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MORPHOLOGICAL BASICS FOR EVOLUTION OF FUNCTIONS

Immunolocalization of Cystathionine β-Synthase, Cystathionine γ-Lyase, Heme Oxygenase-2 and Nitric Oxide Synthase in the Human Fetal Retina in Different Gestational Trimesters

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Abstract—The endogenous gaseous transmitters (GTs) —nitrogen oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S)—make up a special neuromodulatory system which mediates the development, maturation and plastic modification of nervous centers. We addressed immunolocalization of the key enzymes of GT synthesis, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), heme oxygenase-2 (HO-2) and constitutive NO synthase (nNOS), at different ontogenetic stages of the human fetal retina. CBS, CSE and HO-2 were found to be expressed in photoreceptor, bipolar and amacrine neurons, the number of which increases in the first and decreases in the third trimesters of gestation. The number of nNOS-immunopositive amacrine and ganglion neurons demonstrates inverse dynamics with maximal values in the third trimester. Uneven distribution patterns of the marker enzymes are discussed in the light of the modulatory function of GTs in neurogenesis of the human retina and their involvement in cytoprotective mechanisms.

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Key words: neurogenesis, neuromodulators, cystathionine β-synthase, cystathionine γ-lyase, heme oxygenase-2, constitutive NO-synthase.

INTRODUCTION

The concept of gaseous transmitters (GTs)—carbon monoxide (CO), nitrogen oxide (NO) and hydrogen sulfide (H_2O)—arose in the mid-1980s, when there their neuromodulatory effects were clearly demonstrated in the central and peripheral nervous systems [1]. Progress in this direction of studies owes the discovery of the key enzymes of GT synthesis which enabled mapping their topography. In the brain, H_2S synthesis is catalyzed by cystathionine β-synthase (CBS; EC 4.2.1.22)

and cystathionine γ-lyase (CSE; EC 4.4.1.1), CO is synthesized via hemoxygenase 2 (HO-2; EC 1.14.99.3) while NO production involves different isoforms of NO synthase (NOS; EC 1.14.13.39).

Biochemical analysis revealed high indices of GT activity in the retina of the monkey, rat, turtle and salamander [2–5]. The GT distribution across the wall of the human eyeball is usually judged by activities of constitutive and inducible NOS [6, 7]. Levels of NO, H_2S and CO production are well known to be critical for the development of oxidative stress in retinal degeneration, glaucoma, optic nerve atrophy and cataract [8–11]. Nevertheless, the topography of CBS, CSE and HO-2 in the human retina remains obscure.

GTs supplement GABA/glutamatergic neurotransmission and play an important role in differentiation of neural connections during ontogenesis [7, 8]. In the human retina, a major source of gasotransmission is NOergic photoreceptors, amacrine and ganglion cells [12]. Temporal expression of NOS shows continuous changes throughout embryogenesis. Data on the involvement of other GTs in retinal neurogenesis are absent.

The aim of this study was to find out immunolocalization of CBS, CSE, HO-2 and NOS during prenatal ontogenesis of the human retina.

MATERIALS AND METHODS

The retina was sampled from 6 eyes of three human fetuses aged 12–13 weeks, 5 eyes of four fetuses aged 21–22 weeks, and 5 eyes of three fetuses aged 30–31 weeks. 1st and 2nd trimester fetuses were obtained due to miscarriage and abortion performed for medical indications (maternal extragenital disease), 3rd trimester fetuses resulted from intranatal fetal death (asphyxia) without congenital malformation.

