Synaptic Plasticity and Functionality at the Cone Terminal of the Developing Zebrafish Retina

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ABSTRACT: **Previous studies have analyzed photoreceptor development, some inner retina cell types, and specific neurotransmitters in the zebrafish retina. However, only minor attention has been paid to the morphology of the synaptic connection between photoreceptors and second order neurons even though it represents the transition from the light sensitive receptor to the neuronal network of the visual system. Here, we describe the appearance and differentiation of pre- and postsynaptic elements at cone synapses in the developing zebrafish retina together with the maturation of the directly connecting second order neurons and a dopaminergic third order feedback-neuron from the inner retina. Zebrafish larvae were examined at developmental stages from 2 to 7dpf (days postfertilization) and in the adult. Synaptic maturation at the photoreceptor terminals was examined with antibodies against synapse associated proteins. The appearance of synaptic plasticity at the so-called spinule-type synapses between cones and horizontal cells was assessed by electron microscopy, and the maturation of photoreceptor downstream connection was identified by immunocytochemistry for GluR4 (AMPA-type glutamate receptor subunit), protein kinase β_1 (mixed rod-cone bipolar cells), and tyrosine hydroxylase (dopaminergic interplexiform cells). We found that developing zebrafish retinas possess first synaptic structures at the cone terminal as early as 3.5dpf. Morphological maturation of these synapses at 3.5–4dpf, together with the presence of synapse associated proteins at 2.5dpf and the maturation of second order neurons by 5dpf, indicate functional synaptic connectivity and plasticity between the cones and their second order neurons already at 5dpf. However, the mere number of spinules and ribbons at 7dpf still remains below the adult values, indicating that synaptic functionality of the zebrafish retina is not entirely completed at this stage of development.** © 2003 Wiley Periodicals, Inc. J Neurobiol 56: 222–236, 2003

Keywords: **zebrafish; retina; development; photoreceptor synapse; synaptic plasticity; spinules**

INTRODUCTION

Developmental studies on the zebrafish retina have revealed a rapid morphological and functional maturation. Light sensitive photoreceptor outer segments appear already at 55hpf (hours postfertilization), and in the photoreceptor terminals the presence of presynaptic ribbon densities has been described at 60hpf (Schmitt and Dowling, 1999). This photoreceptor development is paralleled by the differentiation of the neurons in the inner retina, that is, the second order horizontal and bipolar cells around 50hpf and 60hpf, respectively, and third order amacrine cells around 60hpf (Schmitt and Dowling, 1999). In correspondence to these morphological findings, the first visual functions, such as the optokinetic response (Easter, Jr. and Nicola, 1997), and the first electroretinograms (ERGs) are detectable (Branchek, 1984) 3 days post-

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fertilization (dpf). However, only small ERG responses to high intensity stimuli can be generated at this developmental stage; in the further course of development retinal functions improve, and at 5dpf a clear ERG can be recorded (Saszik et al., 1999; Van Epps et al., 2001). By that time photoreceptors have developed sufficiently to allow reliable screening for visual behavior anomalies (Brockerhoff et al., 1995; Neuhauss et al., 1999), thus indicating a fully-fledged visual pathway. Besides these studies on retinal development, we know from the work of Connaughton and Nelson (2000) that the adult zebrafish retina contains at least 13 different bipolar cells (BC). Among these, 15% are OFF-BCs, which express exclusively AMPA/kainate-responses in patch-clamp experiments. Another 78% are ON-BCs expressing either a glutamate-gated conductance, a metabotropic APB-type response, or a combination of both. Morphologically, OFF-BCs generally terminate in sublamina a of the inner plexiform layer (IPL), whereas ON-BCs terminate in sublamina b (Famiglietti, Jr. et al., 1977). This classification is also true for the largest part of zebrafish BCs; however, there are several types of multistratified BCs that can be distinguished as ON- or OFF-type by the location of their axon terminals and by their axonal boutons throughout the sublamina of the IPL (Connaughton and Nelson, 2000). Nevertheless, so far only little is known about the development of synaptic connectivity, plasticity, and functionality at the first synapse between photoreceptors and their following second order neurons, the horizontal, and bipolar cells in the zebrafish.

In order to reveal the maturation of synaptic functionality at the photoreceptor synapse, we noted the appearance of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex and vesicle loading proteins at the presynaptic side in order to ascertain the synapses' ability to release neurotransmitter via vesicle fusion and subsequent exocytosis. SNARE complex proteins, like the synaptosomal-associated protein of 25kDA (SNAP-25) and syntaxin, are proteins that are involved in the fusion of transmitter-filled synaptic vesicles with the presynaptic terminal membrane (Calakos and Scheller, 1996) and have already been identified in the adult (Morgans et al., 1996; Brandstatter et al., 1996; Morgans, 2000) and developing (Greenlee et al., 2001) mammalian retina. In addition to the SNARE proteins, we also examined the synaptic vesicle protein SV2 (Feany et al., 1992) to document the transmitter loading of synaptic vesicles during zebrafish retinal development. Furthermore, we examined the formation of the so-called spinule-type synapses on the cone pedicles in the developing zebrafish retina. In the fish retina, spinules can be identified as transitory, finger-like protrusions of the horizontal cell (HC) dendrites invaginating into the cone pedicle laterally to its presynaptic ribbon. Spinules are highly plastic synaptic structures whose formation is triggered by ambient illumination as well as by endogenous factors (Wagner, 1980). They are formed during light adaptation and are persistent in the light, and they are retracted during dark adaptation and vanish in the dark. Pharmacologically, spinule formation is mediated by a decrease of glutamate (Glu) release from cones and by dopamine (DA) release from interplexiform cells (Kohler et al., 1990; Wagner and Djamgoz, 1993). Additionally, it has been shown in recent years that retinoic acid (RA) (Weiler et al., 1998), nitric oxide (NO) (Djamgoz et al., 2000), and nerve growth factor (NGF) (Haamedi et al., 2001) contribute to and/or trigger spinule formation. According to the crucial role of DA for spinule formation, we followed the development of dopaminergic interplexiform cells (ICs) as the source of the spinule triggering DA. ICs represent third order neurons that establish a feedback loop from the inner retina onto the photoreceptor synaptic complex.

It is generally accepted that photoreceptors use Glu as a neurotransmitter and that bipolar cells express non-NMDA receptors to receive that signal (Connaughton and Nelson, 2000). We therefore examined both the development of AMPA-type synapses as a sign of functionality of the glutamatergic synapse to the OFF-BCs, and the development of the mixed rod-cone bipolar cells (Mb-BCs, which never express AMPA-type synapses; Connaughton and Nelson, 2000), the main second order neurons in the fish retina ON-center pathway. Our data show that the ultrastructural maturation at the cone pedicle, accompanied by the development of synapse associated proteins and downstream second order neurons, enable first signal transmission at around 3dpf and virtually full synaptic functionality at 5dpf.

MATERIALS AND METHODS

Fixation

Experiments were carried out in accordance with the European Communities Council Directive for animal use in science (86/609/EEC). Fish were maintained and bred as previously described (Mullins et al., 1994) and kept under a 12:12 h light/dark cycle. Light adapted adult animals were sacrificed at noon and dark adapted adult animals 1 h after onset of darkness. Larvae were always sacrificed at exactly the same daytime: egglay usually takes place immediately after light onset; thus, larvae at the respective developmental stage were sacrificed shortly after light onset (light onset at 7 am, sacrifice between 7:30 and 8:00 am). Larvae at

"half day" developmental stages (e.g., 3.5dpf) were sacrificed between 4 and 5 pm. Earlier studies (Kohler et al., 1990) showed that the difference in the time of sacrifice between adult and larval animals does not affect the parameters (see below) examined in the present study. For the dark adaptation studies, larvae were dark adapted for at least 1 h before the above mentioned, scheduled time of sacrifice. In order to secure comparable developmental stages, all of the larvae in our experiments were examined macroscopically for body length before entering our observation procedures. Unusually small or large fish were sorted out. Prior to fixation, larvae were anesthetized on ice. For light microscopy they were then immediately fixed in 4% paraformaldehyde in 0.2 *M* phosphate buffer, pH 7.4 for 1 h (4°C). Larvae for electron microscopy (EM) were fixed overnight in 1% paraformaldehyde, 2.5% glutaraldehyde made in 0.1 *M* phosphate buffer.

