Differential expression of syntaxin-1 and synaptophysin in the developing and adult human retina

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Synaptophysin and syntaxin-1 are membrane proteins that associate with synaptic vesicles and presynaptic active zones at nerve endings, respectively. The former is known to be a good marker of synaptogenesis; this aspect, however, is not clear with syntaxin-1. In this study, the expression of both proteins was examined in the developing human retina and compared with their distribution in postnatal to adult retinas, by immunohistochemistry. In the inner plexiform layer, both were expressed simultaneously at 11–12 weeks of gestation, when synaptogenesis reportedly begins in the central retina. In the outer plexiform layer, however, the immunoreactivities were prominent by 16 weeks of gestation. Their expression in both plexiform layers followed a centre-to-periphery gradient. The immunoreactivities for both proteins were found in the immature photoreceptor, amacrine and ganglion cells; however, synaptophysin was differentially localized in bipolar cells and their axons, and syntaxin was present in some horizontal cells. In postnatal-to-adult retinas, synaptophysin immunoreactivity was prominent in photoreceptor terminals lying in the outer plexiform layer; on the contrary, syntaxin-1 was present in a thin immunoreactive band in this layer. In the inner plexiform layer, however, both were homogeneously distributed. Our study suggests that (i) syntaxin-1 appears in parallel with synapse formation; (ii) synaptogenesis in the human retina might follow a centre-to-periphery gradient; (iii) syntaxin-1 is likely to be absent from ribbon synapses of the outer plexiform layer, but may occur at presynaptic terminals of photoreceptor and horizontal cells, as is apparent from its localization in these cells, which is hitherto unreported for any vertebrate retina.

1. Introduction

Syntaxin-1 (35 kDa) and synaptophysin (38 kDa) are two membrane proteins found in the nerve endings at synapses (Wiedenmann and Franke 1985; Bennett *et al* 1992). The former is reported to occur along the presynaptic plasma membrane (Bennett *et al* 1992; Yoshida *et al* 1992) and associates with several other synaptic proteins (e.g. synaptotagmin, synaptobrevin and SNAP-25) to form a high molecular weight fusion complex (Sollner *et al* 1993). Although its precise involvement in synaptic transmission is not fully understood, several authors have proposed that the formation of such fusion complex may mediate the docking of synaptic vesicles at the presynaptic active zones, thereby aiding in fusion and subsequent release of neurotransmitters (Bennett *et al* 1992; Blasi *et al* 1993; Sollner *et al* 1993). Synaptophysin occurs in neurotransmitter, containing small synaptic vesicles, located inside the presynaptic terminals (Jahn *et al* 1985; Wiedenmann and Franke 1985; Navone *et al* 1986) and is suggested to have an active involvement in the regulation of vesicular exocytosis (Wiedenmann and Franke 1985; DeCamilli and Jahn 1990; Jahn and Sudhof 1994).

Immunocytochemical studies have shown the relative distribution of syntaxin-1 and synaptophysin in the adult mammalian retinas. The former is reported to be present in the inner plexiform layer (IPL) of adult rat retina (Barnstable *et al* 1985) whereas the latter occurs in both inner and outer plexiform layer (OPL) of rat, rabbit and human retina (Kivelä *et al* 1989; Catsicas *et al* 1992;

Keywords. Development; human retina; immunohistochemistry; synaptogenesis; synaptophysin; syntaxin-1

Abbreviations used: DPX, Dibutylpthalate polystyrene xylene; IPL, inner plexiform layer; PBS, phosphate buffer saline; PD, postnatal day; OPL, outer plexiform layer.

Kapfhammer *et al* 1994; Sheedlo *et al* 1995; Brandstätter *et al* 1996). In rat, synaptophysin appears first in the developing OPL between postnatal day (PD) 2–5, and then in the IPL between PD 8–12 (Kapfhammer *et al* 1994; Dhingra *et al* 1997). The immunocytochemical expression of this synaptic vesicle protein roughly corresponds to the morphological development of synapses in the rat retina: in the OPL, synapses appear at around PD 5 and in the IPL at around PD 10–11 (Weidman and Kuwabara 1968, 1969). Several studies in other parts of the central nervous system have reported the immunoreactivity of synaptophysin to increase in parallel with the formation of morphological synapses (Knaus *et al* 1986; Devoto and Barnstable 1987; Leclerc *et al* 1989), suggesting that the labelling of this synaptic vesicle protein may be a reliable approach to study synaptogenesis at the light microscopical level (Voigt *et al* 1993). Okada *et al* (1994) have employed SV_2, another synaptic vesicle protein, as a marker for developing synapses in the macaque monkey retina and reported by combined light- and electron microscope immunocytochemistry that the SV_2 expression and synapse formation in the foveal IPL appear earlier than in the OPL in this primate, suggesting a clear-cut vitreal-to-scleral sequence of synaptogenesis in the primate retina (see also, Nishimura and Rakic 1985, 1987).

No reports are available on the immunolocalization or developmental expression of syntaxin-1 in the monkey or human retina. As regards to synaptophysin, the report of Seiler and Aramant (1994), although aimed at looking for markers of developing photoreceptors, furnishes some fact on this synaptic vesicle protein in the developing human retina. Except for this report, not much is known about the development of various synaptic proteins in the human retina nor the state of synaptogenesis in the fetal human retina beyond 15 weeks of gestation (Hollenberg and Spira 1972, 1973; Spira and Hollenberg 1973; Van Driel *et al* 1990). On the other hand, information about synaptotagmin (P_{65}, Sarthy and Bacon 1985; Koontz and Hendrickson 1993; SV_2, Mandell *et al* 1990; Okada *et al* 1994), syntaptoporin (Brandstätter *et al* 1996) and synapsin (DeCamilli *et al* 1983; Mandell *et al* 1990; Haas *et al* 1990; Koontz and Hendrickson 1993) in the developing and adult retina of rat and monkey is sufficiently available.

The aim of this study was to examine the developmental expression of syntaxin-1 and synaptophysin in the fetal human retinas of 11 to 25 weeks of gestation, by light microscopical immunohistochemistry. Additionally, this study has examined the distribution of these two synaptic proteins in the postnatal and adult human retina. The results show a differential distribution of these two synaptic proteins in the human retina, since early development. Also, the immunohistochemical data provide some information regarding synaptogenesis across the fetal retina, the detail of which awaits verification by electron microscopy.

2. Materials and methods

2.1 *Tissue samples*

Human fetuses were obtained from patients undergoing hysterotomy for medical termination of pregnancy. Prior clearance from the Institute's ethical committee and consent of the parents involved were sought for use of the fetal specimens. The age of the fetuses was determined by measuring the crown rump length (Hamilton *et al* 1972), biparietal diameter and foot length, and comparing them with the known values against gestational ages. Accordingly, the age of the fetuses ranged from 11–12 weeks to 24–25 weeks of gestation. A total of 10 fetuses of 11–12 weeks (3), 14 weeks (1), 16 weeks (2), 18 weeks (1), 20–21 weeks (2) and 24–25 weeks (1) were employed. Additionally, the eyeballs from a four-month-old infant, died of congenital heart disease and from a 35-year-old man, died of road-accident were used. The eyeballs were available for fixation within 30 min to 1 h (fetal) and 3 h postmortem (adult).

