

The foetal pig pineal gland is richly innervated by nerve fibres containing catecholamine-synthesizing enzymes, neuropeptide Y (NPY) and C-terminal flanking peptide of NPY, but it does not secrete melatonin

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Summary. Innervation of the mammalian pineal gland during prenatal development is poorly recognized. Therefore, immunofluorescence studies of the pineals of 70- and 90-day-old fetuses of the domestic pig were performed using antibodies against tyrosine hydroxylase (TH), dopamine- β -hydroxylase (D β H), neuropeptide Y (NPY) and C-terminal flanking peptide of NPY (CPON). The investigated glands were supplied by numerous nerve fibres containing TH and D β H. The density of these fibres was higher in the distal and middle parts of the gland than in the proximal one. NPY and CPON were identified in the majority of D β H-positive fibres as well as in a small population of D β H-negative fibres localized mainly in the proximal part of the pineal. The immunoreactive fibres were more numerous in 90-day-old fetuses than in 70-day-old ones. The effect of norepinephrine on melatonin secretion by the foetal pineals in the short-term organ culture was studied to determine the role of D β H-positive fibres during prenatal life. For the same purpose melatonin was measured in the blood in the umbilical cords and in the jugular vein of the mother. The pineals of both groups of fetuses did not secrete melatonin in the organ culture, independently of the presence or absence of norepinephrine in the medium. Melatonin concentrations in the blood in the umbilical cords of fetuses from the same litter and in the jugular vein of their mother were similar. The presence of adrenergic nerve fibres in the pig pineal during gestation does not seem to be associated with the control of melatonin secretion.

Key words: Pineal gland, Melatonin, Innervation, Foetus, Pig

Introduction

The mammalian pineal gland is innervated by nerve fibres, having their sources both in the peripheral ganglia and in the central nervous system (Vollrath, 1981; Matsushima et al., 1999; Møller and Baeres, 2002). From a functional point of view, special attention should be paid to the sympathetic nerve fibres originating from the cranial cervical ganglia, which form the last constituent of the multi-synaptic pathway connecting the pineal gland with the retina and the suprachiasmatic nucleus of the hypothalamus (Tecuamariam-Mesbah et al., 1999; Møller and Baeres, 2002). Norepinephrine released from these fibres is the chief neurotransmitter responsible for the nocturnal increase in melatonin synthesis (Simonneaux and Ribelayga, 2003). Density and distribution patterns of the intra-pineal sympathetic nerve fibres differ markedly between mammals, although in all investigated species they represent the main neuronal component of the gland (Møller and Baeres, 2002; Nowicki et al., 2003). The majority of sympathetic nerve fibres innervating the mammalian pineal gland contains neuropeptide Y (NPY) and C-terminal flanking peptide of NPY (CPON).

Our previous studies led to the detailed

Abbreviations. BSA: bovine serum albumin; CPON: C-terminal flanking peptide of neuropeptide Y; DABCO: 1,4-diazabicyclo[2.2.2]octane; DAPI: 4',6-diamidino-2-phenylindole; D β H: dopamine- β -hydroxylase; NPY: neuropeptide Y; PBS: phosphate buffered saline; PVDF: polyvinylidene-fluoride; SDS: sodium dodecyl sulfate; TBS: tris(hydroxymethyl)aminomethane buffered saline; TH: tyrosine hydroxylase; TRIS: tris(hydroxymethyl)aminomethane

characterization of the sympathetic and non-sympathetic innervation of the pig pineal gland during the period of postnatal life (Kaleczyc et al., 1994; Przybylska-Gornowicz et al., 1995, 1997a,b; Nowicki et al., 2002, 2007). It has been demonstrated that the distribution of nerve fibres containing catecholamine-synthesizing enzymes undergoes prominent reorganization during the period of postnatal life (Przybylska-Gornowicz et al., 1995).

The development of the pineal organ during foetal life has been investigated in detail in only a few mammalian species: the human, the bovine, the sheep, the rat, the hamster, the Indian palm squirrel and the Northern Quoll (Clabough, 1973; Calvo and Boya, 1981; Garcia-Maurino and Boya, 1984; Bhaskar et al., 1986; Halder-Misra and Srivastava, 1986; Ueck, 1986; Redondo et al., 1996, 2001; Regodon et al., 1998, 2006; Regodon and Roncero, 2005). The studies dealing with pineal ontogeny were focused mainly on the formation and maturation of the parenchyma, whereas the development of innervation was not intensively investigated. In the most frequently studied laboratory rodents (the rat, the hamster and the gerbil) the sympathetic nerve fibres appear in the pineal gland soon after birth (Machado et al., 1968; Li and Welsh, 1991). In contrast, according to electron microscopic studies, this form of innervation develops in the human pineal gland during the period of foetal life (Hülsemann, 1971; Møller, 1976). Unlike the sympathetic innervation, commissural fibres were observed at early stages of development in foetal pineals in almost all investigated mammals (Vollrath, 1981). A specific feature of some species (the human, the sheep, the rabbit) is the presence of the foetal pineal nerve, which is considered an ontogenetic equivalent to the sensory afferent tract, occurring in lower vertebrates (Møller, 1979; Vollrath, 1981).

It is generally believed that the mammalian pineal gland shows no or a very low secretory activity during the period of foetal life. In rodents, the diurnal rhythm of pineal melatonin content has been first detected during the second week of postnatal life (Tamarkin et al., 1980). Maternal pinealectomy in sheep abolished the diurnal rhythm of plasma melatonin in foetuses, although it did not eliminate completely melatonin immunoreactivity in maternal and foetal blood plasma during late gestation (McMillen and Nowak, 1989). Several data suggest that melatonin produced by the maternal pineal gland regulates many physiological processes in foetuses, providing, among others, information on photoperiod (Weaver and Reppert, 1986; Goldman, 2003). Such a regulatory strategy seems to be very reasonable, because pineal secretory activity may be controlled by environmental light acting via the retina in the mother but not in foetuses. It should be noted that melatonin very easily passes through the placenta.

In the present work we demonstrated the occurrence of a well-developed network of nerve fibres containing catecholamine-synthesizing enzymes - tyrosine

hydroxylase (TH), dopamine- β -hydroxylase (DBH) as well as neuropeptides - NPY and CPON in the pig pineal gland during the period of foetal life. In view of these findings, the question about melatonin secretion by foetal pig pineals and role of norepinephrine in regulation of this putative process had to be answered. To resolve this query we studied the effect of norepinephrine on melatonin secretion by foetal pineals *in vitro* as well as we compared concentrations of this hormone in blood samples taken from the jugular vein of the sow and from the umbilical cords. For the same purpose melatonin was assayed in the amniotic fluid.

