of the pineal gland.

Accordingly, in the mammalian pineal gland, NO is synthesized in both presynaptic nerve fibers and pinealocytes, as well as in blood vessels. However, the anatomical location of NO synthesis varies considerably among species. NO released in the pineal gland, might influence both the pineal metabolism and the blood flow of the gland.

Key words: Nitric oxide synthase, cGMP, Pineal gland, Innervation, Blood vessels

Introduction

The history of nitric oxide (NO) in biology is recent but intense. In 1980 Furchott and Zawadzki showed the presence of a diffusible and unstable substance which caused relaxation of the smooth muscles of the blood vessels. This so-called «endothelium-derived relaxing factor» was later found to be NO (Ignarro et al., 1987; Palmer et al., 1987) and the ability of the vascular endothelium to synthesize NO was demonstrated (Palmer et al., 1987). Nitric oxide and L-citrulline are generated from L-arginine by the enzyme nitric oxide synthase (NOS) (Palmer and Moncada, 1989; Moncada et al., 1991). The presence of NOS in both the central and peripheral nervous system indicated that this molecule also plays a role in cellular communication (Garthwaite, 1991). Nitric oxide stimulates guanylyl cyclase by binding to the heme group of this enzyme (Arnold et al., 1977) with a consequent increase of intracellular cGMP (Knowles et al., 1989).

NO differs from classical neurotransmitter molecules by not accumulating inside transmitter vesicles. NO is also a highly diffusible molecule with a very short half life, and can act retrogradely. Furthermore, it does not bind to membrane-associated receptors to mediate its biological action and it is not inactivated through reuptake mechanisms or enzymatic degradation.

Offprint requests to: Manuel Oscar López-Figueroa, Ph.D., Institute of Medical Anatomy, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen N, Denmark

Nitric oxide synthase characteristics

Following the purification (Bredt and Snyder, 1990) and cloning (Bredt et al., 1991) of NOS at the beginning of this decade, the biochemical mechanisms involved in the generation of NO has been extensively studied. It has been verified that L-arginine is the substrate of the reaction where NOS oxidizes the guanidino nitrogen, utilizing molecular oxygen and NADPH as cosubstrates. The reaction also involves flavin mononucleotide, flavin adenine dinucleotide, and heme iron as prosthetic groups, and requires tetrahydrobiopterin as a cofactor. The products of the reaction are L-citrulline and NO. The exact molecular mechanisms underlying the production of NO is still not clearly understood.

Three subtypes of the enzyme NOS have been described and they have been classified according to their chromosomal location, amino acid composition, location and function (for reviews see Dawson et al., 1994, Forstermann et al., 1994, Knowles and Moncada, 1994, Nathan and Xie, 1994). The numerical nomenclature of the isoforms of the enzymes

corresponds to the order by which they were purified.

Subtype I

This was the first isoform to be purified by use of cerebellar material (Bredt and Snyder, 1990) and later cloned (Bredt et al., 1991). The enzyme has a molecular weight of about 160 kD (Bredt et al., 1991). The gene encoding this subtype is located on chromosome 12 (Xu et al., 1993). Subtype I is also called «neuronal» or «brain» NOS. Neuronal NOS is a constitutive enzyme, the activation of which is highly influenced by the presence of Ca^{++}.

Immunohistochemical studies have located this isoform of the enzyme in neurons from the central and peripheral nervous system, but it is also present in epithelial cells as well as in muscle (Kobzik et al., 1994). In the brain, this subtype of NOS is present in neurons of the cortex, striatum, hippocampus, dorsal raphe, septum, mesopontine tegmentum, supraoptic and paraventricular neurons of the hypothalamus, as well as in cerebellar granule cells (Vincent, 1994). The NO produced by this isoform of the enzyme, is involved in a high number of physiological processes in both the central and peripheral nervous system (see Dawson and Snyder, 1994).

Subtype II

This isoform of the enzyme is also known as «inducible» NOS due to its inducibility by lipopolysaccharides and cytokines (Xie et al., 1992). Chromosome 17 locates the gene encoding this subtype (Xu et al., 1994). The enzyme is a soluble protein of approximately 130 kD in molecular weight. Compared to subtype I and III, the activation of this subtype is independent of Ca^{++}. However, calmodulin has been found to be tightly bound to the enzyme (Cho et al., 1992). This subtype is found in macrophages where it plays a role in the autoimmune response as a toxic agent (Dawson, 1995). However, the enzyme is also found in other cell types, e.g. hepatocytes (Bandaletova et al., 1993).

Subtype III

The last NOS isoform is located in endothelial cells where it is constitutively expressed (Forstermann et al., 1994). The activity of the enzyme is regulated by Ca^{++} and calmodulin. The presence of a myristylated N-terminal allows the attachment of the enzyme to membranes (Pollock et al., 1992). The gene encoding this subtype of NOS is located on the chromosome 7, producing an enzyme with a molecular weight of 133 kD (Xu et al., 1994). Recent immunohistochemical studies have also described the presence of this subtype in pyramidal cells of the CA1 in the hippocampus after strong fixation of the tissue (Dinerman et al., 1994).

The major role of NO produced in the endothelial cells, is vasodilatation and prevention of adhesion of cells in the vascular system (Forstermann et al., 1994).

Nitric oxide in the pineal gland

Physiology of the pineal gland

The pineal gland secretes the hormone melatonin in a circadian rhythm with a peak during the night and a nadir during the day (Reiter, 1991). The circadian secretory pattern is generated by the hypothalamic suprachiasmatic nucleus (SCN), the "endogenous clock" of the brain (for a survey see Aronson et al., 1993) which projects via multiple neuronal pathways to the pineal gland (see Møller et al., 1991). Light reaching the retina is transmitted to the SCN via the optic nerves (Moore, 1983) causing an inhibition of production of melatonin in the pineal gland (Reiter, 1991).

Melatonin binds to target cells in the forebrain. Melatonin receptors are located in the SCN (Masson-Pévet and Gauer, 1994). Via these receptors, melatonin modulates the activity of the suprachiasmatic nucleus. Other receptors are located in the tuberal part of the anterior pituitary gland (Vanecek and Vollrath, 1989; McNulty and Hastings, 1994). Through these receptors, melatonin regulates the secretion of hormones from the anterior pituitary.