2.2 *Fixation*

The eyeballs were enucleated and the cornea and lens removed. The eyecups were then fixed in 4% paraformaldehyde in 0·1 M phosphate buffer (pH 7·4) for 3–7 days at 4°C. After several washes, the retinas were isolated and cryoprotected in 15–30% sucrose overnight. Frozen sections of 20–25 μm thickness were cut with a cryocut (Reichert-Jung), collected in vials containing buffer and stored at 4°C until use.

2.3 *Immunohistochemistry*

Sections were treated with 20% horse normal serum for 2 h at 4°C to block nonspecific binding. Following this, they were incubated in mouse primary antisera against synaptophysin (Boehringer Mannheim, Germany, 1 : 200 dilution) and syntaxin-1 (Sigma Chemicals Company, St. Louis, MO, USA, 1 : 200 dilution) for 2–3 days at 4°C. The antibodies were diluted with 0·01 M phosphate buffer saline (PBS) containing 0·05% Triton X-100 and 5% horse serum. After this, the sections were washed in PBS and incubated in biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) at 1 : 200 dilution overnight at 4°C. They were washed and put in avidin-biotin peroxidase complex (Vector Laboratories; 1 : 200 dilution) for 2–3 h at room temperature. For visu-

alization of the reaction sites, the sections were treated in 0·03% diaminobenzidine tetrahydrochloride (Sigma Chemicals Company) and 0·05% hydrogen peroxide together with 0·3% nickel sulphate (as intensifier), all being dissolved in 0·1 M acetate-imidazole buffer (pH 7·4), for 15–20 s. They were then washed in distilled water several times, mounted onto gelatin-coated slides, dehydrated in ethyl alcohol and coverslipped with dibutylpthalate polystyrene xylene (DPX). For controls, the sections from each representative age group were treated in the same manner as already outlined, except that the incubation in the respective primary antibody was replaced with normal serum.

2.4 *Histology*

To identify the development of different retinal layers and cell types, adjacent cryosections (12 μm thick) were stained with haematoxylin and eosin, dehydrated in ethyl alcohol and mounted in DPX.

3. Results

3.1 *Retinal neurogenesis*

Retinal neurons arise from multipotent neuroblasts in a sequence that is common to most mammals studied thus far. In human, ganglion cells as well as their axons (aligned in the nerve fibre layer) differentiate in the central retina beore 10 weeks of gestation; Müller cells also differentiate simultaneously, and both cell types lie together at this fetal age in the inner neuroblastic zone from where they arise (Mann 1964; O'Rahilly 1975; Rhodes 1979). The amacrine cells are formed in course of development from the innermost rows (2–3) of the outer neuroblastic zone at 11 weeks of gestation (Rhodes 1979). The photoreceptors originate at around 10–12 weeks of gestation from the scleral rows of the outer neuroblastic zone; of these, the cones differentiate first at 10 weeks, followed by rods from 12 weeks of gestaion onward (Hollenberg and Spira 1972; Rhodes 1979). By 18–19 weeks, the cone inner segments are arranged into distinct mosaic patterns (Narayanan and Wadhwa 1998), as seen in the adult retina. The horizontal cells differentiate somewhat late around 16 weeks, from the outer neuroblastic zone (Nishimura and Rakic 1985; Nag and Wadhwa 1996). Bipolar cells are the last to be born (Maslim and Stone 1988; Nishimura and Rakic 1985), and in case of human they differentiate around 17–18 weeks of gestation from the outer neuroblastic zone (Rhodes 1979). Synaptogenesis commences in the central retina at 11–12 weeks of gestation (Hollenberg and Spira 1972, 1973; Linberg and Fisher 1988, 1990). However, the IPL develops earlier (visible even before 15 weeks of gestation, Van Driel *et al* 1990) than the OPL, which is clearly seen only after 16 weeks of gestation (Rhodes 1979). The situation is just reverse in case of rats, in which the OPL appears earlier (PD 5) than the IPL (PD 10; Weidman and Kuwabara 1968, 1969; Horsburgh and Sefton 1987). It is evident that a vitreal-to-scleral sequence of synaptogenesis is operative in the primate retina (monkeys and human, Nishimura and Rakic 1985, 1987; Okada *et al* 1994). The neurogenesis of the vertebrate retina, including that of man, however, proceeds in a centre-to-periphery sequence (Mann 1964; Rapaport and Stone 1982; Provis *et al* 1985). At around mid gestation (20–21 weeks), all retinal layers and constitutent cell types are clearly recognizable in the central and midperipheral parts of human retina. Foveal maturation, however, does occur after several months of postnatal life in human infants (Hendrickson 1992). The different layers and constituent cell types of the fetal human retina at different stages of development are diagramatically represented in figure 1.

3.2 *Immunoreactivity at 11–12 weeks of gestation*

The neural retina consisted of an outer and inner neuroblastic zones. Centrally, the IPL had developed, separating the two neuroblastic zones (figure 2A). Both syntaxin-1 and synaptophysin expressed simultaneously in the developing retinas at this gestational time point. Syntaxin-1 immunoreactivity was expressed moderately in the IPL and weakly in the nerve fibre layer (figure 2B) at central retinal regions, 1-2 mm away from the optic disc. No immunoreactivity was detected in the position of the OPL. In the midperipheral part (distance: 3–4 mm from optic disc, figure 2C), the immunoreactivity in the IPL was patchy and weak. Weakly labelled cells were occasionally seen in the ganglion cell layer and closer (scleral) to the IPL, which seemed to be amacrine cells. No immunoreactivity was developed in the peripheral part of retina (not shown).

Synaptophysin immunoreactivity was localized in the IPL from central (figure 2D) to midperipheral parts of retina. Few cells lying in the ganglion cell layer showed weak immunoreactivity. Fine immunoreactive processes, most probably the bipolar cell axons, were observed to run from the pigment epithelial side of the retina into the outer border of the IPL. A number of round photoreceptor cell bodies, lying buried into the pigment epithelium, were also observed. At the retinal periphery, synaptophysin immunoreactivity (figure 2E) was present in some differentiating cells of the outer neuroblastic zone. Few strongly labelled cells were present close to the nerve fibre layer, which suggested that they were ganglion cells. In the photoreceptor layer, the immunoreactivity was found in the cell bodies (tucked against the pigment epithelium) and

inner fibres (figure 2E). No immunoreactivity was seen in the control sections (figure 2F).

3.3 *Immunoreactivity at 14 weeks of gestation*

Syntaxin-1 was expressed conspicuously in many cell bodies lying in the ganglion cell layer of the peripheral retina (figure 3A). Faint staining was present in the nerve fibre layer. It is thus possible that the cell types labelled in the ganglion cell layer were displaced amacrine as well as ganglion cells, although in rat retina the ganglion cells (identified by Thy-1 labelling) did not label with syntaxin-1 antibody in the colocalization study performed by Barnstable *et al* (1985). In the fetal human retina, the IPL was moderately labelled in the central-to-midperipheral parts of retina (not shown), as in the preceding stage, although no immunoreactivity was evident in this layer at the retinal periphery (figure 3A). In the OPL, there was no sign of immunoreactivity development elsewhere in the retina at this gestational time point.