Material and methods

Pineal glands, samples of blood and amniotic fluid

Sows were purchased from a farm with natural lighting conditions. They were artificially inseminated and the pregnancy was confirmed by ultrasonic examination. On the 70th and 90th day of gestation sows were killed via an intravenous administration of sodium pentobarbital (Vetbutal, Biowet, Poland) at a lethal dose followed by exsanguination (during which blood samples were taken from the jugular vein). Immediately after cardiac arrest, foetuses were removed from the uterus. The pineal glands with adjacent parts of the diencephalons as well as samples of the amniotic fluid and the umbilical cord blood were collected. The samples of amniotic fluid and blood plasma, as well as the pineal glands for immunoblotting, were frozen and stored at -80°C. The study was performed with the permission of the Local Ethics Commission for Experiments on Animals in Olsztyn, Poland.

Histological study

The pineal glands with adjacent parts of the diencephalons (70-day-old $n=4$, 90-day-old $n=4$) were fixed in Solcia's solution, dehydrated, embedded in paraffin and cut into 8 μ m thick sections, which were stained with hematoxylin and eosin.

Immunofluorescence study

The pineal glands with adjacent parts of the diencephalons (70-day-old $n=6$, 90-day-old $n=6$) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h, rinsed several times with phosphate buffer, transferred into 30% sucrose solution and stored at 4°C until sectioning. The tissue blocks were cut in frontal or sagittal planes using a Microm HM 560 cryostat (Carl Zeiss, Germany) at a thickness of 12 μ m and mounted on gelatinized glass slides.

The sections were processed for a double-immunofluorescence staining followed by DAPI nuclear staining. After drying at 32°C for 45 minutes, the sections were rinsed in phosphate buffer containing

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0.8% sodium chloride and 0.02% potassium chloride (PBS, 3x10 min.) and incubated in 10% goat serum in PBS with 0.3% Triton X-100 (Sigma, USA) and 1% bovine serum albumin (BSA; Sigma, USA) for 20 minutes. Then, the sections were incubated overnight at 4°C with primary antibodies (Table 1) diluted in PBS containing 0.3% Triton X-100 and 1% BSA. On the following day, the sections were rinsed (PBS, 5x15 min) and incubated with secondary antibodies (Table 1) at a concentration of 7.5 µg/ml (in PBS containing 0.25% BSA and 0.1% Triton X-100) for 4 hours. Then, the sections were rinsed (PBS, 3x5 min), incubated for 10 minutes in a 500 nM solution of DAPI (Invitrogen, USA), again rinsed (PBS, 3x5 min) and covered with polyethylene glycol / glycerin solution containing DABCO (Sigma, USA). The prepared specimens were viewed and photographed using a fully motorized Axioimager microscope equipped with an AxioCam HRm digital camera and an Apotome (Carl Zeiss, Germany). The microphotographs presented in figures 3 and 4 were captured as z-stacks of Apotome optical sections and merged into "extended focus" images using AxioVision software (Carl Zeiss, Germany).

Control procedures for immunochemical reactions included the replacement of the primary antisera with normal mouse or rabbit serum. Western blot analysis was used for confirmation of the specificity of antibodies against TH and DBH (see below). Specificity of antisera against NPY and CPON was checked using the pre-adsorption tests, in which primary antibodies were incubated at 4°C for 24 h with the corresponding peptides (50 µg of the peptide per 1 ml of the diluted antiserum) before use in the immunochemical procedures.

Immunoblotting

The pineal glands were homogenized in buffer containing 2% sodium dodecyl sulfate, 50 mM TRIS (pH 6.8), 100 mM LD-dithiotreitol and 10% glycerol (two glands per 75 µl of the buffer; all chemicals from Sigma, USA). Homogenate was heated to 95°C for 5

min, cooled to room temperature and centrifuged (2 min., 16 000 g). Protein concentration in the supernatant was measured using the Bradford method (Bio Rad, USA). Aliquots of the supernatant (30-60 µg of protein per lane) were loaded on 10% separating SDS-polyacrylamide gel with 5% stacking gel. Protein molecular weight marker (Broad Range, Bio Rad, USA) was run in one lane of the gel. Electrophoresis was done in a MiniProtean II gel apparatus (Bio Rad, USA) at 200V for 40 min. After electrophoresis the proteins were electroblotted to PVDF membrane (Immobilon, Bio Rad, USA). Next, the membrane was blocked with 10% bovine serum albumin (Sigma, USA) in TRIS-buffered saline (TBS, pH 7.4) at 42°C, washed and incubated overnight with primary antibodies against TH (1:2000 in TBS) or DBH (1:1500 in TBS) listed in Table 1. The membranes were then washed with TBS and incubated with biotin-conjugated anti-rabbit or anti-mouse secondary antibodies (1:1000, Vector, USA) for 30 min. After incubation the membranes were washed with TBS and incubated with alkaline phosphatase conjugated streptavidin (0.5 µg/ml; Sigma, USA). The membranes were washed with TBS, incubated with alkaline phosphatase - activating buffer (100 mM NaCl, 100 mM Tris HCl, pH 9.5) and then soaked in CDP Star Ready-to-use chemiluminescence detection reagent (Roche, USA). The membranes were wrapped in plastic folders and exposed to X-ray film (Retina, Photochemische Werke GmbH, Germany) for 2 min. The X-Ray films were developed with Rodinal developer (AGFA, Germany).

In vitro study

The pineals of 70-day-old (n=12) and 90-day-old (n=12) fetuses were removed under sterile conditions and assigned to one of two groups (six explants in each): the norepinephrine-treated group and the control group. The glands were placed on meshes of tissue culture inserts (Netwell 74 µm, Corning, USA) in appropriate 12-well culture plates, containing 0.5 ml medium BGJb Fitton-Jackson modification (GIBCO, USA) with 1

Table 1. Primary and secondary antibodies used in the study.

Primary antibodies				
Antigen	Host	Dilution	Source	Code
Tyrosine hydroxylase	rabbit	1:800	Chemicon, USA	AB 152
Dopamine, hydroxylase	mouse, monoclonal	1:400	Chemicon, USA	MAB 308
Neuropeptide Y	rabbit	1:1000	Affinity, UK	NA 1233
C-terminal flanking peptide of NPY	rabbit	1:1000	Cambridge Research Biochemicals, UK	VO 27/514
Secondary antibodies				
Antigen	Host	Marker	Source	Code
Mouse IgG	goat	Alexa Fluor 488, green fluorescence	Invitrogene, USA	A11001
Rabbit IgG	goat	Alexa Fluor 594, red fluorescence	Invitrogene, USA	A11012

mg/ml of BSA (Cell culture grade, SIGMA, USA) and incubated in a humidified atmosphere of 70% oxygen and 5% carbon dioxide at 37°C in a Heraeus BB6060 incubator (Thermo Scientific, Germany). After one hour of preincubation, the inserts with pineals were transferred to the new culture plates containing medium with 10 μ M norepinephrine bitartrate (SIGMA, USA) or control medium without this catecholamine. The pineals were incubated for 6 hours. Samples of the culture medium, collected after preincubation and incubation periods, were frozen at 20°C. For control purposes, the same experiment was performed using the pineals of 2 month-old female pigs (n=4). The glands were cut in the sagittal plane into two equal parts (one for the norepinephrine-treated group and one for the control group), which were incubated as described above. At the end of the experiment, the explants were weighed.