For immunohistochemical localization of CBS, CSE, HO-2 and neuronal NOS isoform (nNOS), the isolated eyeball was fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.2) for 4 h at 4°C. The material was then washed overnight in the same phosphate buffer at a 5–8-h interval and saturated thereafter with cold 30% sucrose/0.1 M phosphate buffer. Cryosections (30–35 μm) were then prepared. After inhibiting endogenous peroxidase by 1% H_2O_2 in 1% NGS, sections were incubated for 18 h at 4°C with primary polyclonal antibodies to CBS (Abcam, UK) diluted 1:1000, CSE and HO-2 (Santa Cruz Biotechnology, USA) diluted 1:500, and nNOS (Abcam, UK) diluted 1:200. Sections were washed several times in 0.1 M PBS (pH 7.2) and incubated for 2 h with secondary biotinylated antibodies (Vector Labs, USA) diluted 1:200. After washing, sections were incubated for 1 h with avidin-biotin-HRP (Vectastain Elite ABC Kit, Vector Labs, USA) at 22–24°C in the dark and then washed thrice in PBS. Reaction products were visualized by a substrate kit (Vector VIP Peroxidase (HRP) Substrate Kit, Vector Labs, USA) while controlling the process under a microscope. Sections were then washed thrice in PBS, dehydrated conventionally and embedded in Canadian Balm. For control, primary antibodies were excluded from the incubation medium, yielding regularly no immunopositive reaction. Some sections were stained after Feulgen and Rossenbeck.

Preparations were viewed under a light microscope AxioScope A1 (Carl Zeiss, Germany) and photographed by a digital camera AxioCam ICc3 (Carl Zeiss, Germany). Morphometry and quantification were carried out in the central retina corresponding to the central fovea and 6-mm area thereabout. Immunoreactive cell count was performed on 15 serial sections of the posterior wall of eyeballs obtained from 5–6 eyes of the 1st, 2nd and 3rd trimesters on a standard square of 0.06 mm^2 using a ×40 objective. Images were processed morphometrically using the AxioVision 4.8.1 software package. Statistical analysis was carried out using Statistica 6.0 for Microsoft Windows. Data were presented as means ± SEM obtained from 15 serial sections of each eye. To evaluate the statistical significance of the data obtained, the Student's *t*-test was applied. Differences were considered statistically significant at $p < 0.05$.

RESULTS

In 12–13-week fetuses, the retina comprises six distinct cell layers with a total width reaching 374 ± 2.3 μm. At 21–22 weeks, the width of the retina is 353.9 ± 5.0 while at the end of the third gestational trimester it diminishes to 119.5 ± 3.7 μm. By the time of birth, the outer nuclear