Synaptophysin immunoreactivity was strongly expressed in the IPL of central (figure 3B) and midperipheral parts of retina. Fine immunoreactive fibres of bipolar and photoreceptor cells were observed in the outer neuroblastic zone. The peripheral retina (figure 3C) showed few labelled cell bodies in the ganglion cell layer and in the outer neuroblastic zone (which could be photoreceptor and bipolar cells), with fine stippling in the IPL.

3.4 *Immunoreactivity at 16–17 weeks of gestation*

Histologically, at this time point, the OPL was formed in the central (figure 4A) to midperipheral (3–4 mm away from optic disc) parts of retina. Syntaxin-1 immunoreactivity was expressed strongly in the IPL over the entire retina (figure 4B, C). Also, the nerve fibre layer was strongly labelled over most of the retina. Centrally, the ganglion cell layer showed conspicuous labelling (figure 4B). Some amacrine cells were faintly immunoreactive in the centre (figure 4B) to periphery (figure 4C). The OPL showed syntaxin-1 immunoreactivity in the central-to-midperipheral regions of retina. In the central retina, the immunoreactivity was strong and appeared in a continuous line (figure 4B), as seen in retina of 20–21 weeks of gestation. In the midperiphery, the labelling was patchy and discontinuous (not shown). In the central retina, there were profiles of weakly-labelled horizontal cells, which were tucked against the OPL (figure 4B). It was also evident that the photoreceptor terminals extending into this layer were clearly immunoreactive. No immunoreactivity was seen in the OPL of peripheral retina, but was present in the IPL and nerve fibre layer (figure 4C).

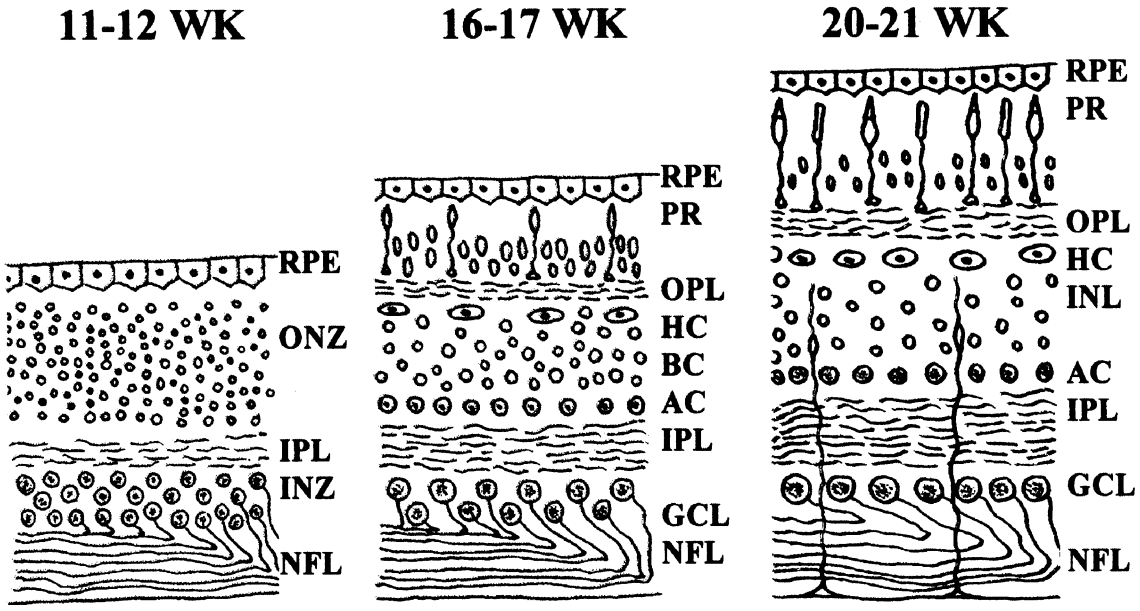

Figure 1. Schematic representation of different layers in the fetal human retina at various stages of development. Cells of the outer retina (photoreceptor, horizontal, bipolar and amacrine) arise from the outer neuroblastic zone (ONZ), whereas those of the inner retina (ganglion cells and Mü ller cells) develop from the inner neuroblastic zone (INZ). The synaptic processes from these neurons constitute the OPL and IPL. RPE, retinal pigment epithelium; NFL, nerve fibre layer; PR, photoreceptor layer; HC, horizontal cells; BC, bipolar cells; AC, amacrine cells; GCL, ganglion cell layer.

Synaptophysin was localized in both IPL and OPL (figure 4D) of the central retina. A few cell bodies belonging to both ganglion and displaced amacrine cells were still immunoreactive in the ganglion cell layer. In the inner nuclear layer, the bipolar cell axons were moderately labelled. The immunopositive photoreceptor inner segments and pedicles in the OPL were distinctly identifiable at this time point. Tangentially cut photoreceptor