Immunocytochemistry

Fixed larvae were cryoprotected in 30% sucrose for at least 4 h. Whole larvae were embedded in Cryomatrix (Tissue Freezing Medium; Jung-Leica, Nussloch, Germany) and rapidly frozen in liquid N_2; 10 μm thick sections were cut at −20°C, mounted on gelatinated slides, and air dried at 37°C for at least 2 h. Slides were stored at −20°C. Before further use, slides were thawed and washed three times in phosphate-buffered saline (PBS; 50 m*M*), pH 7.4, and treated with 20% normal goat serum (NGS), 2% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 (PBST) for 1 h. Sections were then incubated overnight in primary antibody in PBST at 4°C. Rabbit antiprotein kinase C (cPKCβ_1; Santa Cruz, Santa Cruz, CA) 1:1000, rabbit anti-GluR4 (Pharmingen, San Diego, CA) 1:50, and mouse antityrosine hydroxylase (TH; Diasorin, Stillwater, MN) 1:500 were used as primary antibodies for light microscopy, and the immunoreaction was visualized by biotinylated secondary antibody followed by ABC reagent (Avidin DH and biotinylated horseradish peroxidase; Vectastain ABC-Kit; Vector Laboratories, Burlingame, CA) and visualized using hydrogen peroxidase and diaminobenzidine terahydrochloride (DAB) with Ni enhancement. Mouse antisynaptic vesicle (SV2; kindly provided by D. S. Sakaguchi, Iowa State University) 1:100, rabbit antisynaptosomal-associated protein of 25 kDa (SNAP-25; StressGen, San Diego, CA) 1:300, and rabbit antisyntaxin3 (Alamone Labs, Jerusalem, Israel) 1:400 were used as primary antibodies for confocal microscopy, and the immunoreaction was visualized by using Alexa Fluor 488 antimouse or antirabbit IgG (Molecular Probes, Leiden, Netherlands) 1:500 as a secondary antibody. For all immunocytochemical experiments, negative controls were carried out in the same way but without using the first antibody. Slides were either viewed with an Olympus AX-70 light microscope (Olympus, Hamburg, Germany) or a Zeiss LSM 410 confocal microscope (Carl Zeiss, Jena, Germany). The obtained images were processed using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

EM

The EM-fixed larvae were washed in 0.1 *M* phosphate buffer (PB) for 2 h, then postfixed in 1% osmium tetroxide for 1 h 20 min. After rinsing in 0.1 *M* PB, specimens were dehydrated in a graded series of ethanol up to 70% ethanol and incubated in 2% uranyl acetate/70% ethanol at 4°C overnight. On the following day ethanol dehydration was continued. After preinfiltration in 1:1 100% ethanol/TAAB embedding resin (TAAB Laboratories, Aldermaston, UK), larvae were infiltrated in pure embedding resin overnight. Larvae were then positioned in fresh resin in Beem caps (Emitech, Ashford, UK) and the sample polymerized at 60°C for about 16 h. Ultrathin sagittal sections (40 nm) were prepared and stained with lead citrate. Sections were examined and photographed with a Zeiss EM 900 (Carl Zeiss) transmission electron microscope.

Analyses of Spinule Synapses

For spinule analysis, the spinule/ribbon (sp/rib) ratio was determined as the total number of spinules in a given pedicle divided by the number of ribbons in a given pedicle. Sections were taken from at least three retinas, each from different fish. In each retina, approximately 20 pedicles were analyzed. Spinules used for analysis in the present study satisfied the criteria for spinule formation in other fish species (Kohler and Weiler, 1990). Significance between all developmental stages for ribbons, spinules, and sp/rib ratio was determined by an analysis of variance followed by a Tukey's posthoc test performed in GB-Stat (Dynamic Microsystems, Silver Spring, MD) for light and dark adapted animals.

RESULTS

Expression of Presynaptic Terminal-Associated Proteins (SNARE and SV2) in Zebrafish Retinal Development

The presynaptic terminal-associated proteins of the SNARE-complex (Greenlee et al., 1996) together with the synaptic vesicle transmembrane transporter protein SV2 (Feany et al., 1992) are suitable proteins to ascertain potential functionality of synapses because they are imperative for successful neurotransmitter release at the chemical synapse. Therefore, we examined the developmental expression of these proteins in the zebrafish larvae.

SNARE Proteins. Two proteins of the SNARE complex were investigated: the SNAP-25, a SNARE protein that is associated both with conventional and ribbon type synapses, and syntaxin3, a SNARE protein exclusively associated with ribbon type synapses (Morgans et al., 1996).