Melatonin assay

The concentrations of melatonin in blood plasma, amniotic fluid and medium samples were measured by direct RIA using a highly selective R/R/19540-16876 antibody (Stockgrand Ltd, Univ. of Surrey, Great Britain) and 125 I-iodomelatonin (Amersham, UK) according to the procedure described earlier (Lewczuk, 2002). The sensitivity of the assay was 0.7 pg/ml. Intra- and inter-assay coefficients of variation were below 8%.

Statistical analyses

Linear correlation analysis was performed to determine the relationship between concentrations of melatonin in the blood samples taken from the umbilical cords and the jugular vein. The concentrations of plasma melatonin in the samples of umbilical cord blood taken on the 70th and 90th day of the gestation as well as in the samples of culture media were compared using unpaired t-test. The statistical analyses were performed using Statistica 9.0 software (StatSoft, Inc., USA).

Results

Histological structure

The pineal gland of 70-day-old pig foetuses was a round, solid organ, situated between the posterior and habenular commissures, close to the pineal recess of the third ventricle (Fig. 1A). The slightly irregular outer surface of the gland was covered by a thin capsule (Fig. 1A,C). Fine connective tissue septa penetrated from the capsule inside the organ. The pineal parenchyma was composed primarily of cells with round or oval euchromatin-rich nuclei and moderately stained cytoplasm (Fig. 1C,D). Cells with small, usually elongated, dark-stained nuclei and sparse cytoplasm occurred less frequently. Numerous mitotic figures were observed (Fig. 1C). The pineal organ contained abundant

vascular structures, with a lumen diameter ranging from 10 μ m to 100 μ m (Fig. 1A). Their internal surface was covered by round, oval and flat endothelial cells (Fig. 1D).

In contrast to the pineals taken on the 70th day of the gestation, the glands of 90-day-old foetuses were elongated in shape and their outer surface was highly irregular due to the presence of pseudolobes formed by the parenchymal cells in the most outer part of the organ (Fig. 1B). The connective tissue septa originating from the capsule were more prominent than in 70-day-old foetuses. The morphology of cells forming the parenchyma was similar to described above (Fig. 1E). Mitoses were also frequently noted. As in 70-day-old foetuses, the blood vessels were lined by round, oval and flat endothelial cells (Fig. 1E).

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D β H-IR nerve fibres were present in all investigated foetal pineal glands (Figs. 2-4). They were more numerous in 90-day-old than in 70-day-old fetuses, although no prominent age-related differences in the distribution of the positive fibres were observed (Fig. 4). The D β H-IR fibres entered the pineal gland as *nervi conarii* (Fig. 3A) and created prominent bundles in the pineal capsule and just beneath it (Figs. 2A,B, 3C, 4A,D). Smaller bundles of the fibres penetrated from the capsule to the connective tissue septa and ran within them inside the gland (Figs. 2A,B, 3G, 4C,E). They formed plexuses around the blood vessels (Figs. 4B,E). The positive nerve fibres entered from the connective tissue to the parenchyma, where they created a network of variable density (Figs. 3C, 4D,E). The amount of D β H-IR fibres was higher in the distal and middle parts of the gland than in the proximal one (Fig. 2B). Double immunofluorescence staining for D β H and TH revealed that almost all D β H-IR nerve fibres were immunopositive to TH (Fig. 3E,F). TH-positive/D β H-negative nerve terminals were hardly observed (Fig. 3E,F).

The majority of D β H-positive nerve fibres in the pineal glands of 70- and 90-day-old foetuses was also NPY- (Figs. 2A, 3D, 4A-D) and CPON-positive (Figs. 2B, 3G, 4E). These peptides were also found in the nerve fibres forming *nervi conarii* (NPY see Fig. 3B). The nerve fibres immunoreactive to D β H and immunonegative to NPY and CPON were infrequently observed in both investigated groups of foetuses (Figs. 3C,D, 4A,C,E). There were no differences, except for the network density, in distribution patterns between NPY/CPON-positive and NPY/CPON-negative noradrenergic fibres.

The pineal glands of 70- and 90-day-old foetuses contained some NPY-positive/D β H-negative and CPON-positive/D β H-negative nerve fibres (Fig. 2B, 3C,D,G, 4E). They were usually thicker and possessed larger varicosities than D β H-positive fibres. These fibres were much more numerous in the proximal part than in the middle and distal parts of the gland (Fig. 2B). Similar