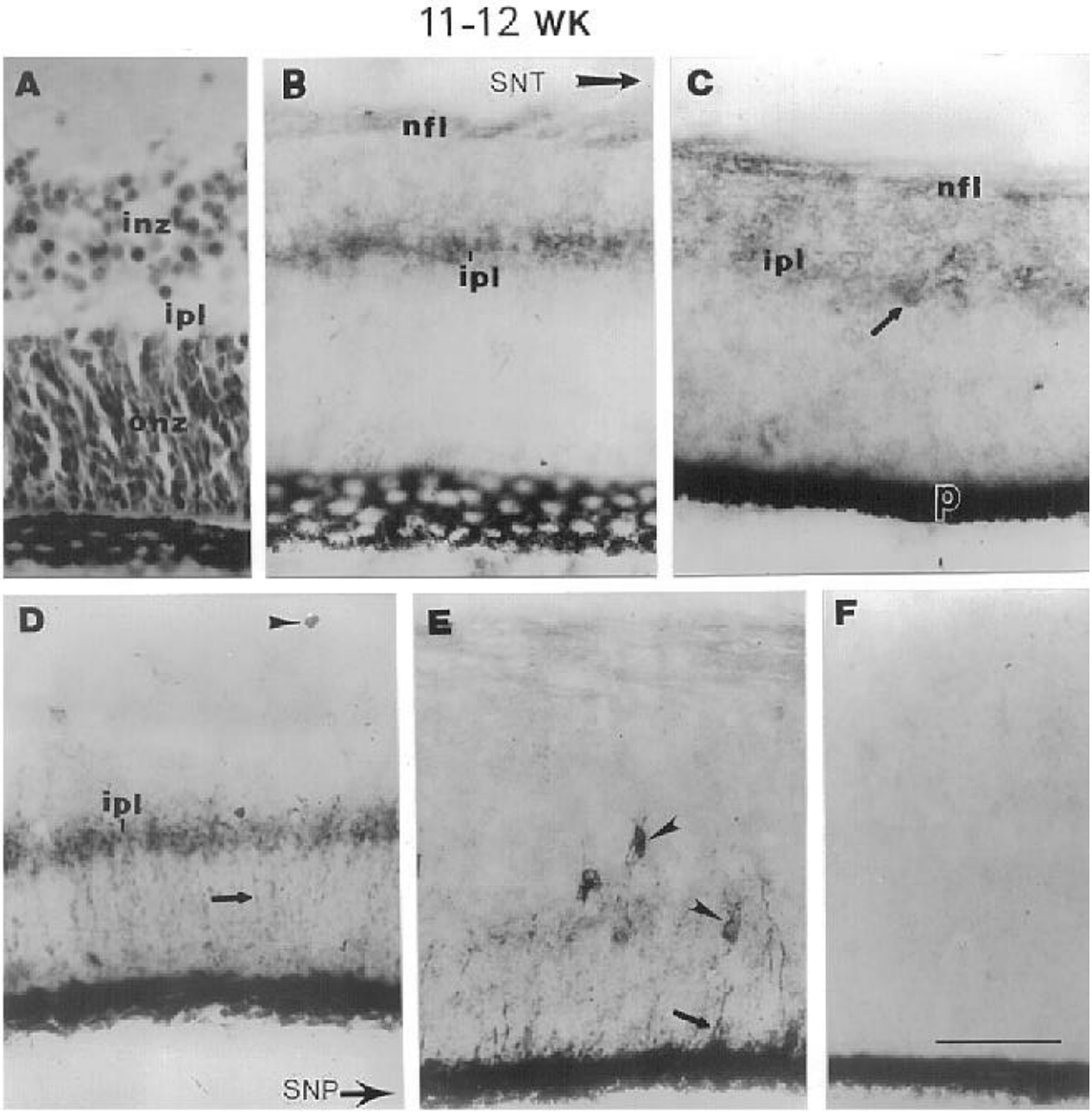

Figure 2. Photomicrographs of 11–12 week-old fetal retina showing SNT (syntaxin-1) and SNP (synaptophysin) immunoreactivity. (**A**) Haematoxylin and eosin stained retina, showing outer and inner neuroblastic zones (onz, inz) and IPL (ipl). (**B**) From the central part of retina, showing SNT-immunoreactivity in the ipl and nerve fibre layer (nfl). (**C**) Showing SNT-immunoreactivity in the midperipheral retina. Weak labelling is present in the nfl, ipl, and in amacrine cell (arrow). p, pigment epithelium. (**D**) Showing SNP-immunoreactivity in the central retina. The ipl and bipolar axons (arrow) are stained. The arrowhead indicates one labelled cell body in the ganglion cell layer. The strongly labelled photoreceptor cell bodies lie embedded in the pigment epithelium. (**E**) From the peripheral part of retina. SNP-immunoreactivity is present in some differentiating neurons (arrowheads) and cone processes (arrow). The immunoreactive photoreceptors lie almost embedded in the pigment epithelium. (**F**) Control section, with omission of the SNP antiserum treatment, showing absence of immunoreactivity. Scale bar = 50 m.

layer revealed that rod as well as cone inner segments were labelled (not shown). In the midperiphery, the staining was present in the IPL and few cell bodies lying in the ganglion cell layer. Fine granular staining extending from the photoreceptor zone to the OPL was noted in the outer part of retina (figure 4E). In the periphery, synaptophysin was localized in the cell bodies lying in the ganglion cell layer (2–3 rows thick), outer neuroblastic zone as well as differentiating photoreceptor cells (not shown).

At 18 weeks of gestation, the immunoreactivity in the OPL was predominantly localized in the photoreceptor terminals for both synaptic proteins. However, syntaxin-1 immunoreactivity was relatively lower than that for synaptophysin in the terminals.

3.5 *Immunoreactivity at 20–21 weeks of gestation*

All the retinal layers were distinctly present by mid-gestation (20–21 weeks, figure 5A). Syntaxin-1 immunoreactivity was observed in a thick homogenous band of staining in the IPL throughout the retina (figure 5B, C). The OPL was labelled as a thin, continuous band from center (figure 5B) to midperiphery (not shown), while in the periphery (figure 5C) no labelling was found. No immunoreactivity was seen in the horizontal cells. There were silhouettes of some weakly-labelled amacrine cell bodies in the inner row of the inner nuclear layer (figure 5B).

With synaptophysin antibody, the photoreceptor cell bodies with their inner fibres and terminals were labelled intensely from central (figure 5D) to midperipheral (not shown) parts of retina. In the central part of the inner nuclear layer, many bipolar cell bodies were inconspicuously labelled. Their immunoreactive axons were observed to run across the inner nuclear layer to terminate in the IPL (figure 5D). In the peripheral part of retina (figure 5E), neither the bipolar cells nor their processes were immunoreactive. The resident photoreceptors were not yet fully differentiated, especially in the zone of their synaptic endings, and a granular staining of their inner fibres extending from the photoreceptor layer to the prospective zone of the OPL was evident. The IPL was moderately stained and reduced in thickness as compared to that of the central part of retina.

3.6 *Immunoreactivity at 24–25 weeks of gestation*

As in 20–21-week gestational retinas, syntaxin-1 was localized in the OPL and IPL. The immunoreactivity was not yet developed in the peripheral part of the OPL (not shown).