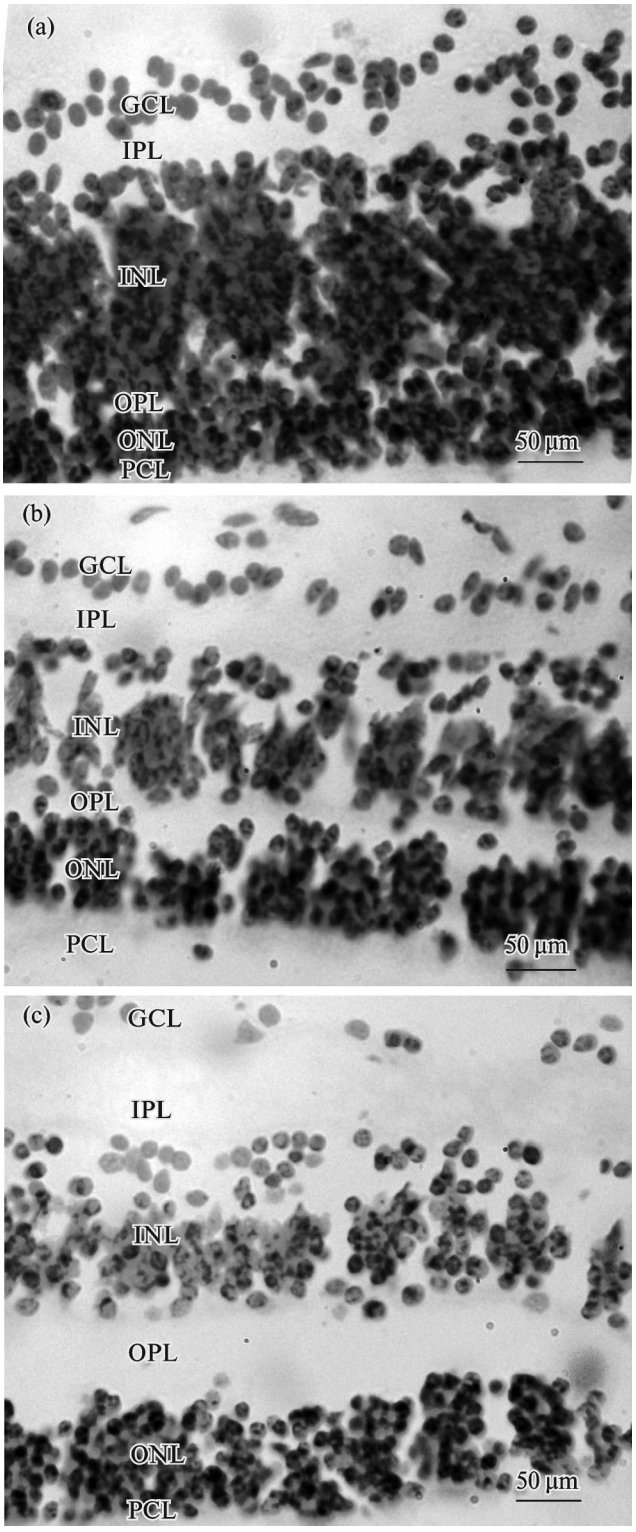

Fig. 1. Stratification of the human fetal retina. Weeks 12–13, (b) weeks 21–22, (c) weeks 30–31. PCL—photoreceptor cell layer, ONL—outer nuclear layer, OPL—outer plexiform layer, INL—inner nuclear layer, IPL—inner plexiform layer, GCL—ganglion cell layer. Feulgen staining.

and inner plexiform layers reach their maximum width, while the ganglion cell (ganglionic) layer is represented by 1–2 rows of neurocytes (Fig. 1). At all developmental stages, CBS, CSE, HO-2 and nNOS are expressed in neurons, their fibers and synaptic terminals, as well as in the retinal pigment epithelium, fibroblasts of the retinal vascular sheathing, and microvascular endothelium. Topography of the above markers varies considerably at different stages of retinal ontogenesis (Table). Peak values of CBS- and CSE-immunoreactivity fall on the first trimester, declining gradually towards the end of the 30rd week. HO-2-immunoreactive cells show the same distribution with only an insignificant increase at the 22nd week. nNOS demonstrates quite a different dynamics. Slight expression of this enzyme shows up already at the 12th week of gestation, increasing monotonously towards the 22nd weeks and reaching its maximum by the end of the third trimester.

CBS marks a heterogeneous cell population in all the nuclear layers. Localization and labeling intensity of the somatodendritic arborization allows discriminating three categories of CBS-immunoreactive cells. Type I neurons demonstrate a high level of enzyme activity: the heavily labeled precipitate fills up the entire cell profile and penetrates into processes. As a rule, these cells have a small diameter and reside approximately in the middle of the inner nuclear layer. Type 2 neurons show up at the interface between the inner nuclear and inner plexiform layers, having a rounded shape, large light nucleus and medium degree of enzyme activity. Type 3 neurons with a low enzyme activity are situated at the interface between the inner nuclear and outer plexiform layers. Their cell bodies contain a small amount of the faintly labeled reaction product which marks their barely discernible primary dendrites (Figs. 2a, 2b).

Neurons in the ganglionic layer do not express CBS, however, the neuropil shows a plenty of dot-like immunoreactive structures which we interpret as interneuronal synaptic terminals (Fig. 2c). CSE-immunoreactive terminals of photoreceptor cells also demonstrate a synaptic pattern of immunoreactivity distribution in the outer plexiform layer (Figs. 2d–2f). Photoreceptors react actively to HO-2. The enzyme is also revealed in neurons of the inner nuclear and ganglionic layers (Figs. 3a–3c).