Regulation of pinealocyte melatonin secretion

The most important physiological circadian neuronal output from the SCN projects via the hypothalamic paraventricular nucleus, the intermedio-lateral column of the spinal cord, and further via the superior cervical ganglion to the pineal gland (see Møller et al., 1991). Sympathetic neurons, with perikarya located in the superior cervical ganglia, innervate the pinealocytes with nerve fibres containing noradrenalin colocalized with neuropeptide Y (Zhang et al., 1991). The signal transduction systems involved in melatonin production of the pinealocytes are displayed in Fig. 1. Accordingly, noradrenalin stimulates ß-adrenoceptors located on the pinealocyte cell membrane which, via a G_S-protein, adenylyl cyclase and cAMP, stimulates N-acetyl-transferase (NAT), the rate limiting enzyme of the melatonin synthesis (see Klein, 1985). Noradrenalin also stimulates an α-adrenoreceptor which, via the inositol-protein kinase second messenger system, stimulates NAT (Ho et al., 1988).

It is of interest that NPY released from the sympathetic nerve terminal, via an Y1-receptor might inhibit the noradrenalin stimulated increase in intracellular concentration of cAMP (Olcese, 1991).

The pineal gland is also innervated by non-sympathetic peptidergic nerve fibres containing VIP and PHI (Mikkelsen et al., 1994; Møller et al., 1994). G-protein coupled receptors for these neuropeptides are present on the pinealocyte cell membrane (Yuwiler,

1983; Tsuchiaya et al., 1987) and binding of the specific peptides to these receptors stimulates the production of melatonin via the cAMP system.

Regulation of pineal NO production and function of NO in the pineal gland (Fig. 2)

Biochemical studies have confirmed the presence of a cytosolic and Ca^{++}-dependent type of NOS in the rat and bovine pineal gland (Lin et al., 1994; Schaad et al., 1994; Maronde et al., 1995). NO increases the intracellular level of cGMP by an activation of guanylate cyclase (GC) (Spessert et al., 1993; Guerrero et al., 1994). However, the role played by cGMP in the pineal gland is still enigmatic. Thus, recent studies in the pineal gland have shown that NO neither affects the intracellular levels of cAMP and NAT nor melatonin synthesis (Lin et al., 1994). Contrarily, an inhibitory effect of certain NO donors in the production of melatonin has been described (Maronde et al., 1995).

In a recent study noradrenaline (NA) binding to the ß-adrenergic receptor has been shown to stimulate NOS activity via a Gs coupled mechanism (White and Klein, 1995). In addition, stimulation of the α-adrenoceptors by NA causes an increase in intracellular Ca^{++}, via the inositol phosphate second messenger system, which might activate NOS (Schaad et al., 1995).

NOS is also activated following stimulation of the glutaminergic NMDA receptor which increases the intracellular concentration of Ca^{++} (Bredt and Snyder, 1992). In this context it is of interest that although glutamate-containing nerve fibres have never been detected in the pineal gland, glutamate binding sites have been reported in the bovine (Govitrapong et al., 1986) and monkey (Mick, 1995) pineal.