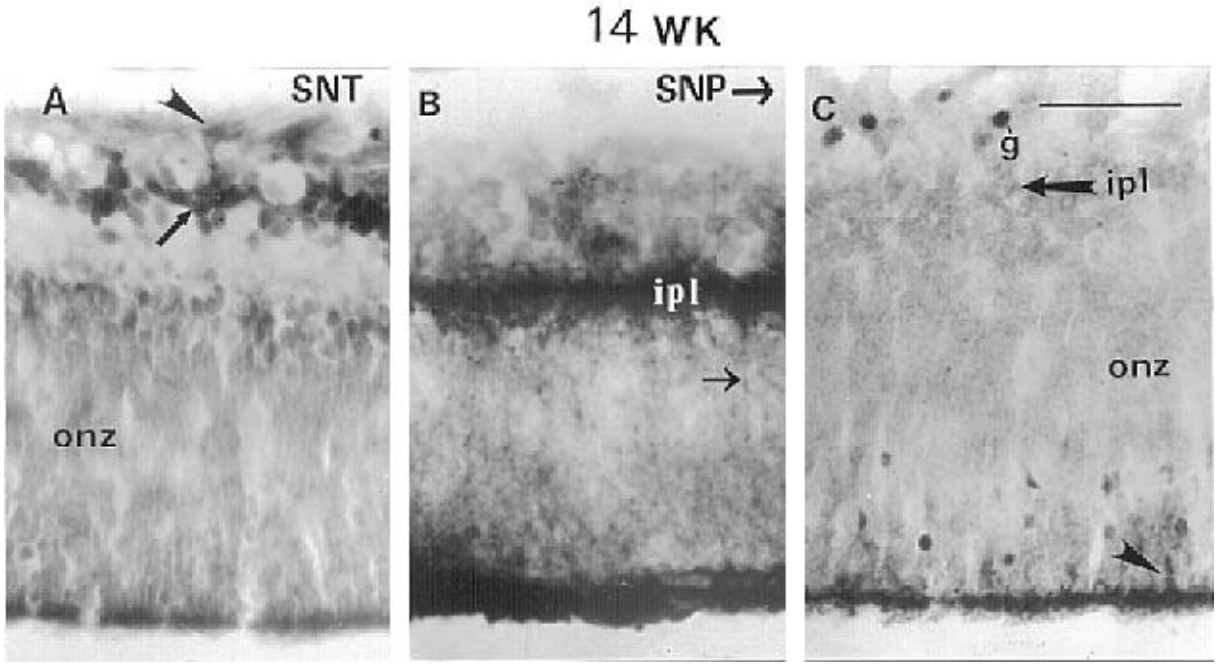

Figure 3. Photomicrographs of 14 week-old fetal retina labelled for SNT (syntaxin-1) and SNP (synaptophysin). (**A**) From the periphery. SNT-immunoreactivity is present prominently in the cells (arrow) of the ganglion cell layer and weakly in the nerve fibre layer (arrowhead). (**B**, **C**) Showing SNP-immunoreactivity in the central and peripheral parts of retina, respectively. Note that immunoreactivity in the central IPL (ipl, **B**) is enhanced at this stage (cf. figure 1D). The arrow indicates labelled bipolar axon. In (**C**), the labelling is present in the presumed ganglion cells (g), and photoreceptor cells (arrowhead). Faintly stippled immunoreactivity in the developing ipl (arrow) is also apparent. onz, outer neuroblastic zone. Scale bar = 50 μm.

The pattern of synaptophysin immunoreactivity did not differ much, except that no labelling was seen in the bipolar axons and displaced amacrine or ganglion cells (figure 5F).

3.7 *Immunoreactivity in postnatal retina*

Syntaxin-1 and synaptophysin immunoreactivities were present in the OPL as well as IPL (figure 6A, B). At the

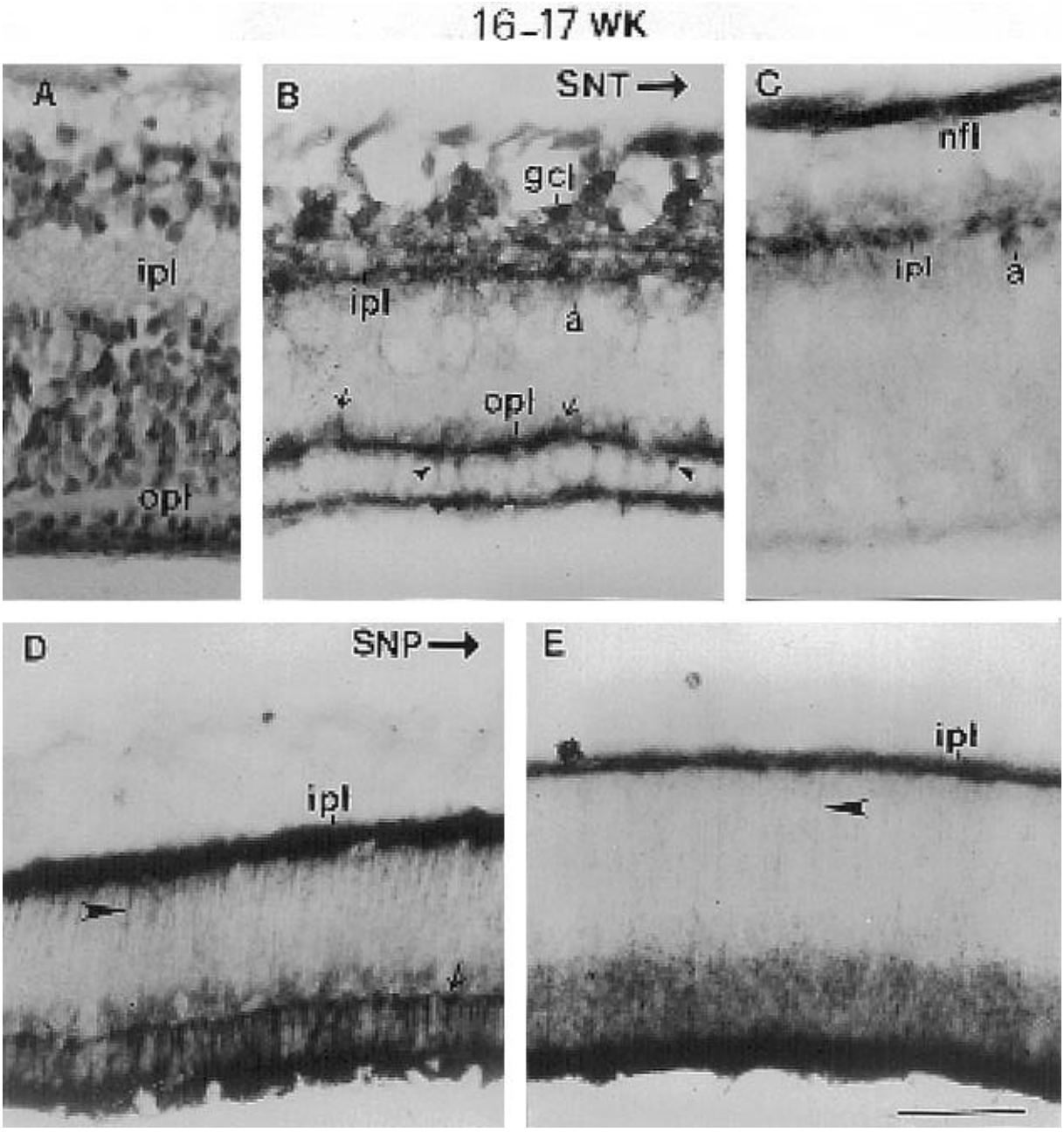

Figure 4. Photomicrographs showing SNT (syntaxin-1) and SNP (synaptophysin)-immunoreactivity in 16–17 week-old fetal retinas. (**A**) Haematoxylin and eosin stained central retina, showing appearance of the OPL (opl). (**B**, **C**) Showing SNT-immunoreactivity in central (**B**) and peripheral (**C**) parts, respectively. In both, the immunoreactivity is present in the ipl and nerve fibre layer (nfl). In the opl, the immunoreactivity is present centrally, but is absent from the periphery. Occasional amacrine cells (a) and cells of the ganglion cell layer (gcl, **B**) are moderately stained. Note that in (**B**), some horizontal cells (arrows) and cone pedicles (arrowheads) are labelled. (**D**, **E**) Showing SNP-immunoreactivity in central (**D**) and midperipheral (**E**) parts. In both, weak labelling of bipolar axons (arrowheads) terminating into the ipl is apparent. The opl is clearly defined in the central (**D**) but not in the midperipheral (**E**) regions. The immunoreactivity in the opl is limited to photoreceptor terminals (**D**, arrow). Scale bar = 50 μm.