Relative density of CSE-, CBS-, HO-2-, and nNOS-immunoreactive cells in the central human fetal retina; 0.016 mm^2 ($M \pm m$)

Retinal nuclear layers	Enzyme	Weeks 12–13	Weeks 21–22	Weeks 30–31
Outer nuclear layer	CSE	32.4 ± 2.7	23.1 ± 2.1	9.1 ± 1.8
	CBS	27.8 ± 2.2	18.8 ± 3.2	11.3 ± 1.6
	HO-2	29.2 ± 3.4	25.8 ± 2.1	8.5 ± 1.4
	nNOS	–	–	–
Inner nuclear layer	CSE	–	–	–
	CBS	38.7 ± 3.2	22.1 ± 2.2	9.9 ± 1.8
	HO-2	47.1 ± 3.,3	38.1 ± 1.3	10.1 ± 2.6
	nNOS	12.4 ± 2.5	21 ± 2.8	30 ± 1.1
Ganglion cell layer	CSE	–	–	–
	CBS	–	–	–
	HO-2	29.1 ± 2.7	17.5 ± 2.2	9.7 ± 1.3
	nNOS	15.3 ± 3.1	22.7 ± 2.1	34.1 ± 2.3

CSE—cystathionine γ-lyase; CBS—cystathionine β-synthase; HO-2—heme oxygenase-2; nNOS—neuronal NO-synthase.

At all developmental stages, the NOergic population is made up of cells of the inner nuclear and ganglionic layers. nNOS-immunoreactive cells in the inner nuclear layer mostly have a large diameter and reside at the border with the inner plexiform layer (Figs. 4a, 4b). Neurons are identified by the presence of a single process which proceeds to a thin horizontal trunk, then is tracked along a short trajectory and finally gets lost in the neuropil. Immunoreactive neurons in the ganglionic layer form small clusters of 3–6 cells (Fig. 4c). In the rest of the ganglionic layer, nNOS defies detection.

DISCUSSION

Here we detected expression of CBS, CSE, HO-2 and nNOS in the developing human retina, which is characterized by a distinct spatio-temporal dynamics and correlates with differentiation of the nuclear layers. First neuroblasts appear in the inner wall of the eyecup in the beginning of the 7th gestational week. Retina develops centrifugally in the horizontal plane and from the inner to outer layers in the vertical one. Definitive layout of retinal neurons and their interconnections forms asynchronously.

The retinal primordium consists of a dense layer of neuroblasts which serve a source for definitive neurons [3]. During the chosen developmental period, we observed a gradual multiplication of retinal layers. This process is initiated by the nascency of the primordial ganglionic layer and penetration of provisional retinopetal fibers into the eyecup. The latter actually pave the way for primary growth of ganglion cell axons [14]. After the end of ganglion cell migration, there occurs gradual differentiation of the cones, amacrine and horizontal neurons. Subsequently, there arise the outer and inner nuclear layers as well as the outer plexiform layer.

It was firmly established that NO plays a determinative role in ontogenesis of the human retina [7]. A major source of NO in the developing human retina is photoreceptor, amacrine and ganglion cells [12, 15]. An appropriate pattern of NOS localization characterizes the postnatal developmental period in humans as well as birds, rodents and monkeys [2, 3, 7]. This resemblance allows NO to be considered as a universal factor that regulates neural network's functioning during ontogenesis.

The revealed immunolocalization of GT-synthesizing enzymes during human ontogenesis reproduces the basic pattern of their distribution across the mammalian retina. In doing this, the traits specific to the GT topography in lower vertebrates appear first. For example, in the turtle and

