Expression of Class-3 Semaphorins and Their Receptors in the Neonatal and Adult Rat Retina

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PURPOSE. Semaphorins comprise a family of molecules that influence the growth and guidance of neuronal processes. Class-3 semaphorins are secreted proteins, and their effects are mediated by neuropilin (NP) and plexin (Plx) receptors. There is considerable information on mechanisms that influence axonal guidance and plasticity in the mammalian visual system, but a role for semaphorins has received less scrutiny. The purpose of the current study was to survey class-3 semaphorin and cognate receptor expression in young and adult rat retinas.

METHOD. The mRNA expression of five class-3 semaphorins (3A, 3B, 3C, 3E, and 3F) and receptor subtypes NP-1 and -2 and plexins A1 and A2 was determined, by using riboprobes and in situ hybridization on cryosections of newborn (postnatal day P0), juvenile (P14), and adult rat retinas. Retinal ganglion cells (RGCs) were identified by retrograde labeling after injection of a fluorescent tracer (Fluorogold, FG) into the superior colliculus. Hybridized sections were also immunostained to identify specific retinal cell classes.

RESULTS. mRNA expression for all five members of the class-3 semaphorin family was seen in adult FG-labeled RGCs. Qualitatively, expression was highest for semaphorins 3B and 3C, and lowest for 3A. Levels of mRNA expression in RGCs were lower in newborn retinas but were adult-like by P14. Expression by different cell types in the inner nuclear layer was also seen, especially at P14. Expression of NP-2 and PlxA2 mRNAs was evident in developing inner nuclear and ganglion cell layers at birth. Expression increased postnatally and was maintained into adulthood. NP-1 and PlxA1 expression was also present, but at comparatively lower levels.

CONCLUSIONS. The presence of class-3 semaphorins and their receptors in neonatal and adult rat retina suggests a potential role for these proteins in retinal development and in the maturation, stabilization, and plasticity of mammalian primary visual pathways. (Invest Ophthalmol Vis Sci. 2004;45: 4554 – 4562) DOI:10.1167/iovs.04-0173

Semaphorins constitute a large family of secreted and membrane-associated proteins. They bind and signal through receptor complexes consisting of members of the neuropilin and plexin families and can also signal through other protein moieties, including L1, Off-track, and integrins. Originally characterized as axonal growth repellants during neural development, these proteins are now implicated in a much wider range of activities, including cell migration, cell death, angiogenesis, and immune system function. Maintenance of expression of semaphorins and their receptors in the adult nervous system, and changes in expression after injury, suggest additional roles for the proteins in plasticity and regeneration.

The rodent visual system is an excellent model in which to study how neural connections are established and organized during pre- and postnatal development. The retinofugal pathway has also been extensively used in studies on degeneration and regeneration in the adult animal. There are many types of molecules, acting at different times and in different combinations, that influence the growth and guidance of retinal ganglion cell (RGC) axons and their growth cones and eventually direct the patterned innervation of central visual target sites. These molecules include extracellular matrix molecules, such as laminin, chondroitin sulfate proteoglycans, and tenascin-R, and adhesion molecules, such as L1, netrins, slit proteins, and the Eph family of receptor tyrosine kinases and their ligands, the ephrins.

Despite the extensive body of knowledge indicating the involvement of semaphorins in a variety of central and peripheral pathways, there are comparatively few studies that have focused on a possible role for semaphorins and their receptors in the developing and mature visual system. Are they involved in central axonal guidance and circuit formation during development and the maintenance/stabilization of synaptic connections within the retina and in retinorecipient areas in the mature brain? Of the studies that have been published, most have focused on the secreted class-3 semaphorins—in particular, semaphorin-3A. Semaphorin-3E may influence the guidance of RGC axons within chick retina and semaphorin-3D is involved in guidance of RGC axons along the dorsoventral axis of the zebrafish tectum. An ensheathing role for the transmembrane semaphorin 5A during optic nerve development has also been reported, and this semaphorin may also inhibit RGC axon growth. In the present study, we used riboprobes and in situ hybridization techniques to survey, in neonatal and adult rat retinas, the mRNA expression of five members of the class-3 semaphorin family (semaphorin-3A, -3B, -3C, -3E, and -3F) as well as members of the neuropilin (NP-1 and -2) and plexin (PlxA1 and -A2) receptor families. Retinal cell types expressing these mRNAs were characterized by combining in situ hybridization with immunohistochemistry. To confirm semaphorin expression in RGCs in adult rats, projecting neurons were labeled with a fluorescent retrograde tracer (FG; Fluorogold; Fluorochrome, Denver, CO) after injection of the tracer into contralateral superior colliculus.
**METHODS**

**Animals**

Neonatal, juvenile, or adult (200–220 g body weight) Wistar rats were purchased either from Harlan (Zeist, The Netherlands), or from the Animal Resources Centre (Perth, WA, Australia). Rats were kept under standard animal house conditions with free access to food and water. Experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were conducted with permission of the University of Western Australia (UWA) Animal Ethics Committee and the Animal Care Committee of the Royal Netherlands Academy of Science.