Studies of the circadian variation of rat pineal NOS-

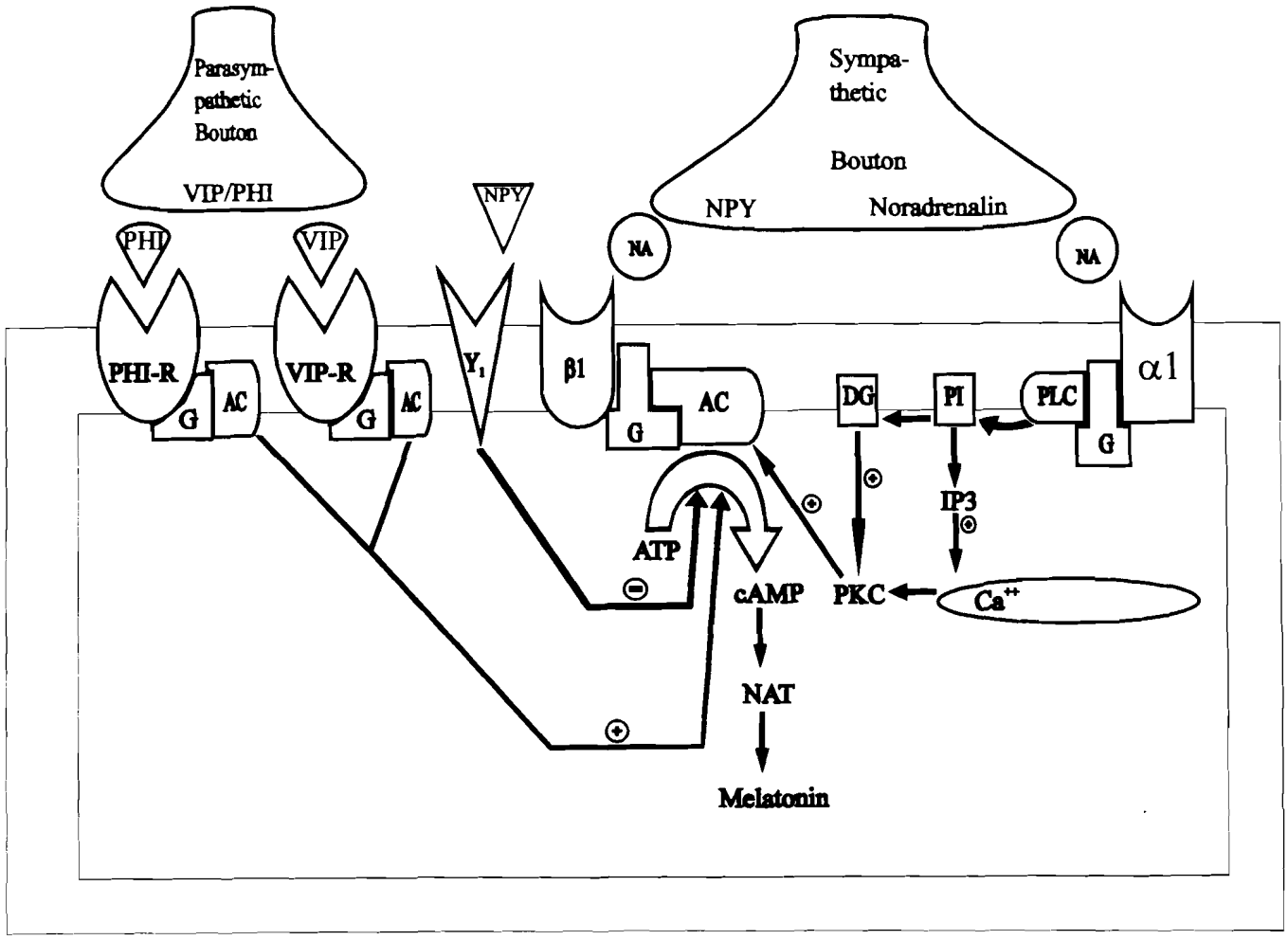

Fig. 1. Scheme showing the mechanism of regulation of melatonin synthesis in the mammalian pinealocyte. α1: α1-adrenoceptor; ß1: ß1-adreneceptor; AC: adenylate cyclase; cAMP: cyclic adenylate monophosphate; ATP: adenosin triphosphate; Ca^{++}: Calcium; DG: diacylglycerol; G: stimulatory G-protein; IP3: inositol triphosphate; NAT: N-acetyltransferase; NA: Noradrenaline; NPY: neuropeptide Y; PHI: peptide histidine isoleucine; PI: phosphatidyl inositol; PKC: protein kinase C; PLC: phospolipase C; VIP: vasoactive intestinal peptide; VIP-R: VIP-receptor; Y1: Neuropeptide Y-receptor.

activity have shown a higher activity during the night than during the day (Schaad et al., 1994). Furthermore, animals kept under constant light conditions have shown a decreased NOS activity compared to animals kept in constant darkness (Schaad et al., 1994), an effect which should involve the ß-adrenoceptor (Schaad et al., 1995). However, a recent study has not been able to verify a difference in NOS activity between day and night (Spessert et al., 1995b), and a down-regulation of a nocturnally elevated guanylyl cyclase activity was shown not to be mediated via adrenoceptors (Spessert et al., 1995a).

Anatomical distribution of NOS

The anatomical distribution of NOS in tissues can be demonstrated by using the enzyme histochemical diaphorase method, as well as immunohistochemically with specific antibodies against the 3 isoforms of NOS. Because NADPH is a co-substrate of NOS, the NADPH-diaphorase enzyme histochemical method can be used as an indirect marker of NOS-activity. It must, however, be emphasized that several diaphorases can be demonstrated by using enzyme histochemistry. Of these, only the NADPH-diaphorase activity can be used as a marker of NOS, the NADP-, and NADH-, and $NADPH_2$-activity are not useful in this context.

Previous histochemical studies have demonstrated diaphorase activity in the mammalian pineal gland (for review see Vollrath and Schmidt, 1969). In lower vertebrates, such as the Larval Lamprey, NADPH-diaphorase activity has been shown to be present in pinealopetal fibres but not in pineal cells (Schober et al., 1994). In the pineal gland of frog, NADPH-diaphorase activity has been described in both pinealocytes and nerve cells (Sato, 1990). The positive cells were located

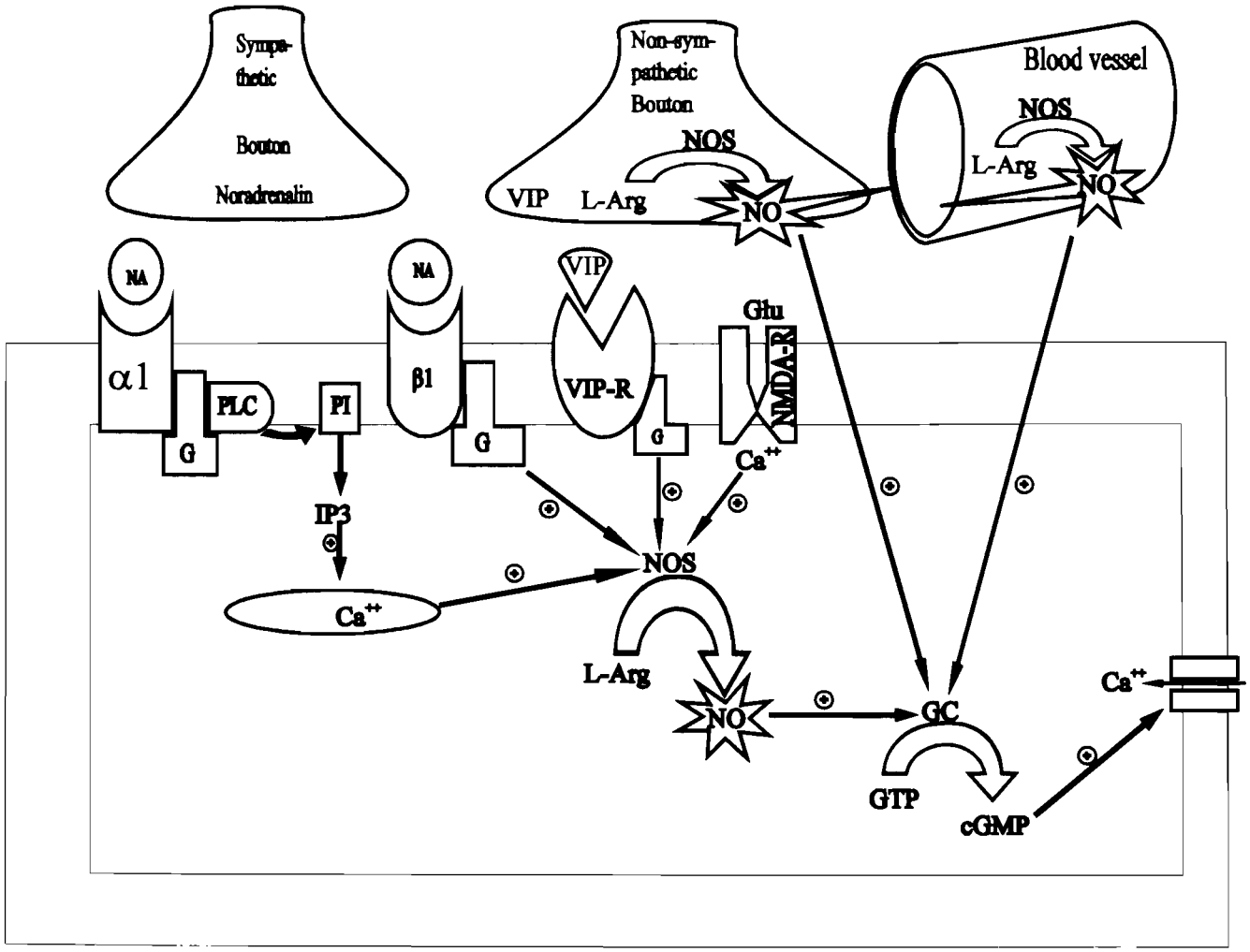

Fig. 2. Suggested regulation of NO synthesis in the mammalian pineal gland. L-Arg: L-arginine; GC: guanylyl cyclase; Glu: glutamate; cGMP: cyclic guanosine monophosphate; GTP: guanosine triphosphate; NMDA-R: N-methyl-D-aspartate-receptor; NO: nitric oxide; NOS: nitric oxide synthase; VIP: vasoactive intestinal peptide. (Spessert, 1993).