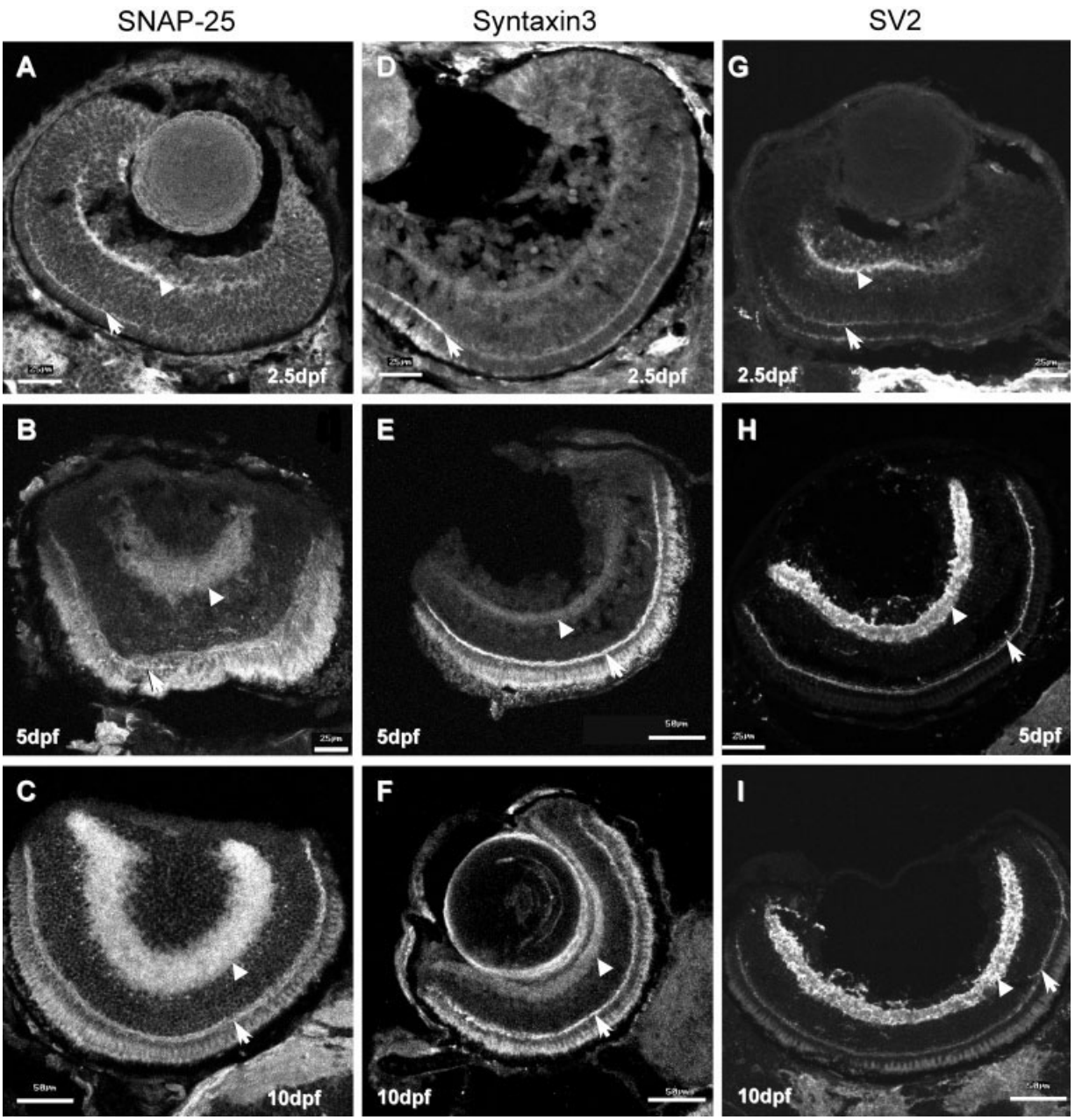

Figure 1 Expression of SNARE-complex proteins [SNAP-25 (A–C); syntaxin3 (D–F)] and synaptic vesicle protein [SV2; (G–I)] in the developing zebrafish retina (2.5 to 10dpf). (A–C) Labeling for SNAP-25 is first observed at 2.5dpf (A) in the OPL and IPL in the ventral patch of the retina. The labeling becomes more intense and the entire retina is labeled with ongoing development [5dpf (B); 10dpf (C)]. (D–F) Syntaxin3 appears first at 2.5dpf (D) in the ventral OPL but not in the IPL. At 5dpf (E), the OPL is strongly labeled and faint immunoreactivity is present in the IPL. (F) At 10dpf, both OPL and IPL express the same labeling intensity for syntaxin3. (G–I) SV2-labeling appears first at 2.5dpf (G) in the ventral patch of the IPL and OPL. The labeling strongly increases with ongoing development [5dpf (H); 10dpf (I)] and has spread over the entire retina by 5dpf (H). The photoreceptor outer segment labeling in (B,E,C,F,I) is nonspecific autofluorescence. Arrow, labeling in OPL; arrowhead, labeling in IPL. Scale bars: (A,B,D,G,H) = 25 μm; (C,E,F,I) = 50 μm.

SNAP-25 first appeared at 2.5dpf in ventral areas of the outer plexiform layer (OPL) and IPL [Fig. 1(A)]. With increasing development of the retina, the labeling became more prominent, and by 5dpf both the entire OPL and IPL expressed clear SNAP-25 labeling [Fig. 1(B)]. The labeling intensity increased

further by 10dpf [Fig. 1(C)] and was then not further different from the adult labeling (data not shown). Thus, the synaptic network can first become functional in the ventral region of the retina at 2.5dpf. In further development, transmitter release via SNAP-25-mediated processes also became possible in dorsal and peripheral parts of the plexiform layers, most probably chronologically linked to the development of the cells in the corresponding nuclear layers.

Syntaxin3 labeling was first observed at 2.5dpf in the ventral region of the OPL but not in the INL [Fig. 1(D)]. At 5dpf [Fig. 1(E)], the entire OPL expressed syntaxin3, whereas the IPL demonstrated only faint labeling. At 10dpf [Fig. 1(F)], like in the adult stage (data not shown), both plexiform layers were positive for syntaxin3. However, the labeling in the IPL appeared somewhat fainter than that in the OPL. Thus, the first ribbon-type synapses may release transmitter at the terminals of the initial cones at 2.5dpf in the ventral retina. The number of potential transmitter-releasing ribbon synapses in the OPL increases then with the developmentally increasing number of functional photoreceptors. In the IPL, potential functional ribbon synapses develop later. This may be due to the fact that bipolar cells are "switched on" only when the first photoreceptor signals run along the vertical signal pathway from the cones to the ganglion cells (between 3 and 5dpf).

SV2 Protein. The SV2 protein was identified as a transmembrane transporter that mediates the loading of neurotransmitters into synaptic vesicles (Feany et al., 1992). Therefore, we decided to use this protein as a marker for functioning vesicle loading and—together with the data from the SNARE-complex proteins—as an indicator of presynaptic maturity in the plexiform layers of the developing zebrafish retina. Similar to our findings for the SNARE component described above, SV2 immunoreactivity was first observed at 2.5dpf in the ventral part of the retina [Fig. 1(G)], where the labeling was clearly present in both IPL and OPL. In later developmental stages [5dpf, Fig. 1(H); 10dpf, Fig. 1(I)], the SV2 labeling had spread out to the dorsal and peripheral parts of the retina, and the OPL and IPL were brightly and entirely stained, just like in the adult stage (data not shown). Thus, we suggest that synaptic vesicle loading starts at 2.5dpf in the OPL and IPL of the ventral retina. Then, between 3 and 5dpf, similar to the SNARE protein development, SV2-mediated vesicle loading becomes possible in the other retinal regions.

Illumination Triggered Synaptic Plasticity

During light and dark adaptation, rod and cone photoreceptors undergo retinomotor movement, accompanied by melanin granule movement. Spinule formation is another common feature of light adaptation in most teleost fish. Spinule formation in the light, and spinule retraction in the dark, requires modulation of Glu release and signal transmission from the cones to their second order neurons and can therefore be used as a hallmark of synaptic functionality at the cone to HC synapse. The synaptic efficiency can easily be quantified by calculating the ratio between the number of postsynaptic spinules of the HCs and the number of presynaptic ribbons of the cone pedicles.

To assess normal pedicle ultrastructure, we first analyzed cone pedicles in the adult retina in light and in dark adapted conditions (Fig. 2). In the light adapted state [Fig. 2(A)], spinules with fingerlike structures and clearly identifiable membrane densities were abundant in the cone pedicle [6.40 $\pm$ 0.48 sp/ped (spinules per pedicle)], whereas spinules were rarely found in the dark adapted state [0.65 $\pm$ 0.18 sp/ped; Fig. 2(B)]. In both light and dark adapted cones, invaginating BC dendrites were observed in the center of the pedicle. Thus, the ultrastructure of the zebrafish cone pedicle during light and dark adaptation is identical with that observed in other teleosts like carp or goldfish (Wagner, 1980).

Maturation of Cone Pedicle Ultrastructure in Zebrafish Retinal Development. In the first developmental stages observed electron microscopically, at 2.5 and 3dpf [Fig. 3(A,B)], neither presynaptic ribbons nor postsynaptic spinules were identifiable in the tangential retinal sections. Ribbons and HC dendritic terminals lateral to the ribbons appeared for the first time at the level of the cone pedicles at 3.5dpf [Fig. 3(C)]. These early HC dendritic terminals did not yet show the mature ultrastructural morphology that characterizes HC spinules. The protrusions of the horizontal cell dendrites were more thumb- than fingerlike, and the membrane densities at the protrusion tips were not as striking as in the adult retina. With increasing development [4–7dpf, Fig. 3(D–F)], the number of ribbons and spinules rose gradually, and ultrastructural morphology became more and more similar to the adult. At 5dpf [Fig. 3(E)], three to five ribbons were present and up to six spinules had formed during light adaptation. Starting at 4dpf, the fingerlike structure and the characteristic membrane densities of the spinules were evident and were clearly more differentiated than at the earlier stages. At 7dpf [Fig. 3(F)], pedicle morphology was virtually identical to the

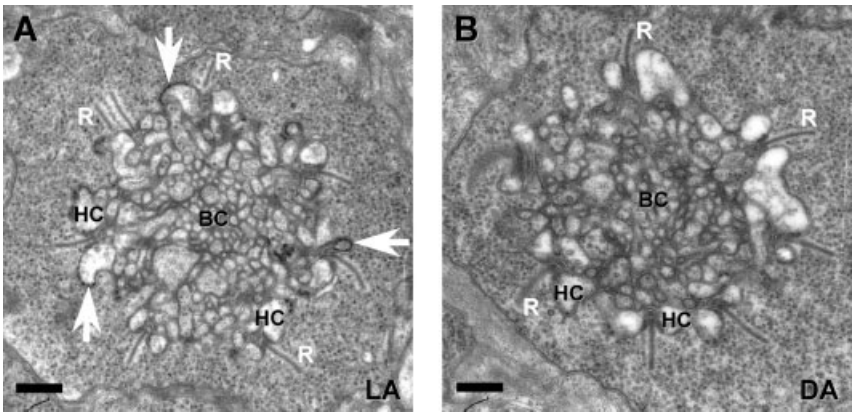

Figure 2 Electron micrographs of tangential sections of light and dark adapted cone pedicles from adult zebrafish. (A) Light adapted condition (LA): numerous ribbons (R) surrounded by vesicles are present in the cone pedicle. Spinules (arrows) on horizontal cell processes (HC) and bipolar cell dendrites (BC) invaginate the cone pedicle. Spinules are characterized by fingerlike protrusions with apical membrane densities. (B) Dark adapted condition (DA): ribbons (R) and vesicles are present in the cone pedicle, and invaginating HC and BC dendrites are observable. Few spinules are present on apical HC dendrites. Scale bars = 0.5 μm.