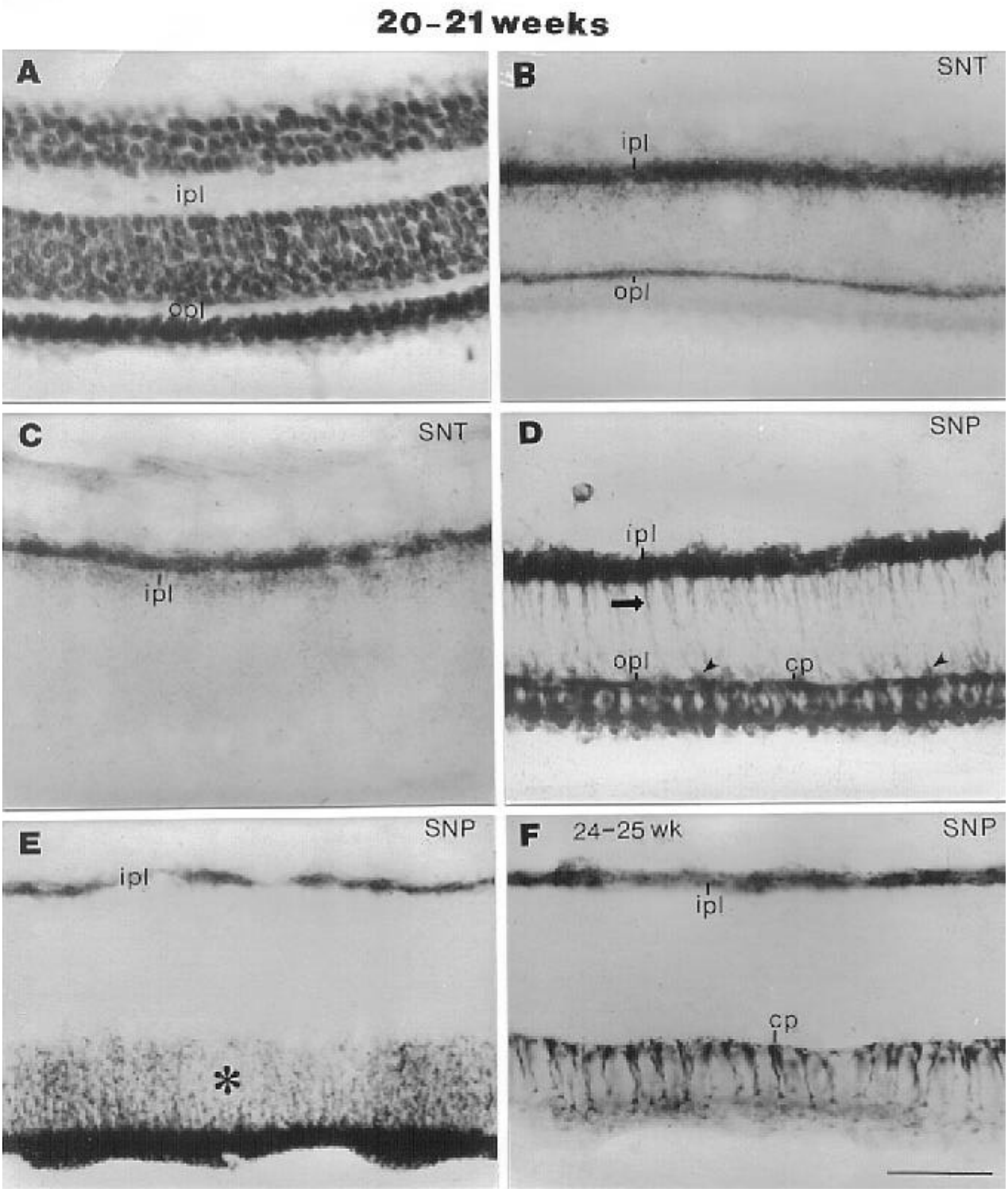

Figure 5. Photomicrographs showing SNT (syntaxin-1) and SNP (synaptophysin)-immunoreactivity in the fetal retinas at 20–21 weeks (**A–E**) and 24–25 weeks (**F**) of gestation. (**A**) Haematoxylin and eosin stained central retina at 20–21 weeks, showing a prominent OPL (opl). (**B, C**) Showing SNT-immunoreactivity in central (**B**) and peripheral (**C**) parts of retina. In both, the ipl is labelled, whereas in (**B**), the immunoreactivity is present in the opl also. Unlike SNP (cf. **D**), photoreceptor cell bodies and terminals are not labelled in the opl in (**B**). (**D**, **E**) SNP-immunoreactivity in central (**D**) and peripheral (**E**) parts of retina, respectively. Bipolar axons (arrow) and somata (arrowheads) are immunolabelled in (**D**). Fine granular staining of the photoreceptor processes (*) is seen in (**E**). Compare the thickness of the labelled ipl in both regions. (**F**) SNP-immunoreactivity in the 24–25 week-old retina. The ipl and cone pedicles (cp) are indicated. Scale bar = 50 μm.

central (distance: 4–5 mm eccentric from fovea) and midperipheral (9–10 mm eccentric from fovea) retinal regions, the photoreceptors were found to be syntaxin-immunoreactive in their ellipsoids (figure 6A, midperipheral) and rarely in terminals. The bulky cones at the midperiphery were more strongly immunolabelled (figure 6A) than the central, thin cones which showed a weak immunoreactivity (not shown). On the contrary, synaptophysin immunoreactivity was strongly present in all cones from central to peripheral retina, especially in their inner fibres and terminals (figure 6B, midperipheral).

3.8 *Immunoreactivity in adult retina (35 year-old)*

Syntaxin-1 was localized in the OPL as well as IPL (figure 6C). However, as in the postnatal retina, there was no labelling of the photoreceptor inner fibres and terminals. Weak immunoreactivity was localized in the amacrine cells and in some horizontal cell bodies lying close to the OPL.

Synaptophysin was present in the photoreceptor inner fibres, and terminals lying in the OPL (figure 6D, E). In the inner nuclear layer, some amacrine cells were weakly labelled, whilst neither bipolar nor horizontal cells were observed to be immunoreactive. The IPL was densely stained across its entire thickness.

No reaction was observed in the control sections omitted with the respective primary antibody treatment. Only one such instance is represented (figure 2F).

As only good fetal specimens were included in this study, the inter individual variation in the staining pattern for both synaptic proteins for the same developmental stage was little and negligible.

4. Discussion

Previous work has reported that syntaxin-1 is selectively present in immature amacrine and displaced amacrine cells, and in the IPL of both embryonic and adult rat retina (Barnstable *et al* 1985). In the OPL, syntaxin-1 immunoreactivity is reported to be absent (Barnstable *et al* 1985; Ullrich and Sudhof 1984) or of low intensity (Dhingra *et al* 1997). However, a recent study (Morgans *et al* 1996; see also Morgans 2000) has demonstrated syntaxin-3, an isoform of syntaxin, to be present in the OPL of rat retina. Our observation suggests that there is syntaxin-1 immunoreactivity in the OPL of developing and adult human retina. This is a species-specific variation and forms an interesting observation of this study. Besides, we found additional immunoreactivity in the nerve fibre layer, which is nonsynaptic, and in some ganglion and horizontal cells, hitherto unreported for any vertebrate retina, during early human retinal development (14–17 weeks of gestation). Syntaxin-1 was also expressed in the photoreceptors of fetal and infant retinas. It was never found in bipolar cells or their axons, which were clearly synaptophysin-positive in the fetal retina from early till midgestation. The horizontal cells, on the other hand, were never immunoreactive for synaptophysin. These data suggest at a glance differential distribution of syntaxin-1 and synaptophysin in the human retina, which is summarized in table 1.