**Processing of Retinal Tissue**

For initial in situ hybridization studies, neonatal, juvenile, or adult rats were deeply anesthetized (pentobarbital, intraperitoneally) and perfused with a saline wash followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Animals were perfused at the following ages: postnatal day (P)0 (day of birth, n = 5), P2 (n = 2), P7 (n = 1), P14 (n = 5), and adult (n = 5). Neonates were perfused through a 26-gauge needle attached to a 10-mL syringe. Eyes were enucleated, and the cornea and lens were removed and postfixed in the same fixative for 50 to 35 minutes. Retinas were left within the eyecup and stored in buffer at 4°C. Eyes were cryoprotected through increasing osmolality, as described earlier.

**In Situ Hybridization**

For retrograde labeling and identification of RGCs, the left superior colliculus (SC) of three adult Wistar rats was injected with FG (6% in dH2O). Rats were anesthetized with 2,2,2-tribromoethanol (intraperitoneally) and perfused with a saline wash followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Animals were perfused at the following ages: postnatal day (P)0 (day of birth, n = 5), P2 (n = 2), P7 (n = 1), P14 (n = 5), and adult (n = 5). Neonates were perfused through a 26-gauge needle attached to a 10-mL syringe. Eyes were enucleated, and the cornea and lens were removed and postfixed in the same fixative for 30 to 35 minutes. Retinas were left within the eyecup and stored in buffer at 4°C. Eyes were cryoprotected through increasing concentrations of optimal cutting temperature compound (Tissue Tek; Miles Inc., Elkhart, IN). Cryostat sections (20 μm thick) were cut through the eyecup in two planes: from dorsal to ventral, with each section containing a complete temporal-to-nasal cross-section of the retina, or from nasal to temporal, and in this case each section contained a complete dorsoventral retinal cross section. Parallel series of sections were thaw mounted onto slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) and stored at −20°C until processed. Every second or third section was mounted. In most cases, approximately 9 to 10 slides were obtained per eye, each slide containing 10 to 12 sections in series across the whole retina. Fewer sections were obtained from the neonatal eyes.

**Fluorescence Labeling**

For retrograde labeling and identification of RGCs, the left superior colliculus (SC) of three adult Wistar rats was injected with FG (6% in dH2O). Rats were anesthetized with 2,2,2-tribromoethanol (intraperitoneally, 250 mg/kg) and placed in a stereotactic holder. A small incision of the left caudal hemisphere was aspirated to reveal the underlying SC. Approximately 1 μL FG was then injected directly into the superficial SC in two to three sites via a pulled-glass micropipette attached to a 100-μL syringe (Hamilton, Reno, NV). Rats were perfused with saline and 4% paraformaldehyde, as described earlier, 3 days after surgery. Eyes were removed and retinas were cut and prepared for in situ hybridization, as described earlier.

**In Situ Hybridization**

Sections were postfixed in 4% paraformaldehyde in diethyl pyrocatechol (DEPC)–treated and autoclaved phosphate-buffered saline (PBS; pH 7.4) for 20 minutes, and washed three times for 5 minutes each in PBS followed by a rinse in H2O. Sections were then acetylated (10 minutes) in 0.25% triethanolamine (TEA; 1 mL in 100 mL H2O) containing 250 μL acetic anhydride. After two washes for 5 minutes each in PBS and a 5-minute wash in 20× SSC, sections were hybridized. Digoxigenin (DIG; Roche Diagnostics, Mannheim, Germany)–labeled cRNA probes were generated by in vitro transcription with T7 RNA polymerase (Roche Diagnostics), using linearized full-length templates18; semaphorin-3A (rat cDNA)19; semaphorin-3B, -3C, -3E, and -3F; NP-2; and PlxAX and -A2 (mouse cDNA, a gift from Andreas W. Pušchel, Institut für Allgemeine Zoologie und Genetik, Westfälische Wilhelms-Universität Münster, Münster, Germany; NP-1 rat cDNA, a gift from Alex L. Kolodkin, Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD). Sense probes were also used as the control (e.g., Fig. 2M).