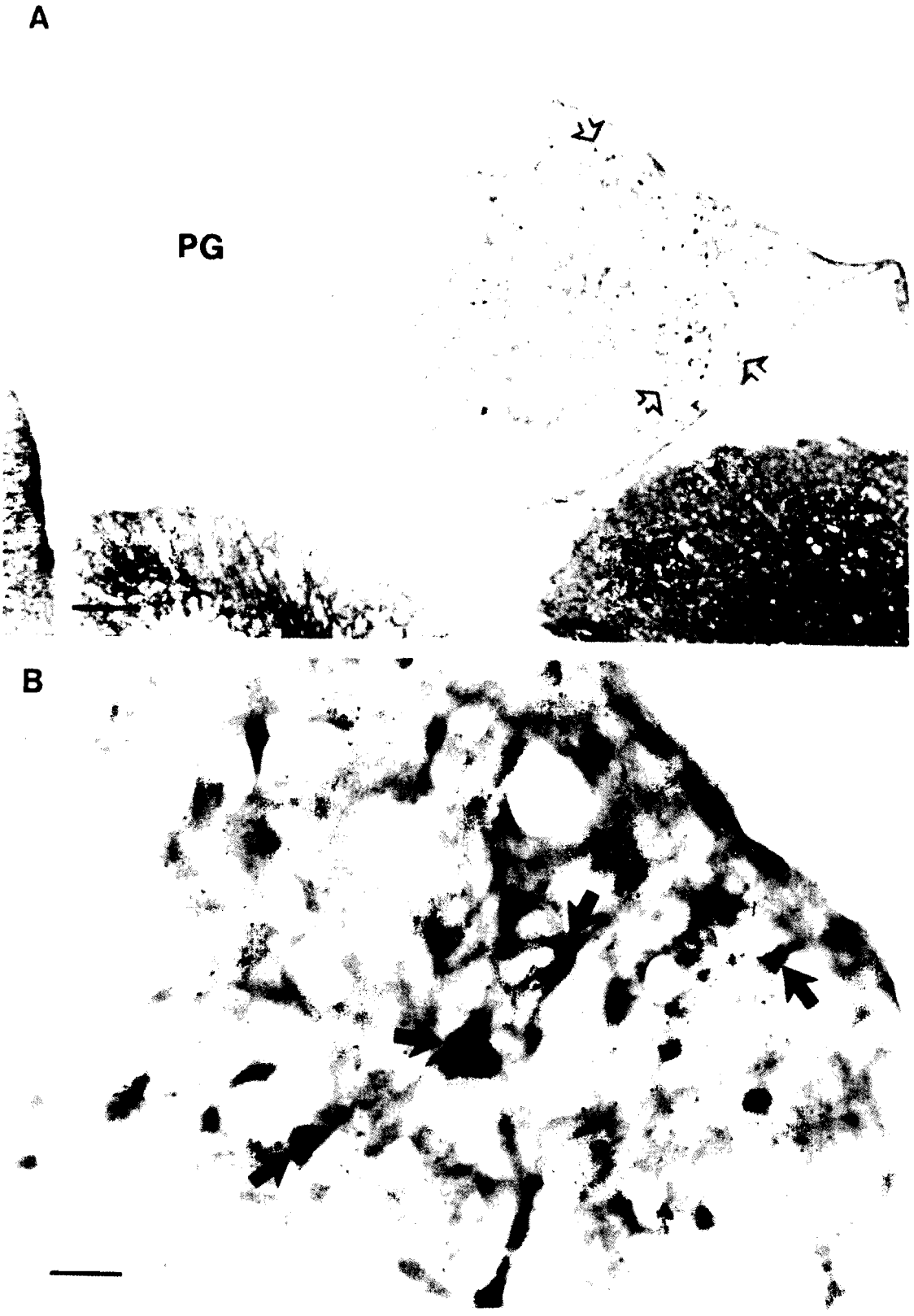

Fig. 3. A. Section of a rat pineal gland (PG) reacted for NADPH-diaphorase activity. The open arrows in the rostral part of the pineal indicate the area with positive cells.
SC: superior colliculus.
Scale bar: 150 μm.
B. High magnification photomicrograph of some of the positive cells shown in figure A (dark arrows). Scale bar: 15 μm.