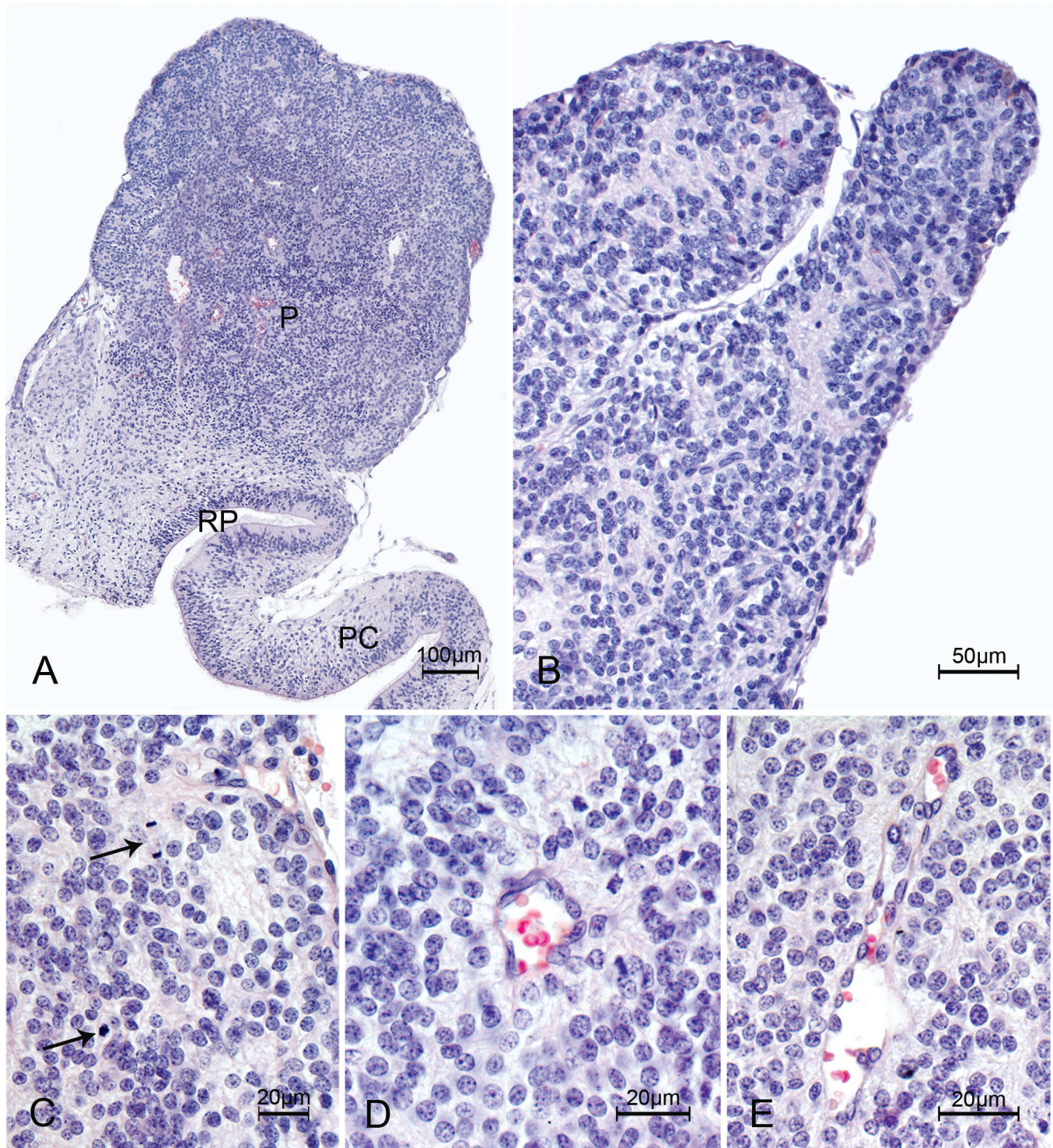


Fig. 1. Histological structure of the foetal pineal gland (HE staining). **A.** Section through the epithalamus of 70-day-old foetus made in the sagittal plane. The pineal gland (P) is an oval structure localized dorsally and rostrally to the posterior commissure (PC), close the pineal recess (RP) of the third ventricle. **B.** Section through the distal part of the pineal gland of 90-day-old foetus. Note irregular outer surface of the gland. **C.** Mitotic figures (arrows) in the pineal parenchyma of 70-day-old foetus. **D, E.** Vascular structures in the pineal gland of foetuses on the 70th (D) and 90th (E) day of gestation. Note that the inner surface of the blood vessels is lined by endothelial cells with round, oval and flat nuclei.

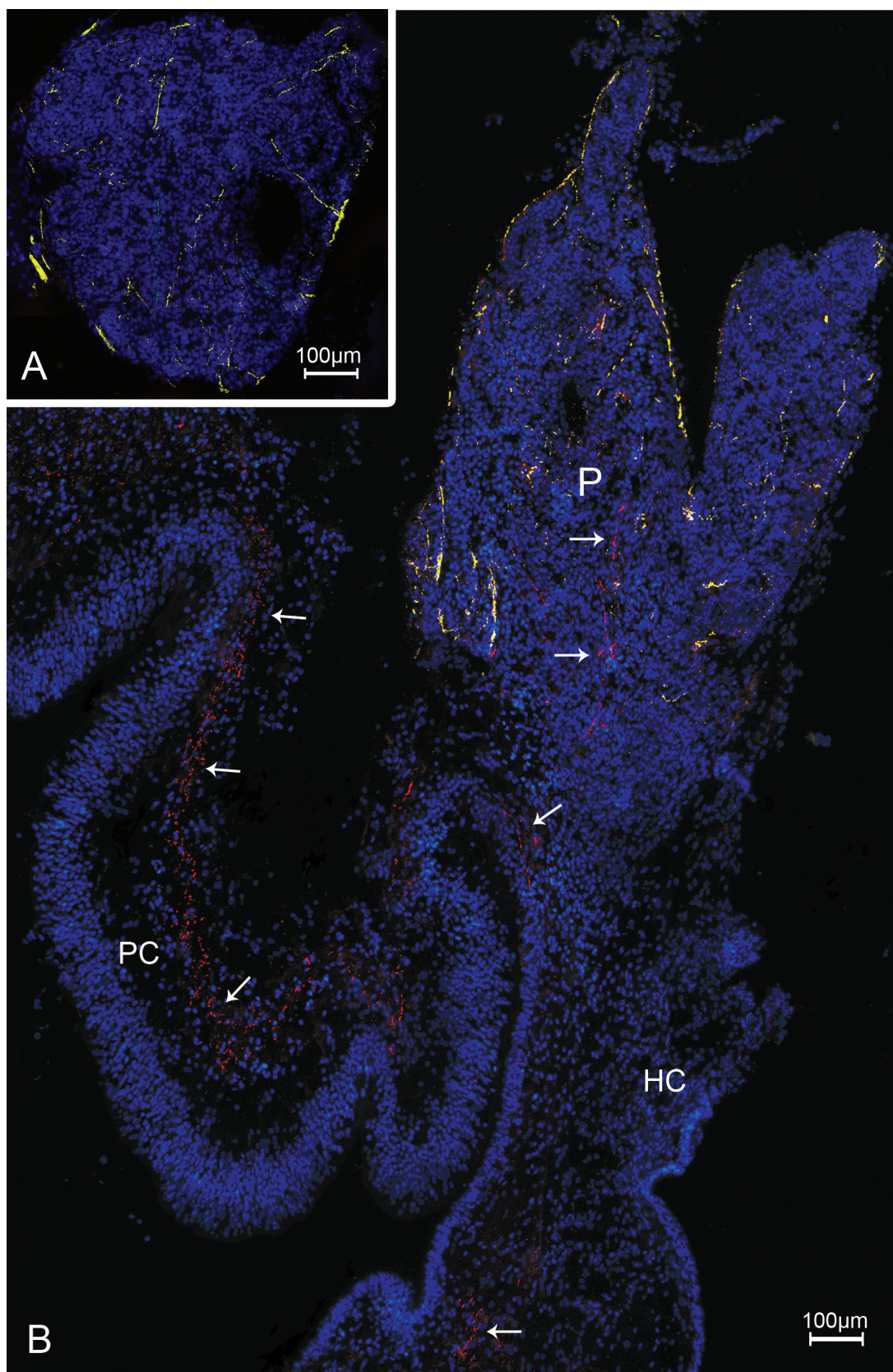


Fig. 2. A. Section through the distal part of the pineal gland of 70-day-old foetus made in the frontal plane and stained for DβH (green fluorescence) and NPY (red fluorescence). Note the presence of numerous nerve fibres immunoreactive with antibodies against both substances (yellow due to merging of red and green fluorescences) forming thick bundles in the capsule and penetrating from the periphery inside the gland. **B.** Section through the epithalamus of 90-day-old foetus made in the sagittal plane and stained for DβH (green fluorescence) and CPON (red fluorescence). Numerous DβH positive/CPON-positive nerve fibres (yellow due to merging of red and green colors) are visible. Nerve fibres positive to CPON, but negative to DβH (red fluorescence, arrows) are present in the posterior commissure (PC) and the habenular commissure (HC) as well as in the proximal and central parts of the pineal gland (P).