For hybridization, 200 ng/mL of each cRNA probe was added to the hybridization solution (on ice). The hybridization solution comprised 5 mL of 50× Denhardt’s (5 g Ficoll, 5 g polyvinylpyrrolidone, and 5 g bovine serum albumin [BSA] in 500 mL H2O), 125 μL 100 mg/mL baker’s yeast tRNA, 12.5 mL 20× SSC, and 25 mL formamide, made up to 50 mL with dH2O. The probe was denatured in this hybridization solution (5 minutes at 85°C) and cooled on ice, and 150 μL of the probe solution was placed on each section and the slide covered (Nescomfilm; Karlan Research Products Corp., Santa Rosa, CA). Slides were hybridized at 60°C in a humidified chamber.

After hybridization, sections were washed for 5 minutes in 5× SSC at 60°C. One minute in 2× SSC at 60°C, 30 minutes in 0.2× SSC in 50% formamide at 60°C, 5 minutes in 0.2× SSC at room temperature, and 5 minutes in 0.1 M Tris buffer at room temperature. Sections were then immersed for 1 hour in 1% blocking reagent in 0.1 M Tris buffer, washed in the same buffer, and incubated for 1.5 hours in 1:3000 anti-DIG-AP-Fab in buffer (400 μL/slide) in a humidified chamber. After two 15-minute washes in 0.1 M Tris buffer, sections were washed (5 minutes) in 0.1 M Tris buffer containing 5 mM MgCl2. For color reaction, sections were incubated for 24 to 48 hours in a dark humidified chamber in Tris/MgCl2 buffer (400 μL/slide) containing nitroblue tetrazolium (NBT) solution (stock, 75 mg/mL NBT in 70% dimethylformamide), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (50 mg/mL BCIP-phosphate in 100% formamide), and levamisole (0.24 mg/mL). The reaction was stopped by washing sections (10 minutes) in 0.1 M Tris buffer containing 10 mM EDTA and sections were coverslipped with an aqueous mounting medium.

**Immunohistochemistry: Double-Labeling Protocol**

To begin to characterize the types of retinal cell that express semaphorins, retinal sections from P14 rats that had been hybridized for semaphorin-3B were subsequently immunostained with a variety of antibodies. After in situ hybridization, the sections were stored in PBS overnight at 4°C. All antibodies were diluted in a solution consisting of 1% BSA, 0.2% Triton X-100 in PBS. Antibodies used were raised against: neuronal class βIII tubulin (clone TUJ1, 1,500 dilution; Covance, Vienna, VA), calbindin D-28K (1:1000 dilution; Sigma-Aldrich, Castle Hill, Australia), parvalbumin (PV-28, 1:1000 dilution; Swant Immunoclonics, Bellinzona, Switzerland), or protein kinase Cα (PKCα, 1:300 dilution; BD Transduction Laboratories, Lexington, KY). Some P14 retinal sections hybridized for semaphorin-3A and some adult retinal sections hybridized for semaphorin-3B were also immunostained for βIII tubulin. Sections were incubated with primary antibodies overnight at 4°C and washed three times for 5 minutes each with PBS. Secondary antibodies were then applied (anti-mouse FITC, 1:100; ICN, Aurora, OH; or anti-rabbit FITC, 1:200; Sigma-Aldrich) and incubated for 2 hours at room temperature. Sections were again washed three times for 5 minutes each with PBS before they were coverslipped (Citifluor; Agar Scientific Ltd., Stansted, UK).

**Cell Measurements in the GCL**

Sections from two P14 and one adult retina hybridized for either semaphorin-3A or -3B were subject to further analysis. Using an image-analysis computer program (Image Pro Express; Media Cybernetics, Silver Spring, MD) regions of the ganglion cell layer (GCL) were randomly sampled across the entire retina, and the diameter of hybridized cells and the number of labeled cells per millimeter length of retinal section were determined.