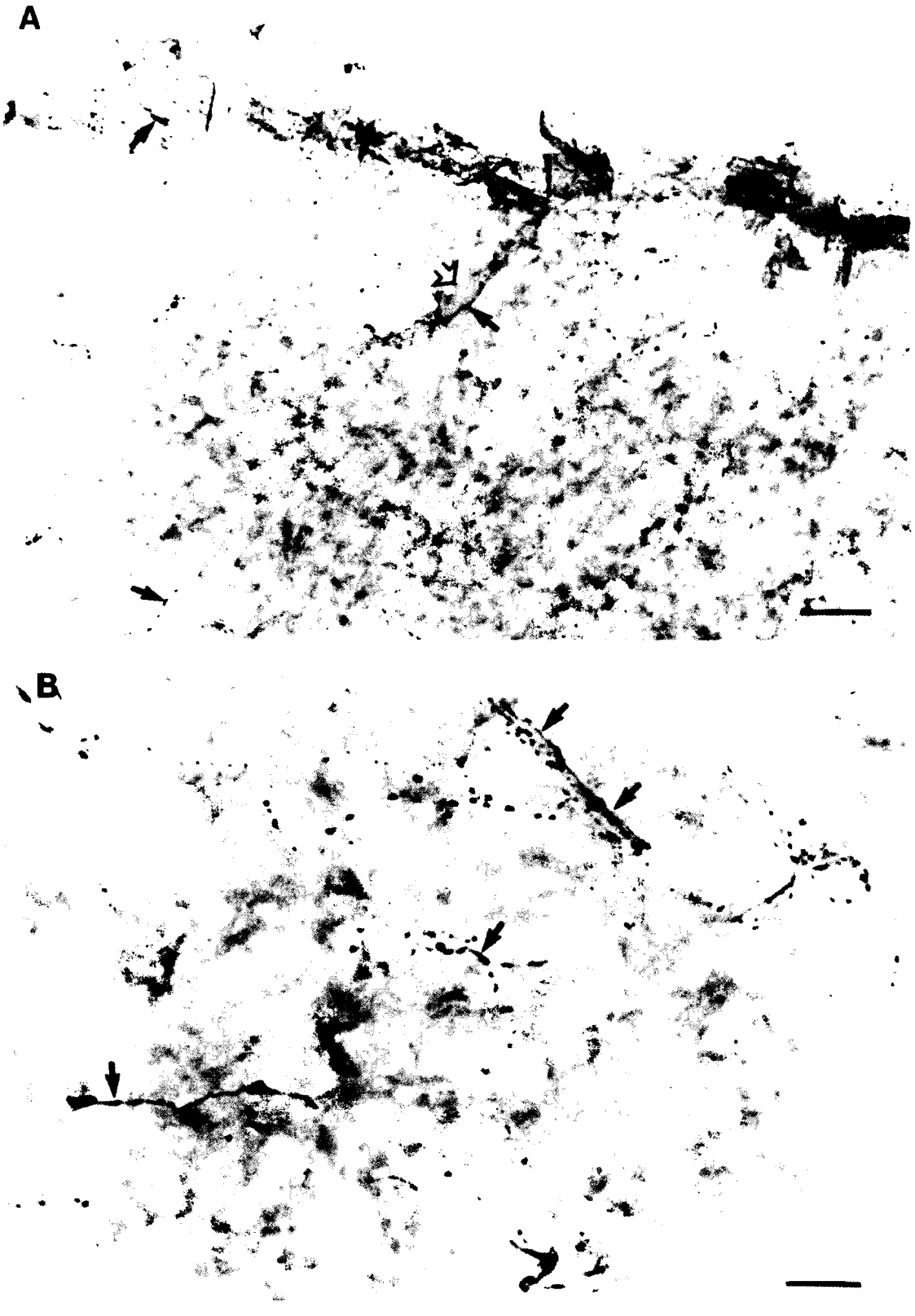

Fig. 4. Photomicrographs of rat pineal sections showing the presence of NADPH-diaphorase-positive nerve fibres and blood vessels. **A.** Nerve fibres (dark arrows) are entering the pineal together with a blood vessel (open arrow) which also expresses NAPDH-diaphorase activity. Scale bar: 30 μm. **B.** Photomicrograph showing both smooth nerve fibres and fibres with boutons en passage. Scale bar: 15 μm.

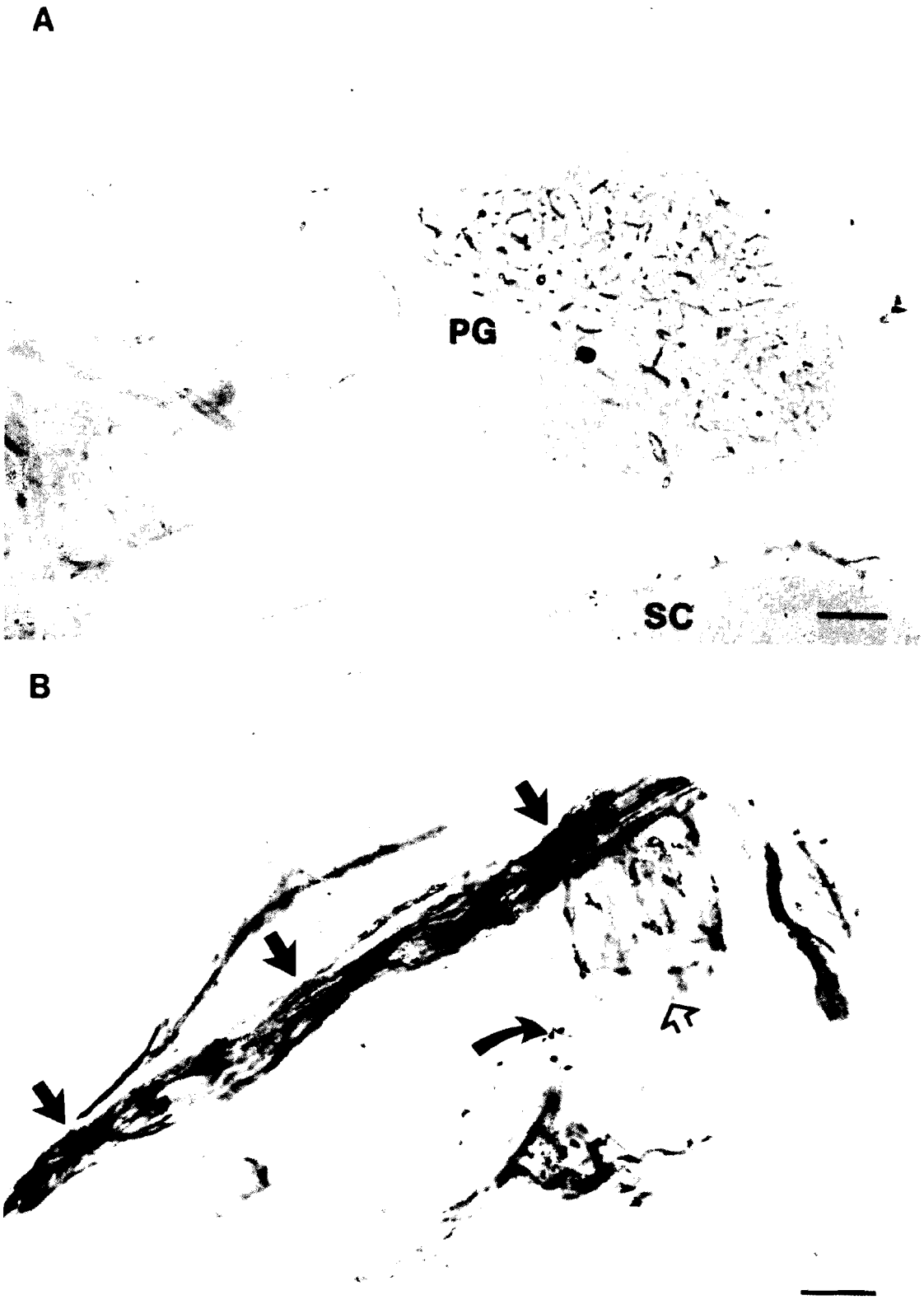

Fig. 5. A. Sagittal section of a sheep pineal gland showing the presence of NADPH-diaphorase activity distributed mainly in the dorsal part. Scale bar: 1 mm. **B.** High magnification microphotograph of a part of a sheep pineal section with bundles of nerve fibres (dark straight arrows) as well as single fibres (dark bent arrow) positive for diaphorase. Notice that also the endothelium of the blood vessels (open arrow) is stained. Scale bar: 15 μm.

mainly in the rostral portion of the frog pineal gland.