adult condition. Thus, the ultrastructure of the cone terminal and the spinules per ribbon ratio (sp/rib) were already very similar to the adult at the 5dpf developmental stage. However, the number of spinules and ribbons remained beyond the adult value. BC dendrites were identified due to their location, round shape in tangential sections, and lack of membrane densities. They were situated opposite to the arciform densities in the more central part of the pedicle and first appeared along with the first ribbons and HC spinules at 3.5dpf, and increased in number with ongoing development [Fig. 3(C–F)]. Thus the cone-BC synapse was established between 3 and 3.5dpf.

Ribbons Develop Prior to Spinules. The quantification of the synaptic structures in the cone pedicle revealed a significant ($p < 0.01$) initial increase of ribbons from a mean value of 1.0 $\pm$ 0.00 ribbon per pedicle (rib/ped) at 3.5dpf to 3.00 $\pm$ 0.15 rib/ped at 4dpf, thus suggesting an early developmental step in ribbon function at this stage of development [Fig. 4(A1)]. The number of ribbons then remained constant during further development. Only in the adult stage, the mean number of rib/ped was again significantly ($p < 0.01$) raised with 4.10 $\pm$ 0.26 rib/ped in comparison to 2.80 $\pm$ 0.16 rib/ped at the 7dpf stage.

Analysis of the number of HC spinules in pedicles at the same developmental stages revealed a later onset of spinule formation in comparison to the developmental appearance of ribbons described above [Fig. 4(B1)]. Only a few sp/ped could be identified at early stages (0.15 $\pm$ 0.08 sp/ped at 3.5dpf and 0.40 $\pm$ 0.15 sp/ped at 4dpf). Subsequently, between 4 and 5dpf, there was a significant ($p < 0.01$) increase in the mean number of spinules to 3.05 $\pm$ 0.21 sp/ped at 5dpf. The number of spinules remained constant at 7dpf, with 3.30 $\pm$ 0.25 sp/ped. Again, there was a significant ($p < 0.01$) increase in the number of sp/ped between the 7dpf and the adult stage, with 3.30 $\pm$ 0.25 sp/ped and 6.40 $\pm$ 0.48 sp/ped, respectively.

Onset of Synaptic Plasticity at the Cone Synapse. To assess the functionality of synaptic plasticity, we calculated the spinules per ribbon (sp/rib) ratio for each developmental stage examined in light (LA) and dark (DA) adapted animals [Fig. 4(C1,C2)]. In the adult zebrafish retina the sp/rib ratio in LA amounted to 1.63 $\pm$ 0.12 sp/rib, in DA to 0.12 $\pm$ 0.04 sp/rib. At the early stages in light adapted zebrafish, the sp/rib ratio was very low, with 0.15 $\pm$ 0.08 sp/rib at 3.5dpf and 0.12 $\pm$ 0.05 sp/rib at 4dpf [Fig. 4(C1)], that is, only 9% (3.5dpf) and 7% (4dpf) of the ratio in the adult fish. Therefore, even though the first spinule-type synapses were visible at these stages of development, the functional plasticity of the photoreceptor synapse had not yet evolved. Only 1 day later, at 5dpf, the sp/rib ratio had increased significantly ($p < 0.01$) to 0.98 $\pm$ 0.08 sp/rib [Fig. 4(C1)], 60% of the sp/rib ratio in the adult; that is, an increase of 53% in 24 h. We presume that this large increase in sp/rib ratio at 5dpf is very likely to be the onset of synaptic function at the cone terminal. In further development, at 7dpf, the sp/rib ratio (1.23 $\pm$ 0.10 sp/rib) gradually increased up to 75% of the sp/rib ratio in the adult. Finally, around 60dpf, the sp/rip ratio reached the adult level of 1.63 $\pm$ 0.12 sp/rib [Fig. 4(C1)]; this

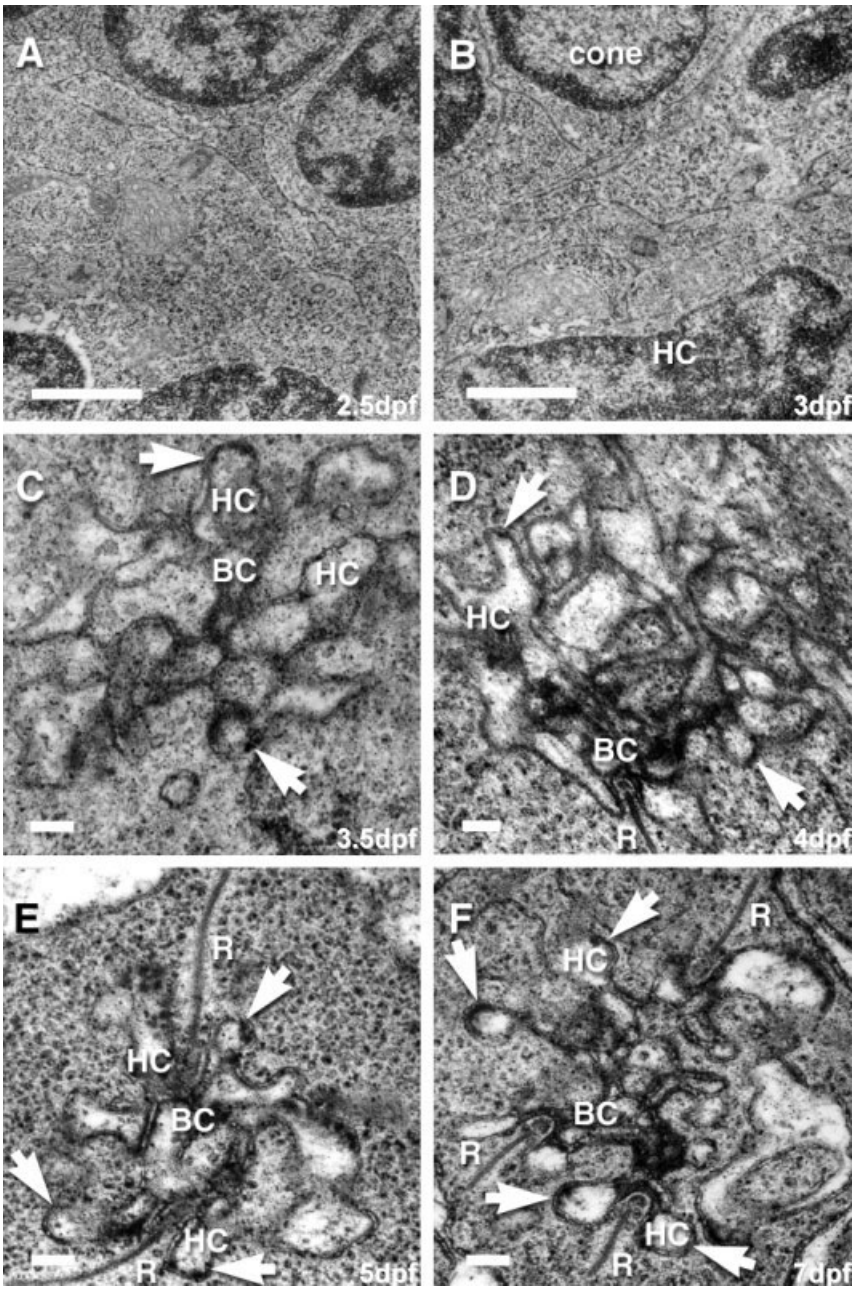

Figure 3 Electron micrographs of tangential sections of cone pedicles from light adapted zebrafish larvae between 2.5 and 7dpf. (A) 2.5dpf; (B) 3dpf; (C) 3.5dpf; (D) 4dpf; (E) 5dpf; (F) 7dpf. At 2.5 (A) and 3dpf (B) there are no synaptic structures observable in OPL tangential section. Beginning at 3.5dpf (C) classical synaptic structures like horizontal cell dendrites (HC) with clear membrane densities (arrows) as well as bipolar cell dendrites (BC) become visible. At 4dpf (D), ribbon synapses (R) and HC spinules (arrows) are clearly identifiable. With increasing development the number of spinules and ribbons in the cone pedicles rises (C–F). At 7dpf (F), spinule and ribbon ultrastructure is identical with the situation in the adult pedicle [see Fig. 2(A)]. Scale bar: (A,B) = 0.05 μm; (C–F) = 0.15 μm.