**RESULTS**

**Expression of Semaphorins**

Representative sections from newborn (P0), P14, and adult retinas hybridized with semaphorin-3 riboprobes are presented.
in Figure 1. Labeling in P2 retinas was essentially similar to that at birth. All five members of the class-3 semaphorin family were expressed in rat retina; however, levels of expression differed between subtypes, and clear maturational changes were seen. Expression was consistently most intense in the GCL, with lower levels of expression in the inner nuclear layer (INL), although occasional cells displayed high levels of expression of some semaphorins. There was little or no consistent semaphorin-3 signal in the receptor or outer nuclear layer (ONL), and there did not appear to be specific label in the pigment epithelium. At all ages studied, we did not detect an obvious gradient of mRNA expression for any members of the semaphorin-3 family across the nasotemporal or dorsoventral axes of the retina.

**Semaphorin-3A.** At all postnatal ages studied, semaphorin-3A mRNA expression was the least intense of all the semaphorin-3 subtypes examined in the rat retina (Figs. 1A–C, 4A). Expression was almost completely confined to the GCL and was more intense at P14 than at birth. Low levels of semaphorin-3A mRNA expression were seen in numerous cells in the GCL in adulthood (Figs. 1C, 3A, 4C).

**Semaphorin-3B.** Overall, the hybridization signal for semaphorin-3B was the highest of any of the class-3 semaphorins (Figs. 1D–F, 4B, 4C). Numerous cells in the developing GCL expressed low levels of semaphorin-3B mRNA at birth. There also appeared to be some hybridization in the neuroblastic layer (NBL). Staining intensity was slightly greater at P7 (not shown) and by P14 a large number of intensely labeled cells was visible in the GCL. At this age, hybridized cells were clearly evident in the INL, especially at the inner margins but also to a lesser extent toward the outer margins of this layer (Fig. 1E). In adult rat retinas, compared with P14 retinas, the intensity of expression appeared slightly lower, but there was still substantial semaphorin-3B expression in most of the cells in the GCL. mRNA expression in the INL was considerably reduced in adult eyes (Fig. 1F; cf. Figs. 3B, 4A, 4B).

**Semaphorin-3C.** In P0 retinas, cells in the GCL were hybridized for semaphorin-3C mRNA (Fig. 1G). The level of expression in individual cells was higher at P14 and this was maintained into adulthood, with many cells in the GCL expressing mRNA for this semaphorin (Figs. 1H, 1I). Some hybridization signal was evident in the INL at P14, but this expression was only infrequently seen in adult retina.

**Semaphorin-3E.** The pattern of expression of semaphorin-3E mRNA was similar to that of semaphorin-3B and -3C although the level of expression was comparatively less in the adult (Figs. 1J–L). Again, note the stronger hybridization in the INL at P14 (Fig. 1K) compared with the adult retina (Fig. 1L), although in the adult there may be some specific hybridization signal in the region of the outer plexiform layer (OPL). Similar to semaphorin-3B, at P14 semaphorin-3E mRNA expression was especially evident at the inner and, to a lesser extent, outer margins of the INL where, respectively, amacrine and horizontal cells are located.

**Semaphorin-3F.** There was minimal hybridization for semaphorin-3F mRNA at birth, but some signal was evident at P7 (not shown). High levels of expression, mostly confined to the GCL, were present in P14 and adult retinas, although the number of strongly labeled cells was less in adult eyes (Fig. 1M–O). Inspection of many retinal sections from different adult animals indicated that the number of hybridized cells and the level of expression of semaphorin-3F in the GCL were lower than for semaphorin-3B and -3C.

**Expression of Semaphorin Receptor Components: Neuropilins and Plexins**

Representative sections from newborn (P0), P14, and adult retinas hybridized with riboprobes specific for NP-1, NP-2, PlxA1, and PlxA2 are presented in Figure 2. An example of a control section hybridized with a sense probe is also shown (Fig. 2L). As for the semaphorins, expression of these receptors was highest in the GCL, but some expression by cells in the INL was evident with probes for NP-2 and PlxA2. No obvious gradients of mRNA expression across the retina were present in developing or adult eyes.

**NP-1.** mRNA expression was very low in the newborn and P2 retinas and was confined to occasional cells in the GCL (Figs. 2A–C). Expression increased with postnatal maturation, and there was low to moderate expression in numerous cells in

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**FIGURE 1.** Retinal mRNA expression of five of the secreted class-3 semaphorins (3A, 3B, 3C, 3E, and 3F) in (A, D, G, J, M) newborn (P0), (B, E, H, K, N) older postnatal (P14), and (C, F, I, L, O) adult rats. The ganglion cell layer is toward the bottom of each photomicrograph. Scale bar: (A, B, D, E, G, H, J, K, M, N) 75 μm; (C, F, I, L, O) 50 μm.
Numerous cells in the maturing INL expressed high levels of NP-2 mRNA, especially in the inner half of this layer (Fig. 2E). Occasional hybridized cells were also seen in the outer parts of the INL. There appeared to be a more scattered distribution of NP-2 mRNA-expressing cells in the INL in adult retina, with reduced signal (Fig. 2F).