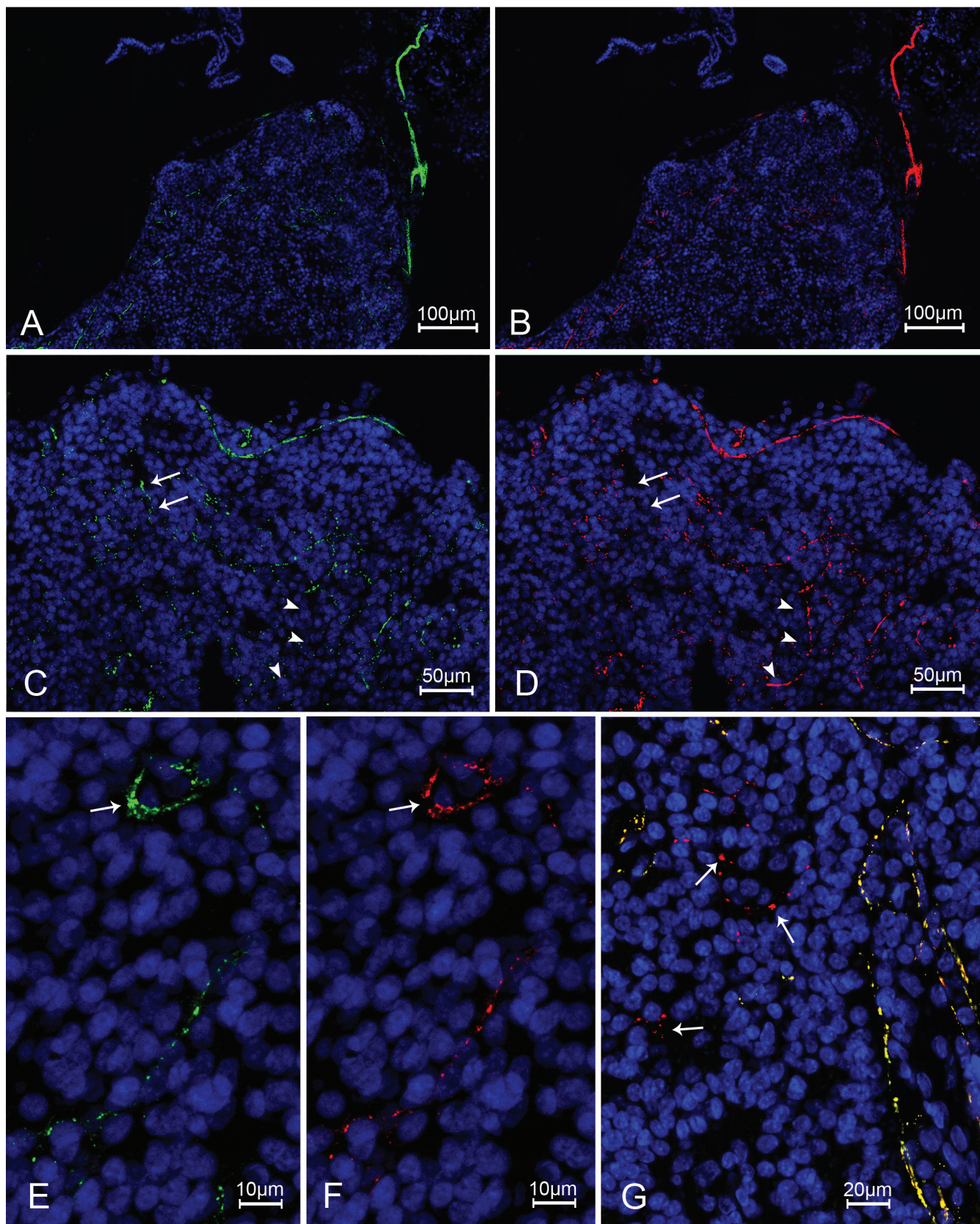
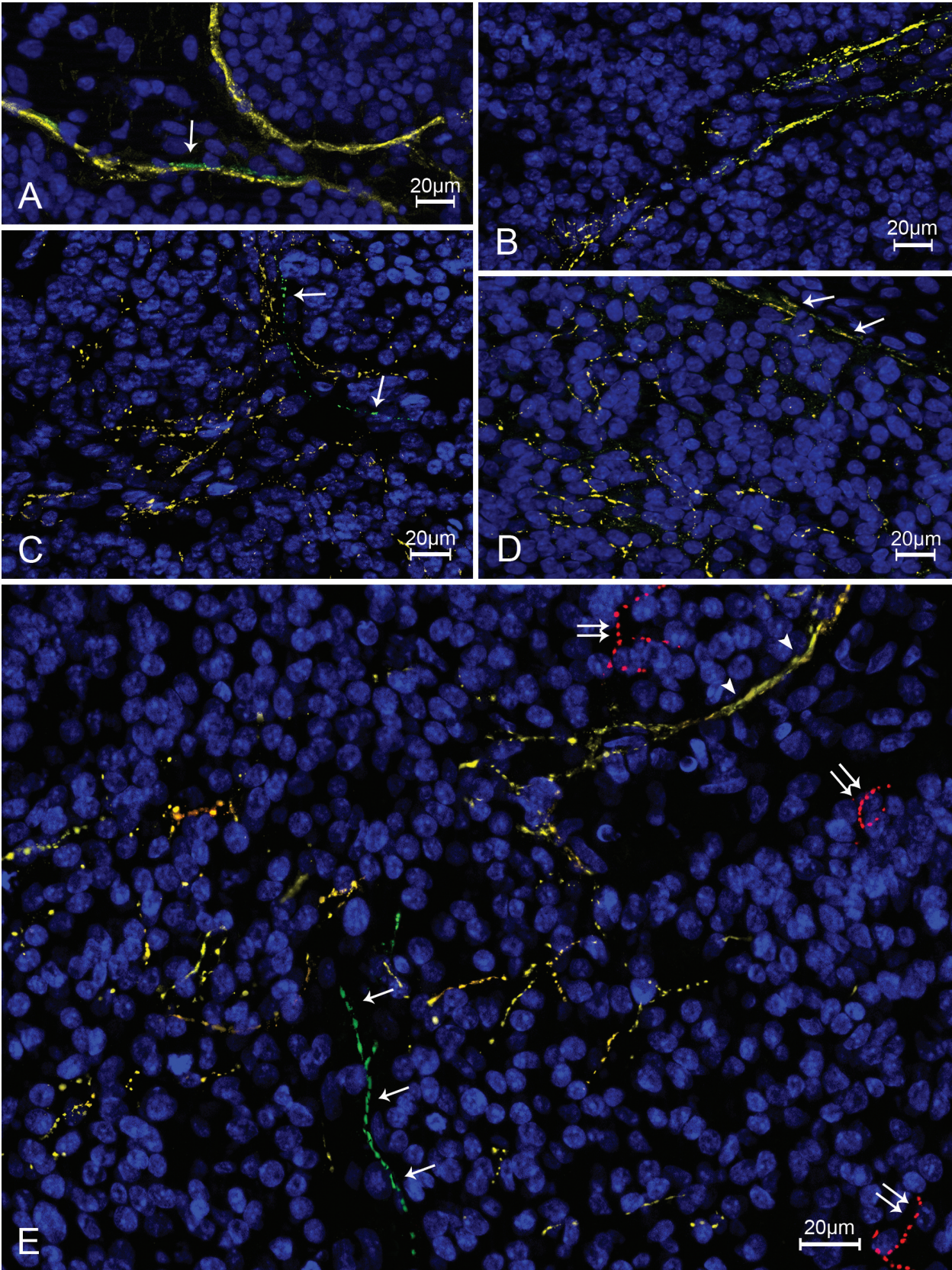