increase between 7dpf and the adult was significant by $p < 0.05$. One factor for this transition from the partial to the full synaptic connectivity and also for partial and full light adaptation between the 7dpf and the adult stage might be the fact that this time interval coincides with the transition from a cone dominated larval retina (at least until 12dpf; Branchek, 1984) to an adult retina, with both rods and cones. Recent data by Bilotta and

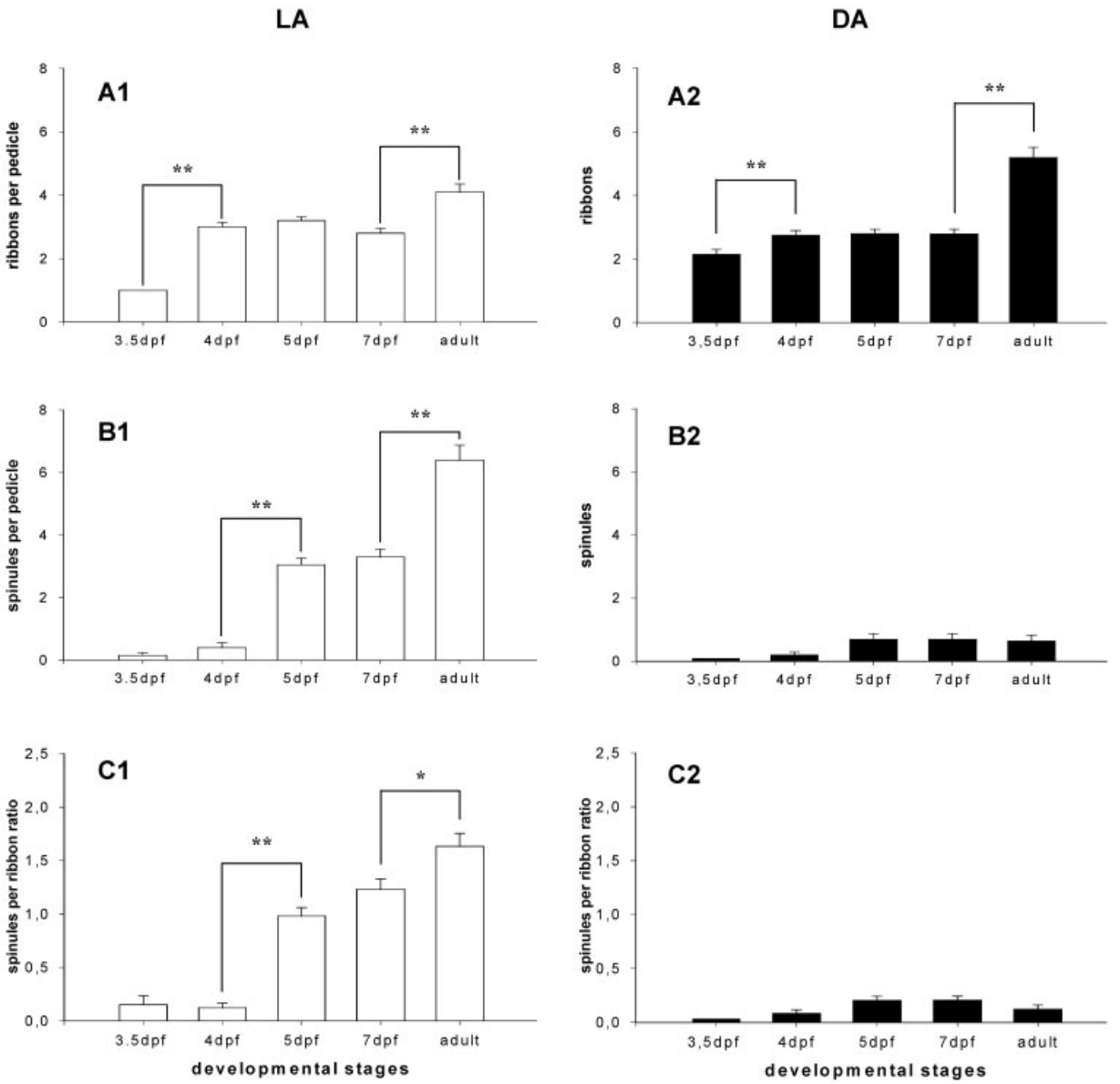

Figure 4 Development of synaptic components and their functionality/plasticity at the cone terminal. Significance was tested among all developmental stages in each histogram. For simplification and better overview, significant differences are only given in the graph if they occur between subsequent developmental stages (e.g., 3.5 to 4dpf in A1). ANOVA results and significances of the Tukey's posthoc test performed among all age groups are given in Table 1. Bars indicate mean number ± standard error of the mean. (A1–A2) Development of ribbons in the light (LA; A1) and in the dark adapted (DA; A2) retina. (B1–B2) Development of horizontal cell spinules in the light (B1) and dark adapted (B2) retina. (C1–C2) Spinules per ribbon ratio in the light (C1) and dark (C2) adapted retina in the different developmental stages. Significant differences between developmental changes are indicated by double asterisks (**) at $p < 0.01$, and a single asterisk (*) at $p < 0.05$.

colleagues (2001) strengthen this hypothesis. They report that the rod contribution to the ERG is still not adultlike in 29dpf old larvae. Thus, the increasing number of rods in the developing retina (>12dpf) may play a triggering role for synapse maturation.

At the early stages in dark adapted zebrafish, the sp/rib ratio was near to zero with 0.00 ± 0.00 sp/rib at 3.5dpf and 0.08 ± 0.03 sp/rib at 4dpf [Fig. 4(C2)]. Due to the immature functionality of the HC terminal dendrites prior to 4dpf, we were unable to calculate a sp/rib ratio prior to that state as the major increase in the number of spinules occurred only at 5dpf [see LA

Table 1 ANOVA Results and Significances of the Tukey's Posthoc Test Performed among all Age Groups

	Light Adapted (LA)					Dark Adapted (DA)				
Ribbons	3.5d	4d	5d	7d	Adult	3.5d	4d	5d	7d	Adult
3.5d	—	**	**	**	**	—	**	**	**	**
4d	**	—	—	—	**	**	—	—	—	**
5d	**	—	—	—	**	**	—	—	—	**
7d	**	—	—	—	**	**	—	—	—	**
Adult	**	**	**	**	—	**	**	**	**	—
ANOVA: $F(4, 25.64) = 50.54$, $p < 0.0001$						ANOVA: $F(4, 35.41) = 51.64$, $p < 0.0001$				
Spinules	3.5d	4d	5d	7d	Adult	3.5d	4d	5d	7d	Adult
3.5d	—	—	**	**	**	—	—	**	**	**
4d	—	—	**	**	**	—	—	—	*	—
5d	**	**	—	—	**	**	—	—	—	—
7d	**	**	—	—	**	**	*	—	—	—
Adult	**	**	**	**	—	**	—	—	—	—
ANOVA: $F(4, 129.79) = 88.51$, $p < 0.0001$						ANOVA: $F(4, 3.72) = 9.43$, $p < 0.0001$				
sp/rib ratio	3.5d	4d	5d	7d	Adult	3.5d	4d	5d	7d	Adult
3.5d	—	—	**	**	**	—	—	**	**	—
4d	—	—	**	**	**	—	—	—	—	—
5d	**	**	—	—	**	**	—	—	—	—
7d	**	**	—	—	*	**	—	—	—	—
Adult	**	**	**	*	—	—	—	—	—	—
ANOVA: $F(4, 8.94) = 57.64$, $p < 0.0001$						ANOVA: $F(4, 0.30) = 8.19$, $p < 0.0001$				

Significant differences between developmental changes are indicated by double asterisks (**) at $p < 0.01$, and a single asterisk (*) at $p < 0.05$.

stage; Fig. 4(B1)]. In the dark adapted retinas at the developmental stages of 5dpf (0.20 ± 0.04 sp/rib), 7dpf (0.21 ± 0.04 sp/rib), and adult (0.12 ± 0.04 sp/rib), the sp/rib ratio always remained in a range between 0 and 0.5 sp/rib, a range that is typical for dark adapted fish retinas (Wagner, 1980; Weiler et al., 1988a). Table 1 gives the ANOVA results and summarizes the significances of the Tukey's posthoc test performed among all age groups.