**PlxA1.** Overall, mRNA for this receptor was expressed at low levels in developing and adult rat retina (Figs. 2G, 2I). Some individual cells expressed moderate signal with this probe, especially in the GCL. These cells were most evident at P14 (Fig. 2H) but very occasional cells in the GCL were also present in adult eyes (Fig. 2I). There was little expression outside the GCL although in the adult, in some sections hybridized for PlxA1, increased signal was seen in the OPL.

**PlxA2.** Moderate levels of PlxA2 mRNA expression were evident throughout the retina at birth (Fig. 2J) and at P7 (not shown). By P14, expression was higher in the GCL and hybridized cells were also present throughout the INL, with particularly intense staining of cells near the inner margin of this layer (Fig. 2K). Expression levels were slightly lower in adult retinas. mRNA expression was now present in many cells in the GCL and in some cells in the inner half of the inner plexiform layer (IPL; Fig. 2L).

**Identification of RGCs Expressing Semaphorin-3 mRNA**

At birth, displaced amacrine cells have yet to migrate into the rat GCL; however, in adults these interneurons comprise a significant proportion of the cells in this layer. Thus, to establish the cellular identity of semaphorin-3 mRNA-expressing cells in the adult GCL, some rats received SC injections of FG, a retrograde fluorescent tracer. For all semaphorins tested (3A, 3B, 3C, 3E, and 3F), unequivocal double-labeling was evident, and mRNA expression was located in FG-labeled RGCs (Fig. 3, arrows). Occasional large, displaced RGCs in the INL also expressed semaphorins. In the adult, not all FG-labeled cells in the GCL were hybridized for a given probe. This was particularly noted for semaphorins-3A and -3F (Figs. 3C, 3O). Note also that we occasionally saw semaphorin-expressing cells in the adult GCL that were not obviously FG positive, suggestive of expression by some displaced amacrine cells.

High-power views of the GCL in P14 retinas that had been hybridized with probes for semaphorin-3A or -3B are shown in Figure 4. Apart from using different probes, these sections were treated identically. Staining intensity for semaphorin-3A (Fig. 4A) was clearly less than for semaphorin-3B (Figs. 4B, 4C). The two images shown in Figures 4B and 4C were taken from the same retinal section but from opposite poles of the retina (in this case, dorsal and ventral margins, respectively). Note the similar number of labeled cells and the similar level of semaphorin-3B mRNA expression.

The number of hybridized cells in the GCL per millimeter of retinal section was counted for each probe. At P14, on average, there were 60.8 semaphorin-3A cells/mm compared with a mean of 62.8 semaphorin-3B expressing cells/mm. Similar values were found across the entire retina. Thus, although in individual cells the level of expression of semaphorin-3A mRNA appeared less, at P14 the overall number of cells expressing semaphorin-3A and -3B mRNA was similar. Indeed, at this age there were very few cells in the GCL that were not labeled with either probe, strongly suggesting that individual neurons express more than one type of semaphorin-3 mRNA.

For semaphorin-3B there were approximately 50 cells/mm in the GCL of adult retina. As a comparison, using similar counting methods, a previous study showed that the number of FG-labeled RGCs in P6 to P8 rat retina averaged between approximately 50 to 55 RGCs/mm, and in adult retina there were approximately 40 to 45 RGCs/mm.30

The size of cells in the GCL labeled for semaphorins-3A or -3B was quantified in P14 retina, when expression was at its peak. Figure 4 shows the size distribution of cells expressing semaphorin-3B in the GCL. The number of cells expressing semaphorin-3A was similar. The distribution of cell sizes was similar for both probes.