Fig. 3. **A, B.** One of two nervi conarii entering the pineal gland of 70-day-old foetus. Positive reactions for D β H (**A** - green fluorescence) and NPY (**B** - red fluorescence). **C, D.** Nerve fibres visualized using antibodies against D β H (**C** - green fluorescence) and NPY (**D** - red fluorescence) in the capsule and parenchyma in the pineal gland of 70-day-old foetus. The majority of fibres shows the presence of both antigens, although some D β H-positive/NPY-negative (arrows) and D β H-negative/NPY-positive fibres (arrow heads) are also present. **E, F.** D β H-positive (**E** - green fluorescence) and TH-positive (**F** - red fluorescence) nerve fibres in the pineal gland of 70-old foetus. Note the presence of the TH-positive/ D β H-negative fibres (arrow). **G.** D β H-/CPON-positive nerve fibres (yellow due to merging to red and green fluorescences) in the connective tissue close to the blood vessels in the pineal gland of 70-day-old foetus. D β H-negative/CPON-positive nerve fibres (red fluorescence) are visible in the parenchyma (arrows).



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nerve fibres were present in the posterior commissure and the habenular commissure (Fig. 2B). NPY/CPON-positive/D β H-negative fibres were more numerous in the pineal glands of 90-day-old than in 70-day-old foetuses.

Cells immunoreactive with antibodies against TH, D β H, NPY and CPON were not found. The positive staining was not observed in the control reactions, in which the primary antibodies were replaced with normal sera. No positive reactions were obtained with the use of anti-NPY and anti-CPON antibodies pre-adsorbed with the corresponding peptides.

Immunoblotting

Western blots (Fig. 5) done on the protein extracts from the pineal glands of 70- and 90-day-old pig foetuses with antisera against TH and D β H detected specific bands for TH (molecular weight ca. 45 kDa) and for D β H (molecular weight ca. 65 kDa).

Melatonin secretion by the foetal pineal glands in vitro

Melatonin was not detectable in the medium samples collected after the preincubation of the foetal pineals taken on the 70th and 90th day of gestation (Table 2). Similarly, the hormone was not detected in the medium samples collected after the incubation of these glands in the presence or in the absence of norepinephrine. In contrast, the pineal glands of 2-month-old pigs released more than 2 ng of melatonin per 1 mg of tissue during

the 1 hour-long preincubation and almost 8 ng of melatonin per 1 mg of tissue during the 6 hour long incubation in the medium without norepinephrine (Table

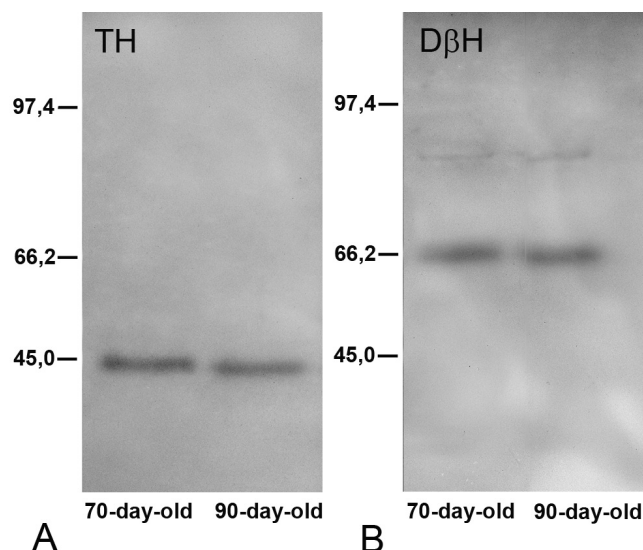


Fig. 5. Western blotting of electrophoretically separated proteins from homogenates of the pineal glands of 70- and 90-day-old pig foetuses immunostained with antibodies against TH (**A**) and D β H (**B**). Horizontal bars on the left side show positions of molecular weight markers.

Table 2. Melatonin secretion from the explants of the pineal glands of 70- and 90 day old foetuses and 2-month-old piglets of the domestic pig.

Sources of the pineal glands Groups	70-day-old foetuses		90-day-old foetuses		2-month-old piglets	
	C	NE	C	NE	C	NE
Melatonin released during the 1 hour long preincubation (mean $\pm$ standard deviation; ng/mg wet tissue)	ND	ND	ND	ND	2.32 $\pm$ 0.15	2.11 $\pm$ 0.27
Melatonin released during the 6-hour long incubation in the absence or presence of norepinephrine (mean $\pm$ standard deviation; ng/mg wet tissue)	ND	ND	ND	ND	7.92 $\pm$ 0.38	33.51 $\pm$ 0.55*

The explants of the pineal glands were randomly assigned to one of two groups: the control group (C) and the norepinephrine-treated group (NE). During the 1 hour-long preincubation explants from both groups were incubated in medium without norepinephrine. Then, they were transferred to the wells containing medium with 10 μ M of norepinephrine (group NE) or without it (group C) and incubated for 6 hours. Medium samples were collected at the end of the preincubation and incubation periods. ND: not detectable; *: significantly different comparing to the explants from the control group.

Fig. 4. A. Bundles of nerve fibres in the pineal capsule of 90-day-old foetus formed mainly by D β H-positive/NPY-positive fibres (yellow due to merging of red and green fluorescences). Note the presence of some D β H-positive / NPY-negative fibres (green fluorescence, arrow). **B.** D β H-positive/NPY-positive fibres forming the perivascular plexus in the pineal gland of 90-day-old foetus (yellow due to merging of red and green fluorescences). **C.** Numerous D β H-positive/NPY-positive nerve fibres in the connective tissue septa in the pineal gland of 90-day-old foetus (yellow due to merging of red and green fluorescences). D β H-positive/NPY-negative fibre is also present (green fluorescence, arrows). **D.** D β H-positive/NPY-positive nerve fibres (yellow due to merging of red and green fluorescences) in the capsule (arrows) and the parenchyma in the pineal gland of 90-day-old foetus. **E.** Different populations of nerve fibres occurring in the pineal gland of 90-day-old foetus: 1) D β H positive/CPON-positive nerve fibres (yellow due to merging of red and green fluorescences) forming the bundle close to the blood vessel (arrow heads) and the dense network between pinealocytes, 2) D β H positive/CPON-negative nerve fibres (green fluorescence, arrows) and 3) D β H negative/CPON-positive nerve fibres (red fluorescence, double arrows).