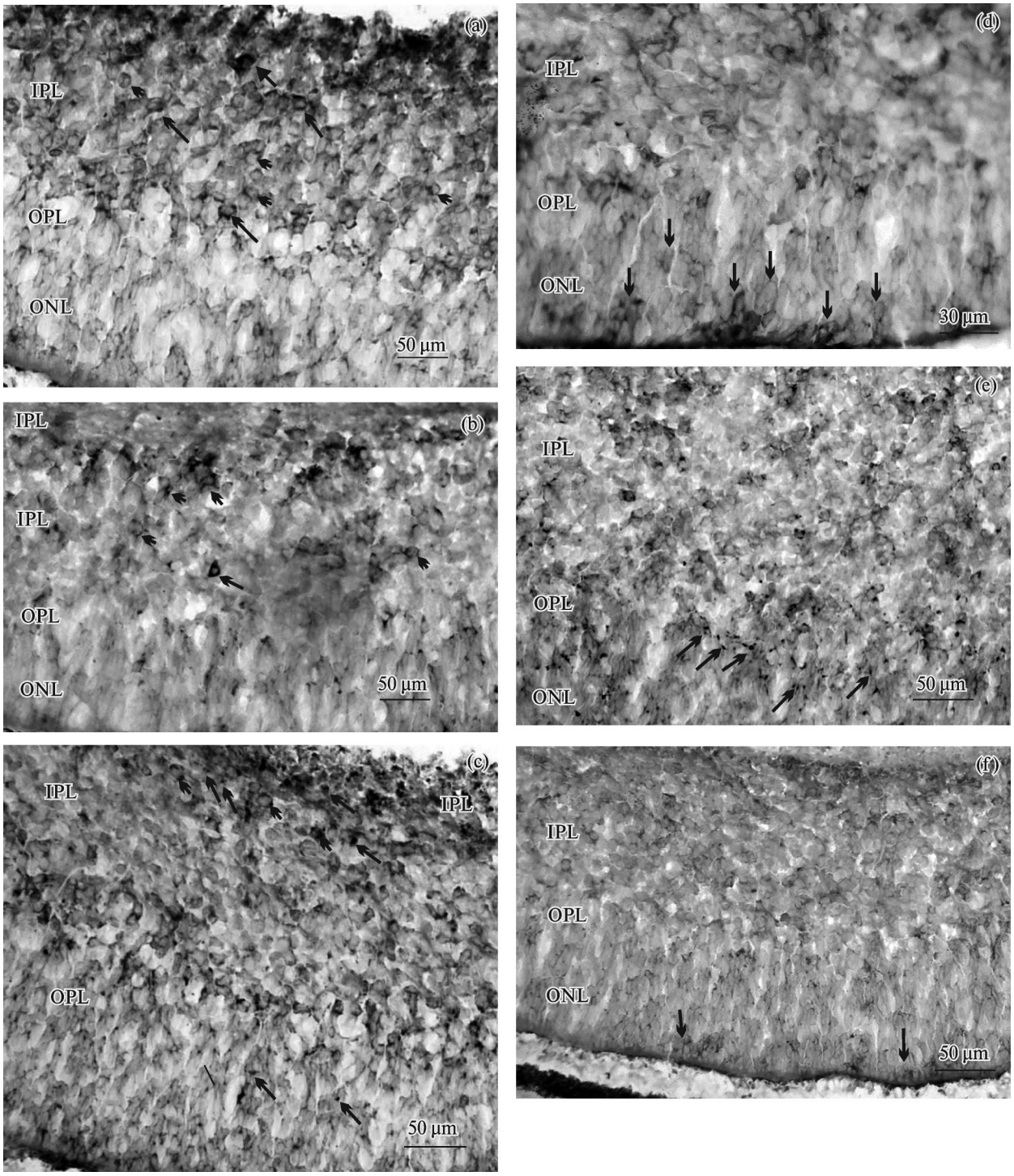

Fig. 2. Typology of CBS- and CSE-immunoreactive neurons in the human fetal retina. (a) CBS-immunopositive neurons in the inner nuclear layer with a high (long arrows) and medium (short arrows) degree of enzyme activity; reaction product is localized to the cytoplasm and cell processes; developmental weeks 12–13. (b) CBS-immunopositive neurons in the inner nuclear layer with an intensely labeled perikaryon (long arrow); large neurons with a large light nucleus and radial processes (short arrow); weeks 21–22. (c) CBS-immunopositive neurons at the border between the inner plexiform and inner nuclear layers (short arrows). In the outer and inner plexiform layers, there are labeled dot-like structures resembling synaptic terminals (long arrows); weeks 30–31. (d) CSE-immunoreactivity in the inner segments of photoreceptor cells (arrows); weeks 12–13. (e) CSE-immunopositive synaptic terminals of photoreceptor cells in the outer plexiform layer (arrows); weeks 21–22. (f) Solitary CSE-immunopositive inner segments of photoreceptor cells; weeks 30–31.