Influence of Interplexiform Cells on Cone Synaptic Plasticity in Early Retinal Development

We know from earlier studies that spinule formation in the fish retina is not only triggered by light, but also by dopamine secretion from the dopaminergic interplexiform amacrine cells (DA-IC) (Weiler et al., 1988b; Kohler et al., 1990; Kohler and Weiler, 1990; Kirsch et al., 1991; Wagner et al., 1992; Wagner and Djamgoz, 1993). As the presence of DA-ICs seems to be necessary for proper light adaptation, we identified these dopaminergic cells by tyrosine hydroxylase immunoreaction (TH-IR) in the developing zebrafish retina [Fig. 5(A–D)]. Immunoreactivity was first identified at 3.5dpf [Fig. 5(B)]. These labeled DA-IC somata in the inner nuclear layer (INL) were of a pear-shape, which is characteristic of immature cells (Becerra et al., 1994). Even though their soma shape was still immature, interplexiform connections from the IPL to the OPL were already present at this early stage of development, and thus, initiation of spinule formation by dopamine from DA-IC axonal processes in the OPL can possibly occur already at 3.5dpf. Furthermore, we observed clearly labeled DA-IC processes in sublayer 1 of the IPL at this stage of development. At 4dpf [Fig. 5(C)], DA-IC somata in the INL were more of round shape, thus suggesting the ongoing maturation of the cells. Axonal processes in the OPL as well as interplexiform processes were labeled at this stage and two labeled bands were found in the IPL, one in the apical part of sublamina a and the other in the distal part of sublayer 1. Furthermore, we identified diffuse TH-IR in the photoreceptor layer beginning at 4dpf. At 7dpf [Fig. 5(D)], the TH-IR in the photoreceptor layer persisted along with the labeling of the somata in the INL, the processes in the OPL and IPL, and the interplexiform connections. Considering these results at 4 and 5dpf, we assume that DA-IC morphology is already mature at 4dpf and, therefore, DA-ICs may enable spinule formation by dopamine secretion via their interplexiform processes in the OPL as early as 4dpf.

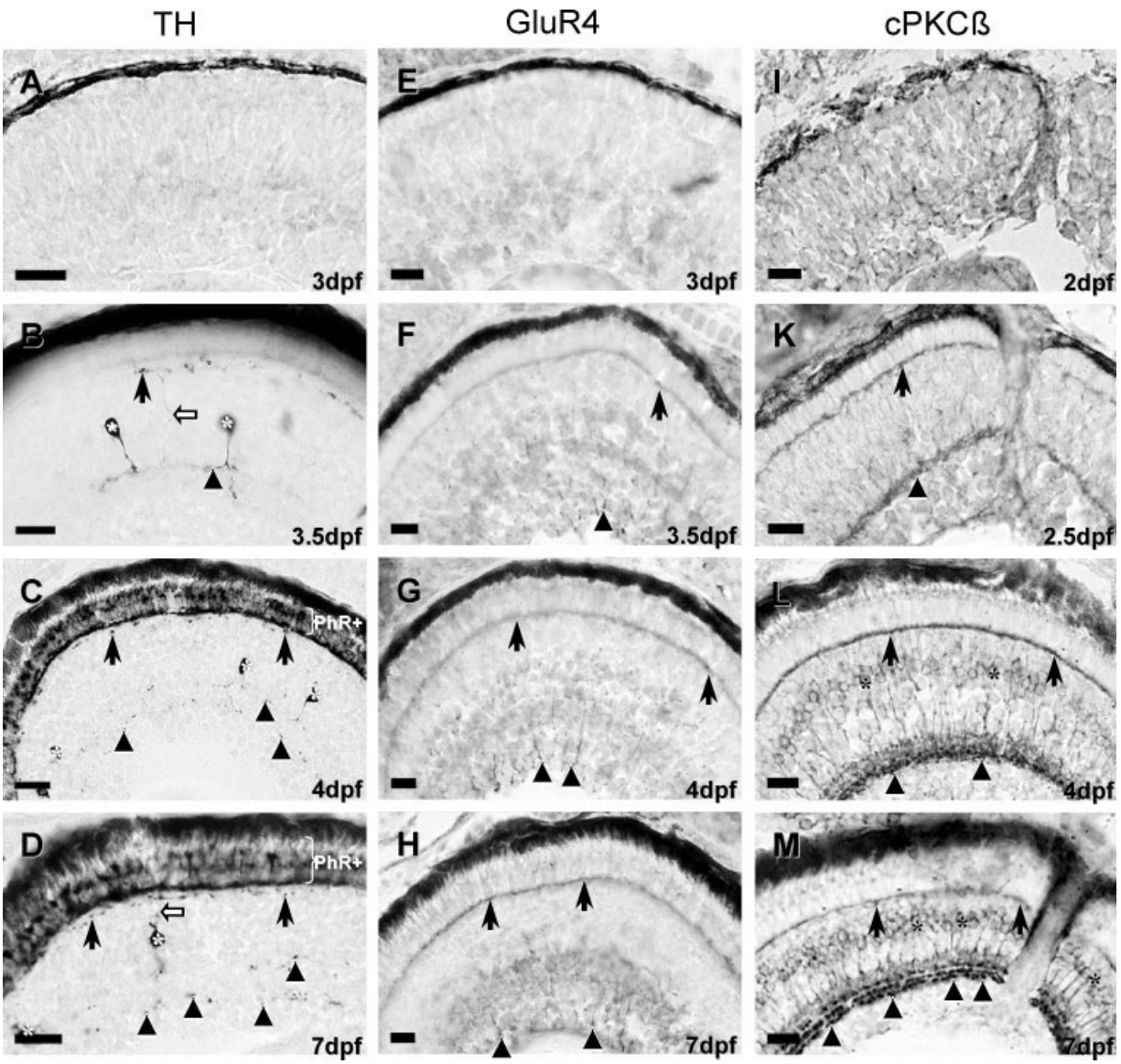

Figure 5 Differentiation of dopaminergic, interplexiform cells (DA-IC) expressing tyrosine hydroxylase [TH; (A–D)], AMPA-type glutamate receptors containing GluR4 (E–H), and Mb ON bipolar cells (Mb-BC) expressing cPKCβ_1 (I–M) in the developing zebrafish retina (2.5 to 7dpf). Scale bar: (A–M) = 10 μm. TH: the first, pear-shaped DA-ICs (asterisks) are present at 3.5dpf (B). They have already elaborated interplexiform processes (filled arrow) to the OPL. DA-IC processes in the OPL (arrow) as well as in the IPL (arrowhead) are present. (C–D) Labeling pattern of somata and plexiform layers becomes more intense with ongoing development. Additionally, diffuse labeling of TH is observable in the photoreceptor layer (PhR+) beyond 4dpf. GluR4: the first AMPA-type receptors are present in the OPL (arrow) and on Muller cell (MC) processes (arrowhead) at 3.5dpf (F). The expression of AMPA-type receptors intensifies with increasing development [4dpf (G); 7dpl (H)]. cPKCβ_1: the first Mb-BC processes are present in the OPL (arrows) and IPL (arrowheads) at 2.5dpf (K). At 4dpf (L), the labeling in the plexiform layers intensifies and Mb-BC somata (asterisks) are labeled as well. Mb-BC processes in the IPL start to ramify in sublamina b. At 7dpf (M), the same structures are labeled, and Mb-BC terminals in the IPL stratify to s3, s4, and s5 of sublamina b. As immunoreactivity of PKC appears 1 day earlier in development than that of TH and GluR4, 2 and 2.5dpf stages were used for the PKC series instead of 3 and 3.5dpf.