2). Adrenergic stimulation induced a significant increase in melatonin secretion from the pineal glands of 2-month-old piglets.

Melatonin in umbilical cord blood and amniotic fluid

Concentrations of melatonin were similar in plasma of the umbilical cord blood in all foetuses from the same litter and in plasma of the blood obtained from the jugular vein of the mother (Fig. 6). Plasma melatonin levels measured in samples from the jugular vein varied between sows and these variations enabled us to demonstrate a close relationship between the pineal hormone concentration in the general circulation of the mother and the umbilical circulation of the foetuses (Fig. 6). No significant differences in melatonin concentrations were observed between samples of the umbilical cord blood taken on the 70th day (10.39 ± 3.41 pg/ml) and on the 90th day (7.38 ± 0.93 pg/ml) of the gestation. The pineal hormone was detectable in the amniotic fluid in the placentas collected from only one sow, which was characterized by the highest concentration of melatonin in the jugular vein blood (Fig. 6A - insert).

Discussion

Our data demonstrate, for the first time, the presence of nerve fibres containing catecholamine-synthesizing enzymes in the mammalian pineal gland during the period of foetal life. Tyrosine hydroxylase and dopamine- β -hydroxylase, the occurrence of which in the investigated material has been confirmed by immunoblotting, are generally accepted as valid markers of the sympathetic innervation of the pineal, because the superior cervical ganglia are sole sources of the

adrenergic nerve fibres innervating this gland in almost all species investigated so far (Møller and Baeres, 2002).

The ontogenic development of the pineal sympathetic innervation has been poorly recognized. In rodents the sympathetic nerve fibres reach the pineal gland after birth. As demonstrated by formaldehyde-induced fluorescence, in the rat pineal gland a few bundles of nerve fibres penetrate from the capsule inside the gland within the first 24 hours of postnatal life (Machado et al., 1968). During the following days the sympathetic nerve fibres become more and more numerous, and reach the adult patterns after 2-3 weeks of postnatal life. In hamsters and gerbils, TH-IR nerve fibres appear in the pineal glands on the second postnatal day (Li and Welsh, 1991). The pineal glands of 7-day-old animals are richly innervated by smooth or sporadically varicose TH-IR fibres. The innervation of the pineal glands of hamsters and gerbils by TH IR processes is fully developed after 4 weeks. The ultrastructural studies of human foetuses performed by Hülsemann (Hülsemann, 1971) have shown that the sympathetic nerve fibres (forming the *nervi conarii*) penetrate the distal pole of the pineal gland between the 60th and the 82nd day of prenatal life. Nevertheless, the fibers surrounding the blood vessels in the gland are not present before the 120th day of prenatal life. The varicosities, with clear and dense core vesicles are present around the 150th day of gestation (Hülsemann, 1971), but according to cytochemical studies they are still immature (Møller, 1976). The nerve terminals with clear and dense core vesicles have also been described in the ovine foetal pineal gland (Redondo et al., 1996). It is reasonable to consider that the differences in the presence of the sympathetic innervation during foetal life between the investigated rodent species and the domestic pig (and probably the human and other

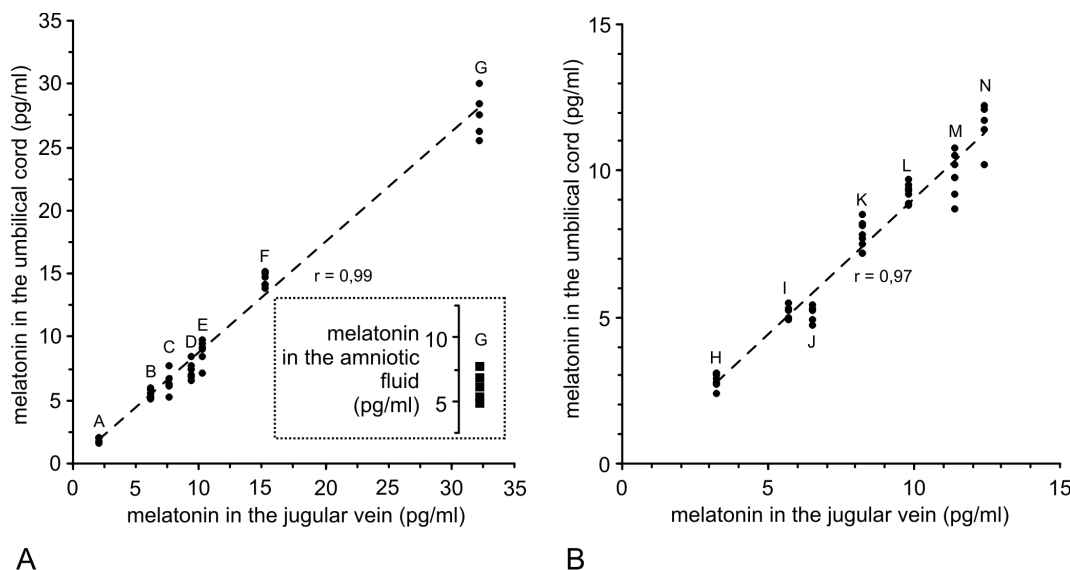


Fig. 6. A, B. Relationship between plasma melatonin concentrations in the jugular vein of the sow and in the umbilical cords of fetuses in age of 70 days (A, the litters signed A - G) and 90 days (B, the litters signed H - N). Insert in A: Melatonin in the amniotic fluid in placentas of the litter signed as G; the pineal hormone was undetectable in the amniotic fluid in placentas of all other investigated foetuses.

ungulates) reflect differences in developmental stages at the time of birth.

Our study showed the presence of NPY and its flanking peptide - CPON in the nerve fibres innervating the pineal glands of the 70- and 90-day-old pig foetuses. Both neuropeptides derive from the same molecule - proNPY, which is post-translationally processed by a cleavage to 36-amino-acid NPY and 30-amino-acid CPON (Simonneaux and Ribelayga, 2003). In the pineal glands of the majority of mammals NPY is present mainly in the sympathetic nerve fibres, since a large portion of NPY-IR nerve fibres disappears after the superior cervical ganglionectomy (the rat - Zhang et al., 1991, the sheep - Cozzi et al., 1992, the cat - Møller et al., 1994 and the European hamster - Møller et al., 1998). A small population of NPY-IR fibres persisting after ganglionectomy represents probably a central innervation of the pineal gland. A prominent exception is the mink pineal gland, where the majority of NPY fibres is of extra-sympathetic origin (Møller et al., 1990). The physiological role of NPY in the pineal gland, despite its common presence, is mysterious (Olcese, 1991; Simonneaux et al., 1994). CPON was much less intensively studied than NPY and its presence has been demonstrated in the pineal glands of the sheep (Cozzi et al., 1992), the pig (Przybylska-Gornowicz et al., 1997a,b), the cat (Møller et al., 1994) and the chinchilla (Nowicki et al., 2003). However, it is reasonable to consider that both neuropeptides co-exist in the nerve fibres. The functional significance of CPON in the pineal gland is still unknown.