An unexpected finding of this study was the presence of syntaxin-1 immunoreactivity along the preterminal ganglion cell axons and cone inner segments during development. It is somewhat difficult to explain the significance of the axonal localization of syntaxin, particularly since this protein is known to be predominantly localized at the presynaptic active zones (Bennett *et al* 1992). Although this pattern of localization may explain, in part, a high turnover and transport of this protein towards synaptic terminals during development, this need not be the only reason for the nonsynaptic presence of syntaxin-1. This localization pattern, however, is reminiscent of the abundant distribution of syntaxin-1 along the parallel fibres (axons of granule cells) of rat cerebellum (Koh *et al* 1993) and its predominant distribution to nonsynaptic portions of the axolemma in rat striatum and cortex (Sesack and Snyder 1995). It seems that syntaxin-1 plays roles other than vesicle docking and fusion at the active zones, which need to be considered.

During development of fetal retina, synaptophysin immunoreactivity was present in the immature neurons

Table 1. Distribution of syntaxin-1 and synaptophysin in the human retina.

Retinal layer	Syntaxin-1	Synaptophysin
Nerve fibre layer	++*	+*
Ganglion cell layer	++*	+*
Inner plexiform layer	+++	+++
Inner nuclear layer		
Amacrine cells	+	+
Bipolar cells and axons	–	++*
Horizontal cells	+	–
Outer plexiform layer		
Photoreceptor terminals	+[p]	+++
Horizontal axon terminals	+[p]	–
Outer nuclear layer	–	–
Photoreceptor layer		
Cell bodies	+[a]	+++
Inner fibres	+[a]	++

–, Absent; +, weak labelling; ++, moderate labelling; +++, strong labelling.
*Present during development.
[p]At presumed presynaptic sites.
[a]Not present in adult retina.

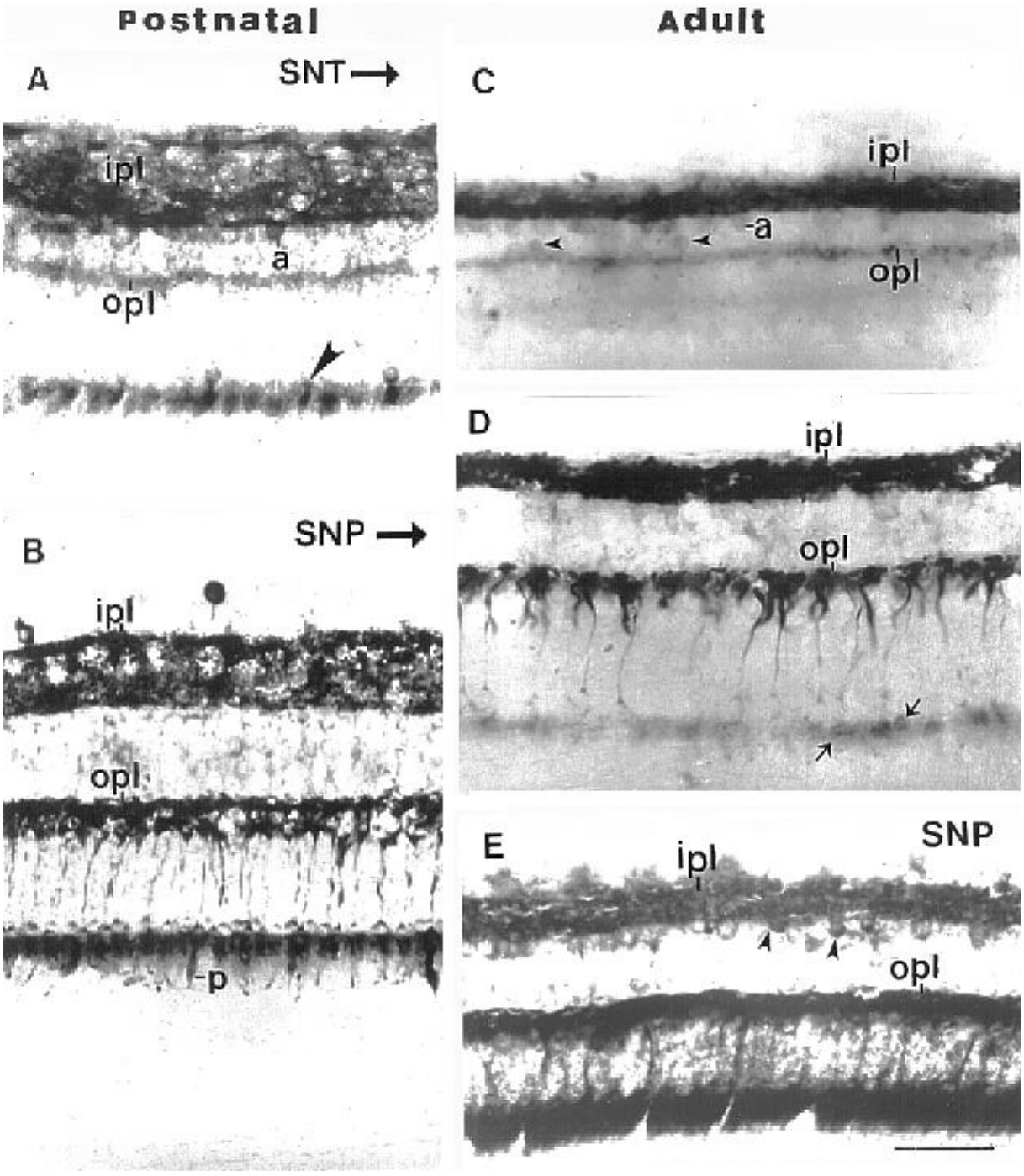

Figure 6. Photomicrographs of postnatal and adult retina, showing SNT (syntaxin-1) and SNP (synaptophysin)-immunoreactivity. (**A**, **B**) From the midperipheral part of retina (approximate distance: 8–9 mm eccentric from fovea) of the postnatal infant. In (**A**), SNT-immunoreactivity is strongly present in the IPL (ipl) and weakly in the OPL (opl). Note that the cone ellipsoids (arrowhead) are labelled. Some amacrine cell bodies (a) are visible. In (**B**), SNP-immunoreactivity is strongly present in both IPL and OPL and photoreceptor layer (p). (**C–E**) From the 35-year-old adult retina. (**C**) Showing SNT-immunoreactivity in the IPL and OPL. Note that a few horizontal cells (arrowheads) are labelled. No labelling is apparent in the photoreceptors (cf. **A**). a, amacrine cell. (**D**, **E**) Showing strong SNP-immunoreactivity in both IPL and OPL. On prolonged treatment in DAB (**E**), labelled amacrine cells (arrowheads) become conspicuous. The arrows in (**D**) indicate cone ellipsoids. Scale bar = 50 μm.

and their processes, located in the peripheral regions. This suggests that synaptophysin is expressed well-before synaptogenesis in the human retina. A similar situation has been reported for this protein in other regions of the CNS, e.g., cerebral cortex, striatum, cerebellum (Chun and Shatz 1983; Leclerc *et al* 1989; Ovtscharoff *et al* 1993; Voigt *et al* 1993) and for other synaptic vesicle proteins, especially synaptotagmin and SV_2 (Sarthy and Bacon 1985; Phelan and Gordon-Weeks 1992; Okada *et al* 1994).

