Developmental expression of synaptophysin, synapsin I and syntaxin in the rat retina

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Abstract

Expression of synaptophysin, synapsin I and syntaxin was studied immunocytochemically in the developing rat retina using indirect immunoperoxidase technique. In the inner plexiform layer (IPL), syntaxin immunoreactivity appeared at postnatal day 1 (P1) whereas synaptophysin and synapsin I staining were first observed at P2. In the outer plexiform layer (OPL), synaptophysin appeared at P4, while synapsin I and syntaxin appeared at P8. In the case of synaptophysin, a punctate pattern of staining was observed from the time of its appearance (P4) in the OPL and from P12 onwards in the IPL. Synapsin I and syntaxin immunoreactivity in the OPL were of a low intensity throughout the development and in the adult stage. These findings are discussed in relation to synaptogenesis in the rat retina.

Keywords: Retina; Development; Synaptophysin; Synapsin I; Syntaxin; Immunocytochemistry; Rat

1. Introduction

The development of various types of synapses in the rat retina has been studied at the ultrastructural level [10,16]. The ribbon synapses have been first observed in the outer plexiform layer (OPL) at postnatal day 5 (P5) and in the inner plexiform layer (IPL) at P12/P13 whereas the conventional synapses appear in the IPL at P11/P12.

Rat retina expresses a number of synaptic proteins [15] which are implicated in various aspects of synaptic functioning [7]. Synaptophysin is an integral membrane protein of the synaptic vesicles and is expressed in all types of neurons and synapses in the adult vertebrate retina [15,18]. It has an important role to play in the neurotransmitter release from the synaptic vesicles by making an exocytotic fusion pore [7]. Similarly, synapsin I is a phosphoprotein associated with the cytoplasmic surface of the synaptic vesicle membrane and is thought to function by increasing the number of synaptic vesicles in the releasable pool [5]. Syntaxin, on the other hand, is an integral protein of the plasma membrane and is associated with synaptic vesicle docking and fusion [3]. Both synapsin I and syntaxin are reported to be expressed mainly by the amacrine cells which make conventional type of chemical synapses in the IPL [2,12].

Attempts have been made to correlate the development of retinal synapses with the appearance of various synaptic proteins in different animal species. The expression of synaptic ribbon specific antigen B16 in the mouse and synaptic vesicle protein SV2 in the monkey retinae are reported to be temporally correlated with the appearance of synapses [1,13]. However, the expression of synaptic vesicle proteins synapsin I and SV48 in the rat retina precedes the synaptic development [8,14]. The present study was carried out to investigate the ontogenic expression of three synaptic proteins, synaptophysin, synapsin I and syntaxin, in the rat retina. The findings are discussed in relation to rat retinal synaptogenesis.

2. Materials and methods

2.1. Animals and tissue preparation

Wistar rats of both sexes ranging in age from P0 to P15 and adults (10 weeks old) were used for the present work. The day of birth was taken as P0. Animals were anesthetized with sodium pentobarbital and perfused transcardially with phosphate-buffered saline (PBS, 0.1 M; pH 7.4) followed by 4% paraformaldehyde in phosphate buffer (0.1
M; pH 7.4). After perfusion, the eyeballs were dissected out, hemisected and post-fixed overnight at 4°C. The eye cups were rinsed thoroughly with phosphate buffer and kept overnight in 30% sucrose for cryopreservation. Sections of 10 μm thickness were cut from the midperipheral region of the eye cups lined with retina, using a cryostat (Jung, CM 3000) and collected on poly-L-lysine coated glass slides.

2.2. Antisera

The monoclonal antisera against synaptophysin and syntaxin were obtained from Sigma Chemicals (USA). The polyclonal antiserum against synapsin I was acquired from Signal Transduction Inc. (USA). The peroxidase conjugated secondary antibodies (IgG fraction, whole molecule) against rabbit and mouse, raised in goat and rabbit respectively, were also procured from Sigma Chemicals (USA).

2.3. Immunocytochemical procedure

The sections were permeabilized by Triton X-100 (0.1%) after which they were incubated in 3% hydrogen peroxide (H₂O₂) for 5–10 min to quench the endogenous peroxidase activity. To avoid non-specific staining, the sections