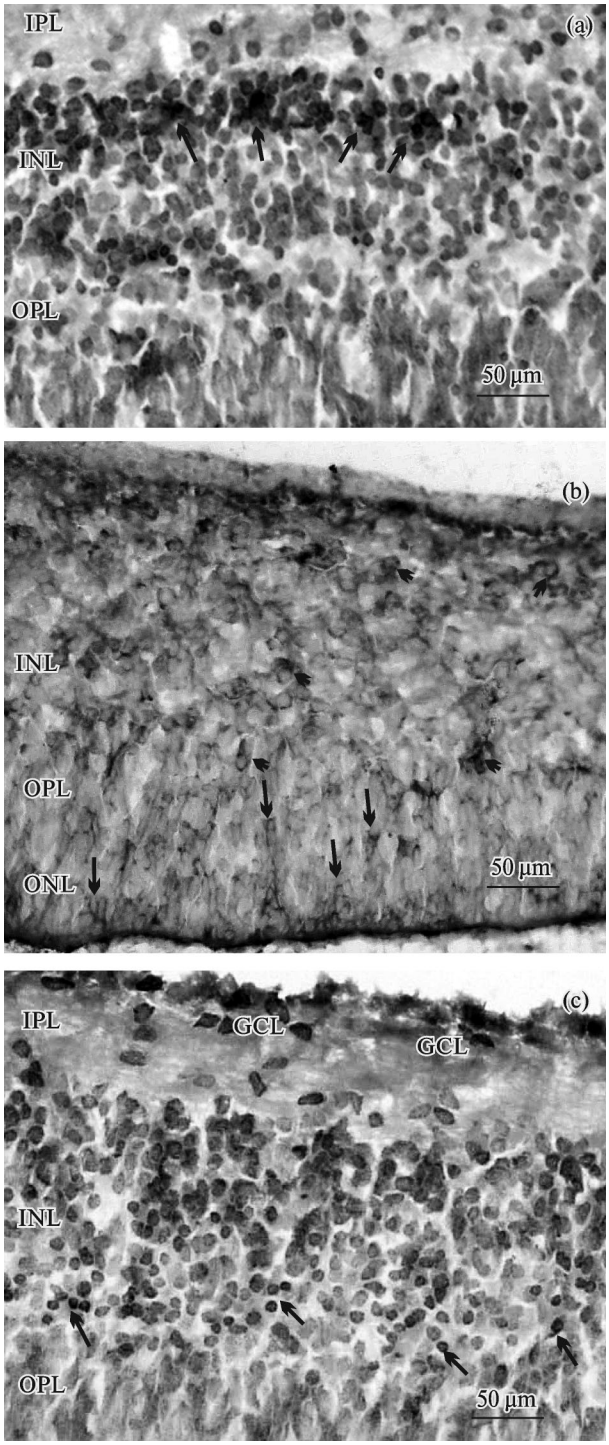

Fig. 3. Topochemistry of HO-2 in the human fetal retina. (a) HO-2-immunopositive neurons in the inner nuclear layer (arrows); weeks 12–13. (b) HO-2 expression in the photoreceptor inner segments and terminals (long arrows) and neurons of the inner nuclear layer (short arrows); weeks 21–22. (c) Immunolocalization of HO-2 in ganglion cells (GC) and interneurons located at the border between the inner nuclear and outer plexiform layers (arrows); weeks 30–31.