In mammals, the results obtained in studies of diaphorase and NOS in the pineal gland vary considerably between the species investigated. Vincent and Kimura (1992) described the presence of nerve fibres in the pineal gland of the rat using the NADPH-d method. In our studies of the rat pineal gland, we can confirm the presence of some NADPH-d positive nerve fibres and single scattered diaphorase positive cells (Figs. 3 and 4) (López-Figueroa and Møller, 1996). Contrarily, in the sheep (López-Figueroa et al., 1996) and bovine pineal in sections reacted both with the NADPH-diaphorase histochemical method (Fig. 5) as well as with specific antibodies against neuronal NOS (Fig. 7), only positive nerve fibres were observed and never NOS-containing cells. The density of the NOS-containing nerve fibres in the sheep was high. Removal of both superior cervical ganglia did not decrease the density of the diaphorase/NOS-positive nerve fibres (Fig. 6). In the sheep, this verifies that NOS is located presynaptically in nerve fibres not belonging to the sympathetic nervous system. Western blot analysis of homogenized sheep pineal glands verified the presence of a 160 kD band corresponding to neuronal NOS.

In our laboratory we have performed double staining for NOS and the neuropeptides VIP and PHI. In this double staining, NOS was shown to be colocalized with both VIP and PHI (López-Figueroa et al., 1996). Such colocalization is in accord with several studies demonstrating NOS to be present in VIP-containing parasympathetic neurons, e.g. the sphenopalatine ganglion (Nozaki et al., 1993). NO might diffuse from the VIPergic nerve terminals to stimulate the guanylyl cyclase in the pinealocytes. However, a presynaptic function must also be considered. Thus, in the hypothalamus NO is able to stimulate release of luteinizing hormone-releasing hormone and somatostatin (Nathan and Xie, 1994). Therefore, in the sheep pineal, NO might also modulate the release of the VIP and PHI from the intrapineal nerve fibres.

In mammals, the presence of NOS in the pinealocytes has been difficult to verify. In the rat, few diaphorase-positive cells are present (López-Figueroa and Møller, 1996) (Fig. 3) and in the sheep, no NOS-containing cells were detected by us (López-Figueroa et al., 1996). Some studies however, have reported both NADPH-d activity and NOS immunoreactivity in cells of additional species. Thus, NADPH-d activity in cultured Guinea pig pinealocytes has been reported (Vollrath and Schmidt, 1969). More recently, Maronde et al. (1995) have described the presence of small, round NADPH and NOS immune-positive cells in bovine cell cultures as well as in sections of bovine pineal gland. Yet, the localization of NOS in the pinealocyte needs further clarification.

By using specific antibodies as well as the NADPH-diaphorase histochemical method, we have identified in our laboratory subtype III or endothelial NOS in the endothelium of blood vessels of rat (Fig. 4.A) (López-Figueroa and Møller, 1996) and sheep (Fig. 8) (López-

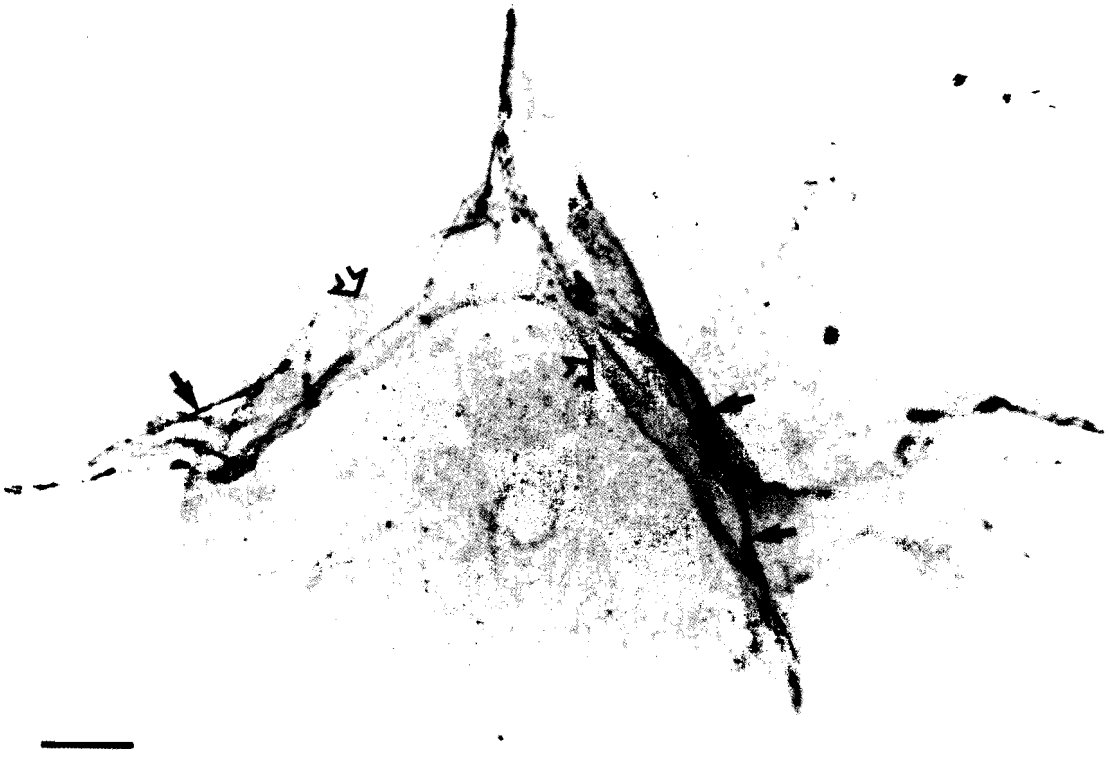

Fig. 6. Nerve fibres (dark arrows) and the endothelium of blood vessels (open arrows) positive for NADPH-diaphorase activity in a sheep. Both superior cervical ganglia were removed 2 weeks before sacrifice. Scale bar: 15 μm.