Fig. 1. Synaptophysin immunoreactivity in the developing rat retina at P1 (A), P2 (B), P3 (C), P4 (D), P8 (E), P12 (F) and adult retina (G). NBL, neuroblastic layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer; INL, inner nuclear layer; and GCL, ganglion cell layer. Synaptophysin appeared in the IPL at P2 (B) and in the OPL at P4 (D). Note the appearance of punctate staining in the OPL and in the IPL (small arrows in D and F). Stained soma in the NBL and GCL are shown by larger arrows in C and D. Scale bar = 50 μm.
were incubated for 1 h in non-immune sera. This included normal goat serum (NGS) for synapsin I and normal rabbit serum (NRS) for synaptophysin and syntaxin, both diluted 1:4 in 5% bovine serum albumin. Subsequently, they were incubated overnight at 4°C with the primary antibodies, diluted in PBS (0.1 M; pH 7.4). The dilutions used were 1:300 for synapsin I and 1:100 for synaptophysin and syntaxin. This was followed by 1 h incubation at room temperature in peroxidase conjugated secondary antibodies, namely, goat anti-rabbit IgG for synapsin I and rabbit

Fig. 2. Synapsin I expression in the developing rat retina at P1 (A), P2 (B), P3 (C), P4 (D), P7 (E), P8 (F) and adult retina (G). Synapsin I expressed in the IPL at P2 (B) and in the OPL at P8 (F). A strong perikaryal staining in the NBL and GCL in early postnatal stages is shown by arrows in B, C and D. Note the low intensity of staining in OPL even in the adult stage. See Fig. 1 for abbreviations. Scale bar = 50 μm.
anti-mouse IgG for synaptophysin and syntaxin, both diluted 1:4000 in PBS. Sections were washed after primary and secondary antibody incubations in PBS containing 0.01% Triton X-100 for $3 \times 5$ min. Finally, they were treated for 5 min with 0.06% 3,3'-diaminobenzidine hydrochloride in 0.05 M Tris-HCl (pH 7.6) containing 0.03%

Fig. 3. Syntaxin immunolabelling in the developing rat retina at P0 (A), P1 (B), P2 (C), P8 (D), and adult retina (E). Syntaxin staining appeared in the IPL at P1 (B) and in the OPL at P8 (D). Syntaxin stained soma in the NBL and GCL are shown by arrows in B, C and E. See Fig. 1 for abbreviations. Scale bar = 50 μm.
of the staining. In substitution controls primary antibody was replaced with non-immune serum.

3. Results

Synaptophysin was localized in the OPL as well as in the IPL, whereas synapsin I and syntaxin staining were mainly seen in the IPL. Control experiments carried out with normal rabbit or mouse sera confirmed the specificity of the staining.

3.1. Synaptophysin

Synaptophysin immunoreactivity was observed from P2 onwards in the IPL and from P4 onwards in the OPL (Fig. 1). The staining in the IPL was diffuse in the early developmental stages and increased in intensity during development. However, a punctate pattern of staining was observed in the IPL from P12 onwards; the discrete puncta of staining first appearing in the inner third region of the IPL (Fig. 1F). In the case of OPL, the staining was punctate right from the beginning (Fig. 1D). A light perikaryal staining was observed in the ganglion cell layer (GCL) and in the neuroblastic cell layer (NBL) close to IPL as well as in the inner portion of the outer nuclear layer (ONL) close to OPL, during the early postnatal stages.

3.2. Synapsin I

Synapsin I immunoreactivity, like that of synaptophysin, appeared in the IPL at P2 (Fig. 2B). The staining was non-punctate throughout the development. A few cell soma were distinctly stained in the NBL and the GCL adjacent to the IPL during early postnatal stages (Fig. 2B,C,D). In the case of OPL, a faint staining was observed from P8 onwards which remained weak in intensity throughout the development and even in the adult stage (Fig. 2F,G). No cell bodies were stained close to the OPL.

3.3. Syntaxin

The staining for syntaxin appeared at P1 in the IPL (Fig. 3B) and at P8 in the OPL (Fig. 3D). The pattern of staining was non-punctate both in the IPL and in the OPL as in the case of synapsin I. A few cell bodies in the NBL and GCL showed a light staining at various developmental stages and in the adult retina. The staining in the OPL was of low intensity and remained so throughout, including the adult stage (Fig. 3D,E), like that of synapsin I.

4. Discussion

We have investigated the developmental expression of three synaptic proteins in the rat retina, namely, synaptophysin, synapsin I and syntaxin. Synapse formation is at its peak in the OPL at P5 and in the IPL at P12 [10,16]. However, synaptic vesicles unaccompanied by pre- or postsynaptic specializations have been noticed in the IPL at a stage as early as P2 [10]. The present study demonstrates that in the IPL, the synaptic vesicle proteins, synaptophysin and synapsin I appear at P2 and the plasma membrane protein, syntaxin, appears at P1, whereas in the case of OPL, synaptophysin appears at P4 and the other two proteins at P8.