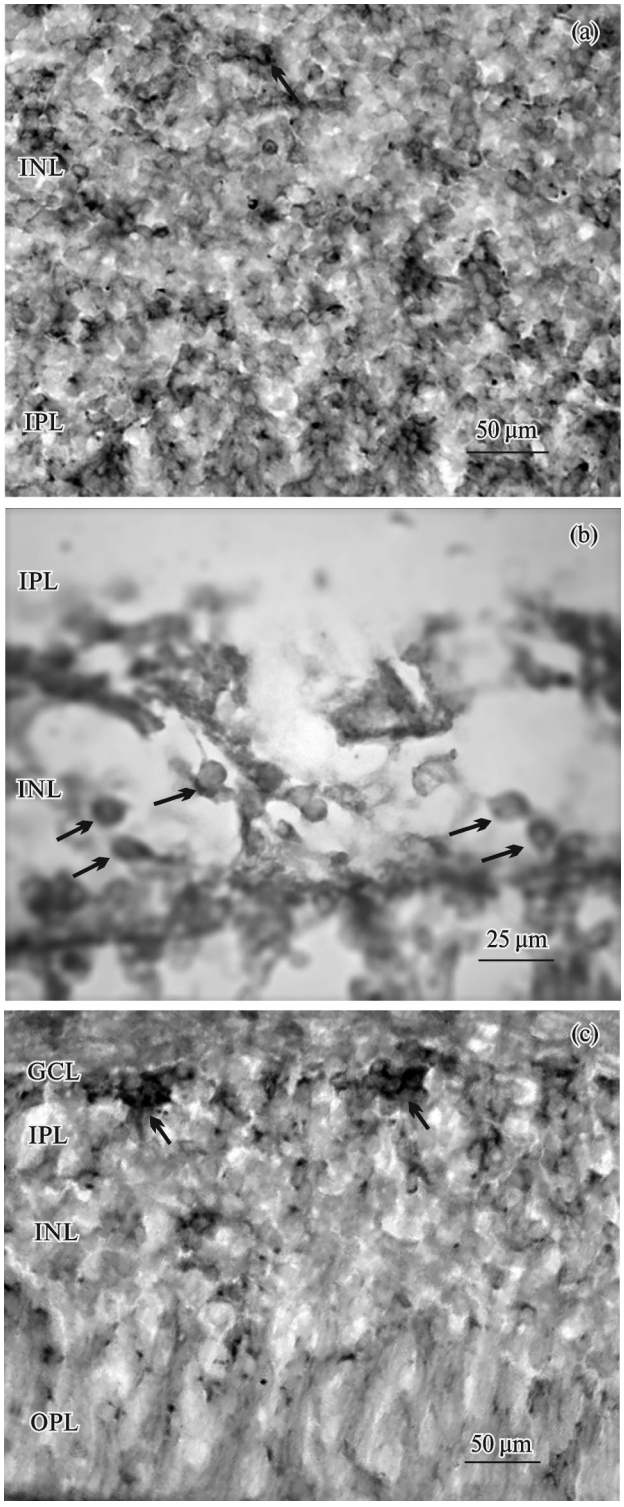

Fig. 4. NO-ergic neurons in the human fetal retina. (a) nNOS-immunopositive cell in the inner nuclear layer with a horizontally arborizing process (arrow); weeks 12–13. (b) nNOS-immunopositive cells in the inner nuclear layer with a large light nucleus (arrows); weeks 21–22. (c) Clusters of nNOS-immunopositive cells in the ganglion cell layer (arrows); weeks 30–31.

salamander, antibodies to HO-2 label all types of photoreceptor, bipolar and ganglion neurons as well as a small population of amacrine cells [2]. The same situation occurs in humans in the 1st gestational trimester. At later stages of gestation, the number of HO-2-immunoreactive photoreceptors decreases dramatically, while the populations of interneurons and ganglion cells begin to dominate. The same scenario was reported for the retina of adult monkeys and rats [2, 3].

During mammalian phylogeny, CBS expression occurs heterogeneously. High CBS levels are expressed in human and porcine ocular tissues [6]. In the mouse retina, one authors report no enzyme expression whatsoever [3], whereas others demonstrate that it is confined mainly to Müller glia [17]. In the prenatal human material, we failed to detect CBS in ganglion cells. However, the temporary absence of CBS in developing neurons can be compensated by other H_2S-synthesizing enzymes. The plausible candidates for this role are cysteine aminotransferase and 3-mercaptopyruvate sulfurtransferase [18]. Notably, CSE is localized mainly to synapses, probably indicating different compartments for H_2S production.