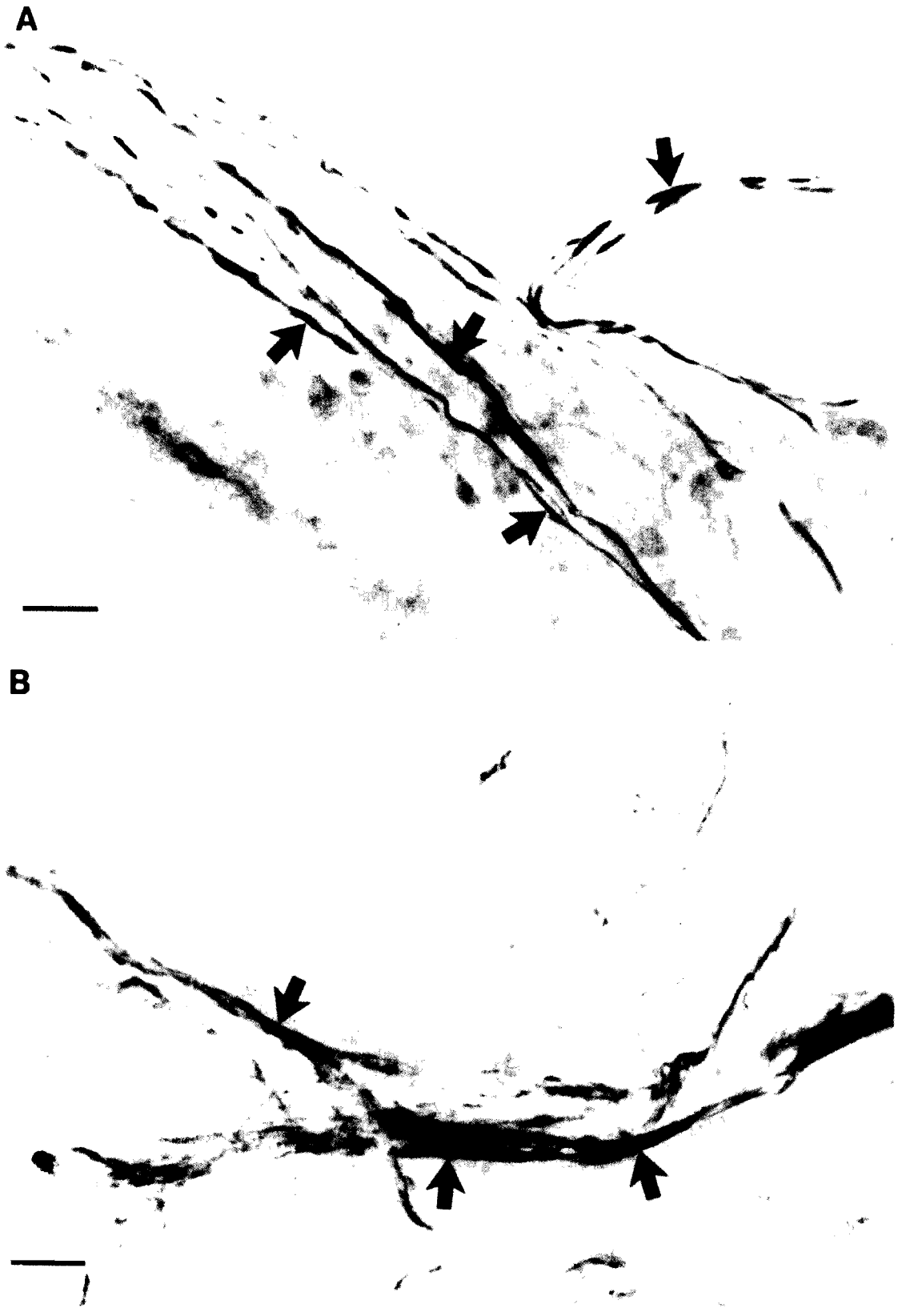

Fig. 7. High magnification photomicrographs of sheep pineal sections reacted with an antibody directed against subtype I of NOS. The arrows shows the immunoreactive fibres positive for NOS. Single fibres (A) and also bundle of fibres (B) can be seen. Scale bars: 15 μm.

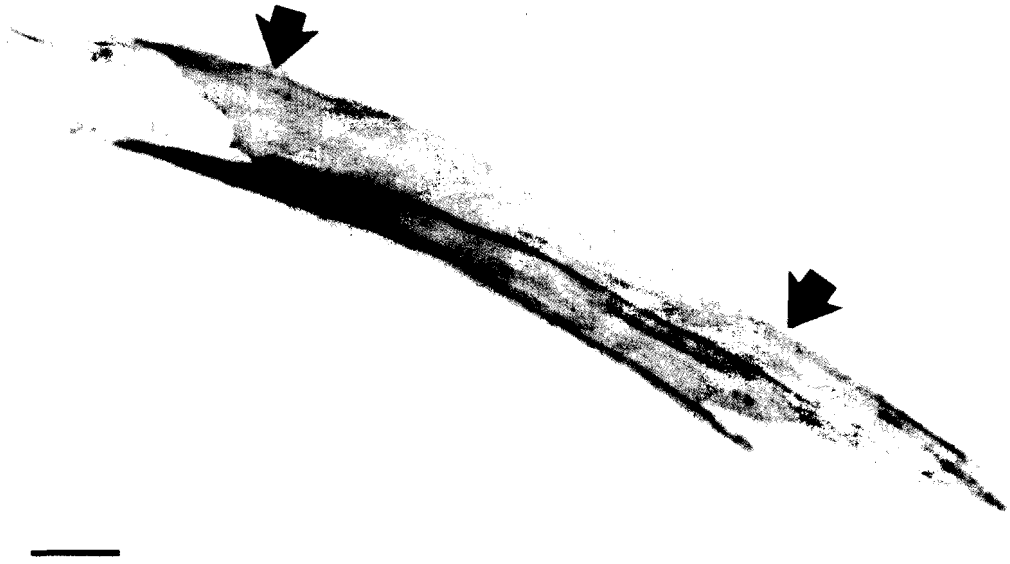

Fig. 8. Endothelium of a blood vessel (dark arrows) in the sheep pineal immunoreacted for NOS subtype III. Scale bar: 15 μm.

Figueroa et al., 1996). NO release from the endothelial cells probably influences the blood flow of the pineal gland, but might also influence the cGMP level of the perivascular-located pinealocytes.