However, it was not clear if syntaxin-1 also shares such a property, i.e., if it is expressed before synaptogenesis or not. In rat retina, it does not express in early embryonic days (E9–15), but begins expression from E17–18 onward in the amacrine cells (Barnstable *et al* 1985). This study, and our finding on syntaxin-1 expression in the immature neurons (amacrine and ganglion cells) of fetal retina during 11–14 weeks of gestation indicate that like synaptophysin, syntaxin-1 is an early protein and appears before synaptogenesis.

This study shows that both syntaxin-1 and synaptophysin appear in the plexiform layers in a centre-to-periphery gradient, which is likely due to the fact that synaptogenesis in the human retina proceeds in a centro-peripheral gradient, as reported for cat and monkey (Rapaport and Stone 1982; Maslim and Stone 1986; Okada *et al* 1992).

Electron microscopic studies have reported that synaptogenesis in the human retina commences at 11–12 weeks of gestation (Hollenberg and Spira 1972, 1973; Linberg and Fisher 1988, 1990). The authors found bipolar monads, and conventional synapses formed by amacrine cells in the central IPL at this stage; by 15 weeks of gestation, both types of synapses become numerous in this layer (Van Driel *et al* 1990). Immunohistochemically, both syntaxin-1 and synaptophysin were simultaneously expressed in the IPL at 11–12 weeks of gestation. This indicates that both proteins are incorporated into respective synaptic elements in parallel with morphological differentiation of synapses at this time point. Synaptophysin is presumed to be present in the synaptic vesicles of bipolar and amacrine cell terminals, whereas syntaxin-1 presumably occurs along the presynaptic membranes at chemical synapses. However, a recent study has shown that syntaxin-1 is associated with synaptic vesicles and even colocalized with synaptophysin in the rat brain (Kretzschmar *et al* 1996). It is not known whether such a situation occurs in retina also.

Whereas expression of both syntaxin-1 and synaptophysin in the IPL was prominent as early as 11–12 weeks of gestation, the same for both proteins in the OPL was totally different. Syntaxin-1 did not express at all in this layer, whilst synaptophysin was expressed in photoreceptor cell bodies and bipolar axons, but clearly not in a layered fashion. Linberg and Fisher (1990) reported the emergence of a thin OPL in the central retina by 11 weeks of gestation, which we could not confirm in our material. The OPL, however, becomes clearly visible by 16 weeks of gestation (Rhodes 1979). No reports are yet available on the synaptic development in this layer beyond 12 weeks of gestation, and therefore the contribution of different cellular elements to this process remains speculative. Interestingly, our immunolocalization study provides some information on the OPL development in the human retina. In the central retina, the differentiated photoreceptor terminals, which showed immunoreactivity to both proteins, were aligned in the OPL by 16–17 weeks of gestation. A differential distribution of both proteins was also evident; the horizontal cells which were tucked against this layer showed clearly syntaxin-1 immunoreactivity. Because these interneurons differentiate in the central retina by 16–17 weeks of gestation, as was evident by their selective labelling with calcium-binding proteins (Nag and Wadhwa 1996, 1999), it was interesting to find syntaxin-1 expression in them at this timepoint. It is thus probable that from this period onward, the horizontal cell processes approach the photoreceptor terminals to form synaptic triad with bipolar dendrites in the OPL. Examination of the late gestational retinas (20–25 weeks) has indicated synaptophysin immunoreactivity to be increased in photoreceptor inner segments and terminals, whilst syntaxin-1 immunoreactivity in the OPL appeared as a thin band of staining, but clearly, not in the photoreceptor inner segments and horizontal cells, as shown for 20–21 week-old retina. This immunolocalization pattern tends to suggest that by this time period, syntaxin-1 is transported to the photoreceptor and horizontal cell terminals.

It raises the vital question, then, as to which synaptic elements in the OPL are syntaxin-positive. Ullrich and Sudhof (1994) and Brandstätter *et al* (1996) have reported that the ribbon synapses of mammalian retina lack syntaxin-1, although another isoform, syntaxin-3, is reported to be present in these synapses (Morgans *et al* 1996; Morgans 2000). The antibody that we employed in this work was HPC-1 (Sigma), recognizing syntaxin-1, and it's low immunoreactivity pattern noted in the OPL tends to indicate that syntaxin-1 may be absent from ribbon synapses of human retina. Other studies have reported that syntaxin-1 is present along the presynaptic membranes at synapses (Bennett *et al* 1992; Sesack and Snyder 1995). In the human retina, the axon terminals from the type 1 horizontal cells are found to be presynaptic onto rod spherules within the invaginations (Linberg and Fisher 1988). Also, both clustered vesicles and electron density have been demonstrated on the presynaptic axon terminals of these horizontal cells. Because horizontal cells expressed syntaxin-1 in the developing as well as adult human retina, it is highly probable that their axon termi-

nals (of type 1 cells) at presynaptic sites contain syntaxin-1, which warrants confirmation by immunoelectron microscopy. Additionally, the cone pedicles, which are known to make conventional synapses with dendrites of flat bipolar cells (Missotten 1965; Dowling 1987) may contain syntaxin-1 along their basal (presynaptic) membranes. In this instance, it is worthwhile to mention that another synaptic protein, synaptotagmin, is reported to be present densely at conventional synapses along the basal membrane of cone pedicles in monkey retina (Koontz and Hendrickson 1993).

Clearly, the subcellular distribution of syntaxin-1 at the level of the OPL remains to be looked for in other mammalian retinas, especially by immunoelectron microscopy. Human retina is not suitable for such a study because of unavoidable postmortem changes. However, our light microscopic immunolocalization points to its possible occurrence along the presynaptic membranes of photoreceptors and axons of type 1 horizontal cells.

5. Conclusion

The present study reveals that both syntaxin-1 and synaptophysin are early proteins and appear before synaptogenesis in the human retina. The cellular distribution of both proteins differs widely, and this differential pattern suggests that mechanisms of synaptic vesicle exocytosis and neurotransmitter release may not be same among the different retinal neurons in human. The nonsynaptic localization of syntaxin-1 in the human retina points to the idea that this protein performs many more functions other than vesicle exocytosis at the active zones, which are at present largely unknown.

Acknowledgements

Financial support from the Council of Scientific and Industrial Research (CSIR), New Delhi, to SW is gratefully acknowledged. TCN was a recipient of CSIR Research Associateship.

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MS received 28 November 2000; accepted 19 February 2001

Corresponding editor: VERONICA RODRIGUES