Ganglion cells represent the only type of neurons that synthesize a widest spectrum of GTs throughout the human prenatal development. Given their early nascency, we suggest an instructive role of GTs in the processes of neurogenesis and cell migration towards their due positions in the retinal layers. For example, NO facilitates growth cone formation in lengthening retinothecal projections [7]. However, in gas hyperproduction, there is an inverse effect: NO blocks cell cycling in immature retinal neurons and launches their apoptosis [12, 19]. This phenomenon has also been described in postnatal ontogenesis and interpreted as a selection factor for already established connections [20].

Heterogeneous and heterochronic maturation of gasotransmitting systems may be coupled in some types of neurons with the formation of their mediatory function. There is evidence that in the developing retina of vertebrates slow and fast neurotransmitters modulate proliferation of precursor cells. In doing this, GABA and glutamate have mutually exclusive effects on the cell cycle of migrating neuroblasts [15]. It cannot be ruled out that similar relationships also form among different GTs.

The retina has a densely packed neuropil which abounds in synaptic contacts and gap junctions between neurons. At all ontogenetic stages, expression of the above enzymes occurs simultaneously, indicative of a combined effect of GTs on target cells. For example, in the salamander retina, the cooperative effect of NO and CO manifests itself in a considerable increase in the cGMP level [4]. Diffusion and physiological effects of GTs spread at least 100 μm away from their source and definitely have areas of their mutual influence. Presumably [21], CO promotes NO production and sensitizes different NO-binding domains in guanylate cyclase. H_2S does not affect guanylate cyclase directly but can modify cGMP-dependent mechanisms obliquely. Besides, H_2S enhances the Ca^{2+} dynamics and induces their release from intracellular stores [22–24]. Although a buildup of cytoplasmic Ca^{2+} is an indispensable prerequisite for NOS activation, H_2S can inhibit the NO-dependent accumulation of cGMP [5, 25].

It is noteworthy that GTs are widespread in pigmentocytes, fibroblasts of the vascular sheathing, and microvascular endothelium, thus indicating their involvement in the regulation of virtually all components of the eye wall. For example, the nascency of the pigment epithelium serves a signal to induce neuromesenchyme differentiation and correct orientation of photoreceptor outer segments. It is not ruled out that GTs can mediate these signaling processes. Anyway, this function of GTs has been demonstrated for NO molecules in the nascent avian and amphibian retina [3]. CBS and CSE activities in ocular tissues often correlate with the neuroprotective effect of H_2S. H_2S donors inhibit ATP-dependent K^+/Na^{2+} channels and positively affect glutathione synthesis, a key component of the antioxidant defense system [26]. Active H_2S production suppresses sympathetic influences on muscle cells in the retinal vascular sheathing and restricts the spread of glutamatergic neurotransmission and related cytotoxic effects [27, 28]. However, depending on their concentration and identity of target cells, GTs can change their protective effect to the opposite [29]. The interrelationship between GT production and oxi-

dative stress due to accumulation of reactive oxygen species is considered as a recapitulation of the evolutionary process in transition from anaerobic metabolism to oxygen-dependent types of cell respiration [30].

Thus, the formation of gaseous transmission is an important component of differentiation of early neural systems during ontogenesis of the human retina. GTs modulate the establishment of due connections and provide their viability and adaptation to various plastic rearrangements.

COMPLIANCE WITH ETHICAL STANDARDS

The material was obtained at the Vladivostok Pathological Anatomy Bureau according to the orders of the Ministry of Health of the Russian Federation no. 354n of 06.06.2013 "On conducting pathological autopsies" and no. 179n of 24.03.2016 "On the regulations for conducting pathological anatomical studies", and the order of the Ministry of Health and Social Development of the Russian Federation no. 346n of 12.05.2010 "On the organization and conducting forensic medical expertise in state forensic examination institutions of the Russian Federation".

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