Up to now, subtype II has never been demonstrated in the mammalian pineal gland. However, recent studies have demonstrated the presence of macrophage/microglial cells, which are able to express class II major histocompatibility complex, in the pineal gland of the rat (Pedersen et al., 1993). In such cells subtype II might be induced after activation by cytokines.

In conclusion, NO is synthesized in the mammalian pineal gland and stimulates the intracellular level of cGMP. The function of cGMP in pineal metabolism has to be elucidated. The anatomical structures synthesizing NO vary considerably among the few species investigated. Future studies must be performed to elucidate the physiological role of NO in pineal metabolism and blood flow.

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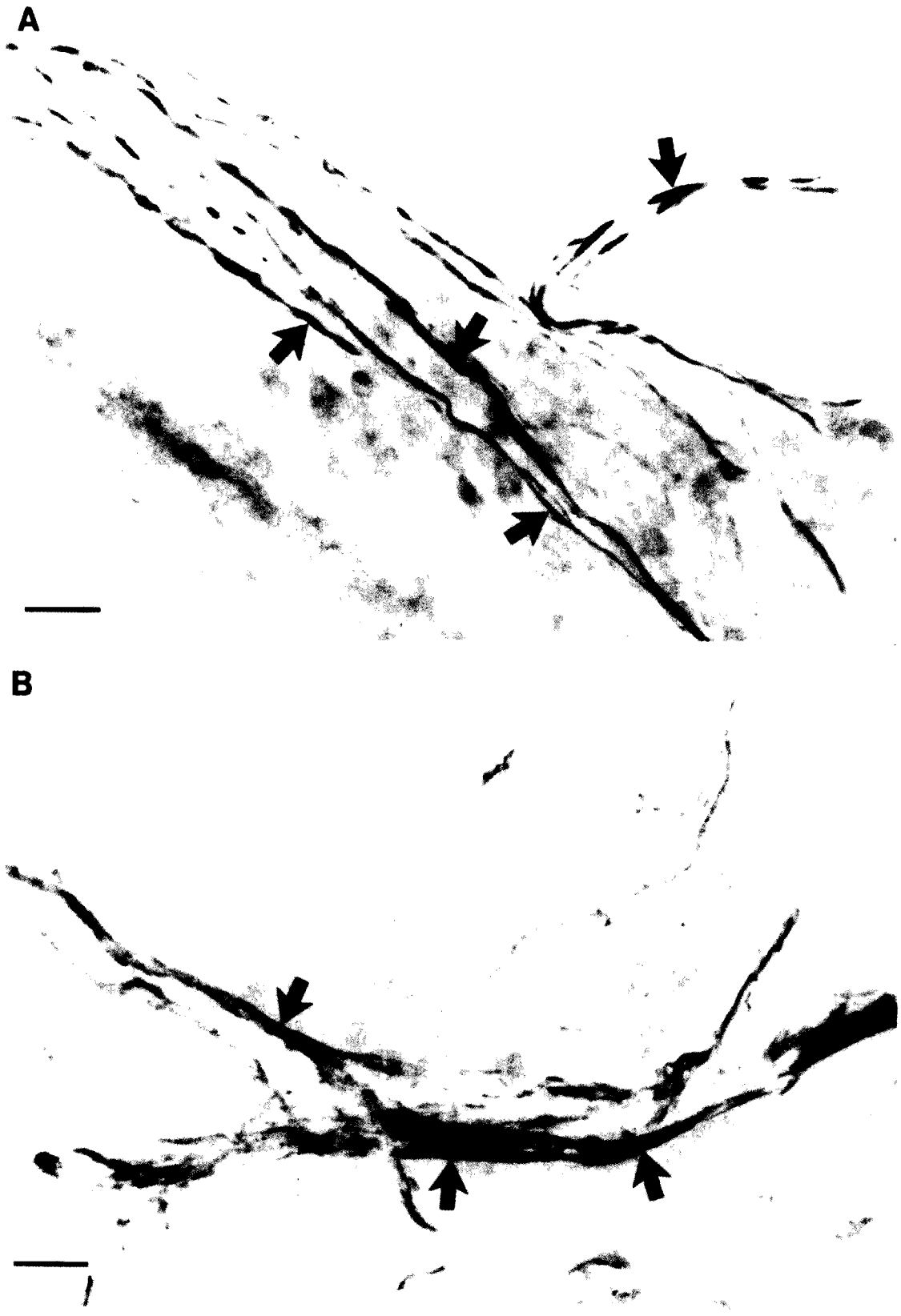

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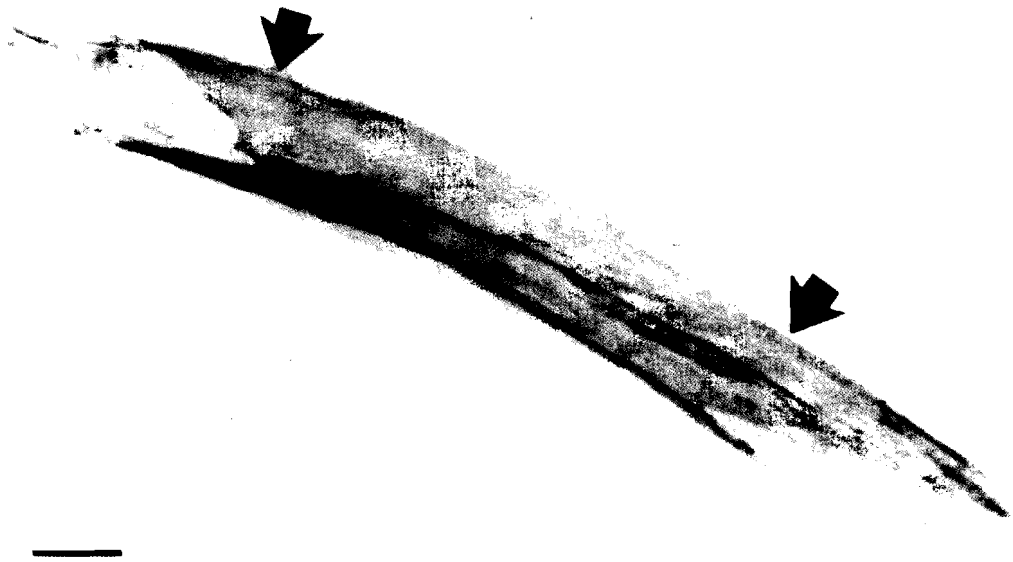

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