Double immunohistochemical staining performed in the present study demonstrated the presence of two populations of NPY- and CPON-positive fibres in 70- and 90-day-old foetuses. The first one is formed by DBH-positive fibres, the density of which is higher in the apical and middle parts than in the proximal part of the pineal. These fibres could be considered as originating from the superior cervical ganglia, because they reach the gland as *nervi conarii*. The second population includes the DBH-negative fibres localized in the proximal part of the gland. They are thicker than the NPY-/DBH-positive and CPON-/DBH-positive fibres. Nerve fibres with similar morphological and immunochemical characteristics were observed in the posterior commissure and the habenular commissure, therefore it is reasonable to suspect that they are of central origin. Similarly, two populations of NPY-positive fibres were also found in the pig pineal during postnatal life (Kaleczyc et al., 1994; Przybylska-Gornowicz et al., 1997a,b).

The presence of neuropeptides in the pineal during foetal life has been investigated only sporadically: VIP-positive nerve fibres were found in ovine fetuses and NPY-positive nerve fibres were demonstrated both in ovine and bovine fetuses (Regodon et al., 1998, 2003, 2006; Regodon and Roncero, 2005). NPY-positive fibres enter (through the capsule) the bovine pineal gland around the 135th day of gestation (Regodon and Roncero

2005). Initially, they occupy perivascular location and later penetrate between pinealoblasts (Regodon et al., 2006). Among rodents, NPY-positive fibres inside the pineal gland were found only after birth - on the 2nd postnatal day in the gerbil and on the 3rd postnatal day in the hamster (Li and Welsh, 1991).

The occurrence of numerous nerve fibres containing catecholamine-synthesizing enzymes, NPY and CPON in the pineal gland of pig fetuses leads to the question concerning their functional significance. Our *in vitro* study has shown that the pineal glands of both 70- and 90-day-old foetuses do not secrete melatonin, independently of the presence or absence of norepinephrine in the culture medium, whereas the pineal glands of 2-month-old pigs incubated in the same condition release large amounts of melatonin and clearly react to the adrenergic stimulation. The data obtained in 2-month-old animals are in agreement with our earlier studies demonstrating that the explants of the pineal glands of 3 - 4 month-old pigs removed during the photophase and kept in the organ culture produced melatonin at a level higher than 20 pg/min/mg of wet tissue and quickly responded to adrenergic stimulation (Lewczuk, 2002). So far, melatonin secretion by the foetal pineal gland *in vitro* has been studied only in the lamb (McMillen et al., 1989). It was demonstrated that the pineals collected during the last two weeks of pregnancy secrete melatonin and respond to adrenergic stimulation, although when compared to the glands of newborn lambs they produce 9-10-fold less melatonin, both before and during the treatment with isoprenaline (McMillen et al., 1989). It should be noted that the ovine pineals used in the cited study were taken in a very late gestation (between 135. and 143. days of 150-day-long pregnancy), whereas in our experiment the glands were removed from foetuses at earlier stages of the development (70. and 90. days of 115-day-long pregnancy), which could be considered a possible explanation of the differences in melatonin production between the ovine and swine pineals. Kennaway and Seamark (1975) found that the activity of hydroxyindole-O-methyltransferase (HIOMT) in the pineal glands of ovine foetuses increased twofold at the beginning of the last week of gestation, but a sudden rise in HIOMT activity (elevation by ca 400%) was observed on 148. day of gestation.

The majority of studies dealing with the ontogeny of melatonin secretion by the mammalian pineal gland was performed in rodents. The day-night fluctuations in the pineal melatonin level did not occur until rats reached 8 days of age and Syrian hamsters - 12 days of age (Tamarkin et al., 1980). The pineal glands of 3-day-old Syrian hamsters kept in organ culture secreted very low amounts of melatonin and did not respond to adrenergic stimulation (Kaufman and Menaker, 1991). The youngest age at which norepinephrine stimulated melatonin secretion from hamster pineals was 4 days. The level of norepinephrine-stimulated melatonin secretion was about 20-fold higher in 11-day-old animals

compared to 4-day-old ones (Kaufman and Menaker, 1991). It is generally believed that the absence of melatonin secretion by rodent pineals during the first days of postnatal life is related to the lack of sympathetic nerve fibres, because the diurnal rhythm of the rat suprachiasmatic nucleus activity was noted already on the first day after birth (Fuchs and Moore, 1980) and β -adrenoceptors were detected in the rat pineal gland on 16th day of gestation (Schlumpf et al., 1987).

According to our assumption, melatonin concentrations in the umbilical cord blood should vary among individual fetuses in the litter, if their pineal glands secrete melatonin. Moreover, in this case some differences in levels of the hormone between the blood samples from the jugular vein of the sow and from umbilical cords could be expected. Our study showed similar concentrations of melatonin in the umbilical cords of all fetuses from one litter and in the jugular vein of their mother. Moreover, the variations in day-time concentrations of melatonin between individual sows, which are well-documented in the domestic pig (Lewczuk and Przybylska-Gornowicz, 1997), enabled us to demonstrate a close relationship between the pineal hormone concentrations in the jugular vein of the mother and the umbilical vessels of the fetuses. These data suggest that the maternal pineal gland is a sole or the main source of melatonin in the foetal blood. Another support for this idea was provided by the study of melatonin in the amniotic fluid. The pineal hormone occurred at a detectable level in the samples of fluid taken from the amniotic cavities of only one litter, the mother of which was characterized by the highest melatonin concentration in the jugular vein among the studied sows.

Our data strongly suggest that the pineal glands of 70-day-old and 90-day-old pig fetuses do not secrete melatonin. Therefore, the physiological role of the pineal sympathetic innervation during foetal life is obviously not related to the regulation of pineal hormone synthesis and the question about the functional significance of this neuronal input remains unanswered. It is reasonable to consider that these fibers may be involved in the control of cell differentiation and organ development. Araki and Tokunaga (1990) demonstrated that norepinephrine suppresses the differentiation of cells expressing rhodopsin and the growth of processes formed by serotonin-like immunoreactive cells in cultures obtained from the pineals of neonatal rats. The number of rat pineal cells showing the ability to differentiate into rhodopsin-immunoreactive cells in culture decreases dramatically after the first four days of postnatal life (Araki, 1992). Tosini et al. (2000) showed that pineal cells obtained from newborn rats and kept in culture for 7 or 9 days in the absence of norepinephrine are light sensitive. The presence of norepinephrine in culture medium abolishes the development of photosensitivity. It should be noted that we did not find any rhodopsin-positive cells in the pineals of 70-day-old and 90-day-old pig fetuses (unpublished data).

Summing up, the pineal gland in pig fetuses during the last trimester of the pregnancy is relatively well-innervated by nerve fibres containing catecholamine-synthesizing enzymes, NPY and CPON. The presence of these fibres is obviously not associated with the control of melatonin secretion, therefore further studies are required to determine the function of innervation in the pig pineal gland during prenatal life.

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