Delayed Development of AMPA-Type Glu Receptors on Off Bipolar Cell Dendrites

To assess the development of OFF-bipolar cell (OFF-BC) synapses in the OPL, we examined the developmental expression of GluR4-containing, AMPA-type Glu receptors during development [Fig. 5(E–H)]. These receptors have previously been identified on OFF-BC dendrites in the goldfish retina (Schultz et al., 1997).

AMPA receptor expression on OFF-BC dendrites first became observable at 3.5dpf [Fig. 5(F)]. At 4dpf, the labeling of the AMPA-type receptors had slightly increased [Fig. 5(G)], and, finally, at 7dpf [Fig. 5(H)], AMPA receptor labeling had become more distinct on the presumably OFF-BC dendrites in the OPL. Thus, OFF-BC dendrites in the OPL identified by AMPA-type Glu receptor expression are detectable later than the PKCβ_1-labeled Mb-BC dendrites in the OPL. Therefore, signal transmission to OFF-BCs via AMPA receptors is largely immature prior to 3.5dpf.

In addition to their location on OFF-BCs, AMPA-type Glu receptors have been identified on Muller cell (MC) endfeet in the goldfish retina (Schultz et al., 1997). We found an initial, faint labeling of the MC endfeet at 3.5dpf [Fig. 5(F)], which increased slightly by 4dpf [Fig. 5(G)]. At 7dpf [Fig. 5(H)], AMPA receptors were clearly labeled on the MC endfeet in the ganglion cell layer (GCL). Thus, in parallel to the situation observed in the OFF-BC dendrites, the first presumably functional AMPA receptors can be identified on MC radial endfeet at 3.5dpf.

Functionality of Mb-BCs

The population of the Mb-BCs represents a major part of the ON-pathway in fish retinas. To assess functional synaptic transmission from the cones to Mb-BCs, we identified this cell population by PKCβ_1-labeling [Fig. 5(I–M)], which specifically labels this type of BCs. At 2.5dpf [Fig. 5(K)], the first Mb-BC processes were labeled in the developing bands of the OPL and INL. Even though the processes were already labeled, no Mb-BC somata were observable at this stage. At 4dpf [Fig. 5(L)], Mb-BC dendrites in the OPL were intensely labeled as well as their somata in the INL. Furthermore, the labeled axonal processes in the IPL started to show stratification in sublamina b of the IPL. At 7dpf [Fig. 5(M)], the labeling of Mb-BC dendrites and somata remained unchanged compared to the 4dpf stage; however, within 3 days of development, the stratification in the IPL had become more precise and synaptic terminals were identifiable in s3, s4, and s5 of sublamina b.

Thus, we suggest the differentiation of Mb-BCs between 2.5 and 7dpf. Initial signal transmission may be possible as early as 2.5dpf, but full signal integration may not be possible prior to 7dpf, as the Mb-BC axon terminals only reach their targets in the substrata of sublamina b in the IPL at this stage and, thereby, make connections to ON-type ganglion (GC) cells to complete the vertical pathway from the cones to the GCs.

DISCUSSION

In this study we showed that the classical ultrastructural components at the cone terminal, namely cone synaptic ribbons, BC dendrites, and horizontal cell spinules, form very early in zebrafish retinal development. Within the first 5 days of development they reach morphological maturity, and, therefore, enable adaptational processes triggered by the ambient light, such as spinule formation and retraction. The appearance of transmitter-filled synaptic vesicles and SNARE-complex proteins in the OPL and IPL between 2.5 and 5dpf indicates the elaboration of synaptic functionality during this time interval. Furthermore, we document the maturation of the interplexiform amacrine cells in parallel time course to spinule development. The interplexiform processes of these cells are likely to be responsible for initial spinule formation at the 3.5dpf stage. Finally, we showed that one major population of the fish retina vertical signal pathway, the Mb-BCs, is fully developed by 5dpf and that the expression of AMPA-type Glu receptors at the apical dendrites of OFF-BCs in the OPL is also present at this stage. Therefore, signal transmission from the photoreceptors to second order neurons may start around 3.5dpf and seems to be functional and virtually mature at 5dpf. However, the mere number of spinules and ribbons at 7dpf still remains below the adult values indicating that synaptic functionality of the zebrafish retina is not entirely completed at this developmental stage.

Plasticity and Functionality of Spinule Type Synapses during Development

Spinules in Adult Zebrafish. Along with other teleost fish, adult zebrafish express horizontal cell spinule formation during light adaptation (Allwardt et al., 2001; this study) and spinule retraction during dark adaptation. Furthermore, the ultrastructure of zebrafish HC spinules (fingerlike structure, apical densities) is identical with spinule morphology in the goldfish and carp retina described in several studies (Wagner, 1980; Weiler et al., 1988a, 1991; Kohler et

al., 1990; Kohler and Weiler, 1990; Schmitz et al., 1995). In addition, sp/rib ratios in the adult zebrafish (LA = 1.63: DA = 0.12) are similar to the values analyzed in other fish, for example, the goldfish (LA = 1.88; DA = 0.05; Wagner, 1980). Thus, spinules in the adult zebrafish retina correspond not only morphologically, but also functionally with spinules in other fish species. However, the total number of ribbons and spinules per pedicle is reduced in the zebrafish, as 20 to 25 spinules amount to around 10 ribbons in the carp and goldfish retina (Weiler et al., 1991; Wagner and Djamgoz, 1993) and only six to eight spinules to around four ribbons in the zebrafish retina. Thus, even though the total number of spinules and ribbons per pedicle was reduced in the adult zebrafish retina, ultrastructural morphology as well as synaptic functionality and plasticity visualized by the sp/rib ratio were at the same level as in other fish.

Morphological and Functional Maturation of the Cone-HC Cell Synapse during Development. In the last 10 years several investigators have examined the development of zebrafish retinal morphology. Mostly, their studies have focused either predominantly (Easter, Jr. and Nicola, 1996; Schmitt and Dowling, 1996, 1999) or exclusively (Branchek and Bremiller, 1984; Kljavin, 1987; Raymond et al., 1995) on photoreceptor development. However, synaptogenesis at the cone synapse has only been reported twice so far: first, in an extensive study on retinal development by means of light and electron microscopy (Schmitt and Dowling, 1999), and second in a recent study in order to draw a comparison to ultrastructural synaptogenesis in a zebrafish mutant (Allwardt et al., 2001). Neither of the two focused exclusively on photoreceptor synapse maturation and plasticity and thus on the ability to transfer the visual signal from the light sensitive structure to the neuronal network during zebrafish retinal development.

Schmitt and Dowling (1999) reported that the first HC processes in the OPL can be found by 48hpf, followed by the formation of rod and cone synaptic terminals at 60hpf. Synaptic ribbons can be first identified in the ventronasal patch at 65hpf, then spread out to most retinal regions around 72hpf. However, they note that the ribbons observed at 72hpf are likely not functional at that developmental stage as many of them appear to float in the cytoplasm, unassociated with postsynaptic processes. In a more recent article, Allwardt and coworkers (2001) specified these findings on the ultrastructural maturation of the cone pedicle. They observed the first invaginating postsynaptic processes in cone terminals at ≈65hpf, followed by first precursors to the synaptic ribbons—so-called small round precursor bodies (srPBs) (Hermes et al., 1992)—in the cytoplasma of the cone pedicle at ≈67hpf. Shortly after, by 69hpf, these densities aggregate and build up the first, short ribbon synapses. Finally, by 75hpf, all terminals in the central retina appear mature (Allwardt et al., 2001).