4.1. Synaptic proteins in IPL

A previous study [11] showed the absence of synaptophysin immunoreactivity in the IPL of rat retina until P12. However, we have observed this protein to be appearing in the IPL at a stage as early as P2. The difference can probably be attributed to the reduced staining sensitivity in the earlier study [11] caused by the use of rat adsorbed secondary antibody. The problem of non-specific staining in early developmental stages was addressed in the present work by using secondary antibodies at a very high dilution.

The staining was diffuse in the early postnatal stages. However, a punctate pattern of staining was observed in the IPL from P12 onwards. Since P12/P13 represents the time of synaptic development in the IPL of the rat retina, the punctate staining probably reflects the spurt of synaptogenesis occurring in this layer. Alternatively, this may represent the development of ribbon synapses only, since this pattern of staining was not observed in the case of synapsin I and syntaxin which are reported to stain only the conventional synapses [2,12,15]. The latter view is further supported by our finding that synaptophysin puncta appeared in the inner before the outer IPL, as has been observed in the case of synaptic ribbon antigen B16 in the mouse retina [1]. This finding also indicates that synaptophysin expresses in the synapses of the “on” pathway before that of the “off” pathway.

The immunoreactivity for synapsin I has been reported to be positive at P4 and negative at P0 in the IPL of the rat retina [8]. Since the stages between P0 and P4 were not investigated in this study, the day of first appearance of synapsin I could not be established. The present study shows that P2 represents the time of first appearance of synapsin I in the IPL, similar to that of synaptophysin.

The immunostaining for syntaxin in the IPL was first detected at P1 in the present study. However, Barnstable et al. have reported that this protein could be detected even at embryonic day 17 in the migrating amacrine cells of the rat retina [2]. The reasons for this discrepancy could possibly be attributed to the difference in the sensitivity of the methods.

An interesting finding in the present study was a difference in the time of appearance between the synaptic vesicle proteins and the plasma membrane protein in the IPL. The plasma membrane protein, syntaxin, appeared at P1, 1 day earlier than the synaptic vesicle proteins, synap-
topophysin and synapsin I. A synaptic vesicle protein, SV-48, has also been shown to appear at P2 in the IPL of the rat retina [14], similar to what we have observed for synaptophsin and synapsin I.

A somal staining was observed in the NBL and GCL close to IPL in the early postnatal stages, which probably represents the newly synthesized protein in the soma, as suggested earlier [8].

4.2. Synaptic proteins in OPL

The OPL which mainly comprises of photoreceptor ribbon synapses starts expressing synaptophsin at P4, as has been reported earlier [11]. This temporarily coincides with the expression of synaptic ribbon antigen B16 in the OPL of the mouse retina [1] and the development of ribbon synapses in the OPL of the rat retina [16]. Moreover, the staining pattern of synaptophsin in the OPL was punctate, like that of ribbon protein B16, from the time of its appearance supporting the view that punctate pattern of staining represents the sites of ribbon synapses.

Synapsin I, on the other hand, has been found to be absent in the ribbon synapses of the vertebrate retina [12]. However, a faint synapsin I staining has been observed in the OPL of the adult rat retina [8,12]. In our study, synapsin I appeared in the OPL at P8 and the intensity of the staining remained low even in the adult stage. The identity of the cells expressing synapsin I in the OPL is not yet established although they are presumed to be the horizontal or interplexiform cells [12].

The synaptophein immunoreactivity in the OPL during development was found to be similar to that of synapsin I, with regards to the intensity of staining and the time of its appearance. It is possible that both of these synaptic proteins are expressed by the same cell type in the OPL. It would be of great interest to establish the identity of these cells at the ultrastructural level especially because these proteins could be used as markers of this particular cell type at least in the in vivo rat retina.

In the present study, we have observed that various synaptic proteins are expressed well before the reported development of morphologically mature synapses in the IPL of the rat retina. It appears from our findings that the punctate pattern of staining in the case of synaptophsin represents and is well correlated with the development of ribbon synapses both in the IPL and in the OPL. Accordingly, we suggest that the conventional and not the ribbon synapses express various synaptic proteins before their actual morphological appearance.

The mechanisms which lead to synaptic formation and its maintenance are still unclear. The synaptic proteins which have a key role to play in synaptic transmission [7] and synaptic plasticity [4] could be important even in synaptogenesis. Synaptophsin and synapsin I are expressed even before the differentiation of axons in cultured hippocampal neurons [6]. Similarly, synapsin I is expressed in the striatal neurons in vitro before the synaptic contacts are made by them [17]. In fact, the overexpression of synapsins in the neuroblastoma cells has been shown to initiate the formation of synaptic vesicle clusters [9]. Our findings that synaptophsin, synapsin I and syntaxin appear in the conventional synapses of the rat retina before the actual development of these synapses are in accordance with the above reports. It would be of interest to examine the role of these proteins in the formation of synapses.

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References