These data from earlier publications suggest that the mature ultrastructure at the cone pedicle, including functional ribbons as well as BC and HC dendritic processes, is likely not present until developmental stages beyond 3dpf (≈72hpf). This is precisely what we found in our study, as we observed synaptic ribbons and initial, thumblike HC spinules only after 3.5dpf and later. In addition, we found first evidence for functional fusion of transmitter-filled synaptic vesicles with the presynaptic terminal membrane at the ventronasal patch just prior to that time of development (2.5dpf). This is exactly the time when first synaptic ribbons, dyads, and triads can be observed within the ventronasal patch (Schmitt and Dowling, 1999). Thus, we suggest that first signal transmission from cones to second order neurons becomes possible at this stage of development. Reports on zebrafish electrophysiology strengthen this hypothesis because the first ERGs could be measured at 3dpf (Branchek, 1984). Additionally, behavioral findings further this hypothesis as the visual startle response as well as the optokinetic nystagmus (OKN) can be first observed at this stage (Easter, Jr. and Nicola, 1996). Nevertheless, synaptic function at the cone pedicle remains immature at developmental stages prior to 5dpf as it is only at this stage, when HC dendritic terminals reach adult, fingerlike spinule ultrastructure and the sp/rib ratio exceeds 50% of the adult value, that synaptic transmission becomes largely functional. Our results on the SNARE proteins and the SV2 protein, which are present in the entire retina at 5dpf, further this hypothesis, as do behavioral results concerning the OKN, because, even though already measurable at 3dpf, the OKN can not be applied for reliable screening (more than 97% reliability of the test) prior to 5dpf (Brockerhoff et al., 1995). Thus, we suggest that spinule-type synapses differentiate between 3 and 5dpf. By 5dpf they may be largely functional even though the number of spinules and ribbons is still increasing at 5dpf.

We do not know which factors trigger this ongoing increase in the number of synaptic structures. One factor may be that the zebrafish retina remains cone-dominated at least up to 12dpf, when the first scotopic ERGs document the initial rod contributions to retinal function (Branchek, 1984). Recent work on rod contributions to the dark adapted spectral sensitivity function of the ERG b-wave component by Bilotta and colleagues (2001) showed that the zebrafish retina remains cone-dominated until 15dpf. Rod contribu-

tion then increases gradually, but is still not adultlike by 29dpf, the last developmental stage observed in their study. Thus, the integration of the rod terminals into the neuropil of the OPL and their connections to the cones may contribute to the final developmental processes at the cone synapse, and possibly the entire cone pathway.

Development of DA-ICs

DA-IC Influence on Spinule Formation and Development. What could be the trigger for initial spinule formation? It has been known for some time that dopamine from DA-ICs is one of the main triggers for spinule formation (Weiler et al., 1988a; Kohler and Weiler, 1990; Wagner and Djamgoz, 1993). Thus, the differentiation of these cells and the appearance of interplexiform processes in the OPL may trigger initial spinule formation during development. We found that DA-ICs are first present at 3.5dpf. Even though the DA-IC somata appear rather immature (pear-shaped) at this stage, interplexiform processes to the OPL can be easily identified, and, therefore, DA-IC may indeed trigger initial spinule formation by dopamine release via the latter processes.

DA-IC Development in the Zebrafish and Other Teleosts. In a recent study, Holzschuh and coworkers (2001) examined the development of catecholaminergic neurons in the developing zebrafish. They cloned cDNAs encoding TH and the dopamine transporter (DAT). Subsequently, they identified TH- and DAT-expressing cells in the developing zebrafish by *in situ* hybridization. The first retinal cells expressed TH RNA by 2.5dpf, whereas DAT RNA first became evident at 3dpf. Our immunocytochemical results suggest that the TH protein is present in DA-IC somata and processes at 3.5dpf, just 1 day after the beginning of TH RNA biosynthesis (Holzschuh et al., 2001).

So far, DA-IC development has only been closely observed in one other fish species, the brown trout (*Salmo trutta fario*) (Becerra et al., 1994). In parallel to our observations in the zebrafish, DA-IC in the trout can be first observed shortly after photoreceptors have begun to differentiate (16 mm stage in the trout; 3dpf stage in the zebrafish). In addition, these first immature DA-ICs in the brown trout have the same pear-shaped somata as the immature DA-ICs in the zebrafish retina. However, from the beginning of their appearance in the retina, maturing zebrafish DA-ICs express TH-labeling on their somata and on all of their processes whereas in the brown trout only somata and IPL processes are labeled in early retinal development.

These findings suggest that DA-IC differentiation in the zebrafish retina occurs very early in retinal development—between 2.5 and 3.5dpf—and that, even though the somata exhibit an immature morphology, all processes are already present by this stage, and, thus, signal integration via these neurons becomes possible at this early developmental stage. Ongoing development then leads to a growth in the number of DA-IC somata and processes and, therefore, may increase their importance as integrating neurons between the two IPLs and as a putative trigger for spinule formation.

Signal Transmission via Distinct Second Order Neurons

Developmental Expression of AMPA-Type Glu Receptors. AMPA-type Glu receptors play an important role in mediating light-induced synaptic transmission (Grunder et al., 2000). So far they have been located on BC dendrites in the OPL and on MC radial processes in the goldfish retina (Schultz et al., 1997) and in two specific groups of OFF-BCs in the zebrafish retina: (group a) OFF-BCs that express AMPA/kainate OFF-center responses and terminate exclusively in sublamina a of the IPL; and (group a/b) OFF-BCs that express similar AMPA/kainate OFF-center responses but differ morphologically because they are characterized by a single axon terminal in sublamina b and one or more axonal boutons ramifying throughout sublamina a (Connaughton and Nelson, 2000). We confirmed both of these results in the developing zebrafish. Even at 3.5dpf, when AMPA receptors were first observed in our study, they were already present on both presumptive OFF-BC dendrites in the OPL and MC radial processes. Therefore, beginning at this stage, functional AMPA receptors may contribute to the signal transmission via OFF-BCs. Recent experiments on 6–10dpf-old larvae (Connaughton, 2001) show that the larval BCs at these stages express exactly the same channel and current characteristics in whole-cell patch recordings as the respective BC-types in the adult zebrafish retina. Thus, these results strengthen our hypothesis of the early functional vertical signal pathway throughout the retina.

Development of Mb-BCs. Mb-BCs were first identified in the goldfish retina (Suzuki and Kaneko, 1990); they possess synaptic contacts to rods, cones, and HCs in the OPL and represent one of the major vertical pathways through the fish retina. However, the development of Mb-BCs in the fish retina has not been examined. In this study, we found that PKCβ_1-positive dendrites and terminal processes of Mb-BCs are present as early as 2.5dpf, and that the entire cell,

including the soma, is labeled by 4dpf, suggesting completed differentiation at this stage of development. As BC differentiation as well as cone outer segment (OS) formation only begin at 2.5dpf, we propose that signal transmission via Mb-BCs is rather unlikely at this stage. It could be at approximately 3dpf, when the first OS are present and first BC ribbon synapses begin to form in the IPL (Schmitt and Dowling, 1999), that Mb-BCs can conduct the first signals from cones to ganglion cells. The fact that the first measurable ERGs are found at about 3dpf (Branchek, 1984), and that 6–10dpf-old larvae already show the same channel and current characteristics as the respective BC-types in the adult zebrafish retina (Connaughton, 2001), further support this hypothesis.

In conclusion, our data suggest that the onset of signal transmission from cones to their second order neurons and their putative neuroreceptors first becomes possible at 3.5dpf, the approximate time of hatching. Then, synaptic ultrastructure and plasticity at the cone synapse as well as second order neuron morphology become largely differentiated over the next day and a half, thereby creating a largely differentiated and functional retina in the 5dpf zebrafish larvae. However, even though the cone synaptic structures are mature by 5dpf, the final step in development takes place between 7dpf and the adult stage. So far, we do not know which signals are necessary for this final step in synaptogenesis.

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