**Figure 2.** Retinal mRNA expression of members of the neuropilin (NP-1, A–C, and NP-2, D–F) and plexin (PlxA1, G–I; and PlxA2, J–L) receptor subtypes in newborn (P0), older postnatal (P14), and adult rats. The GCL is toward the bottom of each photomicrograph. (M) Negative control; section of adult retina hybridized using sense mRNA. Scale bar: (A, B, D, E, G, H, J, K) 75 μm; (C, F, I, L, M) 45 μm.
Cells hybridized for semaphorin-3A ranged from 9.8 to 21.8 μm in diameter (n = 59, mean of 14.3 μm), whereas the size of semaphorin-3B–expressing cells ranged from 9.4 to 24.9 μm (n = 68, mean of 14.4 μm). These soma sizes, which may include some small displaced amacrine cells, encompass the entire range of RGC diameters, suggesting that all classes of RGC can express semaphorin-3 mRNAs.

Immunohistochemical Characterization of Cells Expressing Semaphorin-3B

A number of sections from P14 retinas were hybridized for semaphorin-3B mRNA and then immunostained with antibodies to establish the phenotype of some of the semaphorin expressing cells (Fig. 5). In the GCL, semaphorin-3B mRNA expressing cells were immunoreactive for β-III tubulin (Figs. 5A, 5B, arrows), a cytoskeletal protein highly expressed by RGCs. Similar observations were made in retinal material from adult eyes. In P14 and adult retinas, occasional semaphorin-3B–expressing cells were seen that were not obviously β-III tubulin labeled, suggestive of displaced amacrine cells. We obtained similar data with this antibody in sections of P14 retina hybridized for semaphorin-3A (not shown).

The calcium-binding protein calbindin is highly expressed by axon-bearing horizontal cells and weaker labeling of some amacrine cells and RGCs has also been reported. Although, even at P14, the hybridization signal for semaphorin-3B is weaker in cells in the INL than in the GCL (Fig. 1E), there nonetheless appeared to be colocalization of semaphorin-3B expression with many but not all calbindin-positive horizontal cells (Figs. 5C, 5D, long arrows). Some of the more weakly stained calbindin immunoreactive cells in the GCL (perhaps RGCs) also expressed semaphorin-3B mRNA (Figs. 5C, 5D, short arrows); however, the weakly stained population of calbindin-positive cells in the inner part of the INL, presumably amacrine cells, did not express semaphorin-3B.

Rod ON bipolar cells are immunoreactive for PKCa. Some amacrine cells also express this serine/threonine isoenzyme. In our double-labeled sections, a large number of bipolar cells were labeled (Fig. 5F), but only a few appeared to coexpress semaphorin-3B mRNA (Figs. 5E, 5F, long arrows). Even in these cells the hybridization signal was relatively weak. Occasional PKCa immunopositive amacrine cells expressed semaphorin-3B (Figs. 5E, 5F, short arrows).

In rat, parvalbumin is a marker for the AII type amacrine cell. Most of the parvalbumin immunoreactive amacrine cells did not express semaphorin-3B mRNA, but in some cases there appeared to be colocalization of the two markers (Figs. 5G, 5H, arrows).

DISCUSSION

We have presented the first detailed analysis of mRNA expression of five members of the secreted class-3 semaphorins (3A, 3B, 3C, 3E, and 3F) as well as members of the neuropilin (NP-1 and -2) and plexin (PlxA1 and -A2) receptor families in the postnatal and adult rat retina. mRNAs for all class-3 semaphorins (with the exception perhaps of semaphorin-3F) and for the investigated receptor components were expressed at low levels at birth. Expression of mRNA specific for these chemorepulsive molecules increased during the first 14 days of postnatal development and then persisted into adulthood. The greatest expression was found in the GCL, but expression was also consistently seen in different cell types in the INL, especially at P14. Qualitatively, mRNA expression at P14 and in the adult was highest for semaphorins-3B and -3C, and for the receptors NP2 and PtxA2. Semaphorin mRNA expression in RGCs was confirmed by immunohistochemistry and by double

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933228/ on 01/23/2019)
labeling adult neurons with FG after injection of the retrograde tracer into the contralateral SC.

Expression of members of the semaphorin-3 family of molecules and their receptors by the same population of neurons has been reported in other cell populations. We did not costain individual retinal neurons for semaphorin-3 and NP or Plx receptors; however, for RGCs at least, based on the large number of cells expressing signal for most semaphorins and their receptors in P14 retinas, coexpression of ligand and receptor in individual neurons seems likely. The functional significance of this is not clear, but there may be clues from other studies. In chick embryos spinal motor neurons express semaphorin-3A, and at the same time are repelled by this molecule in in vitro assays. Based on ephrin studies in RGCs, it is possible that coexpression allows semaphorin ligands to modulate their own receptor function. Finally, in endothelial cells, receptor expression and semaphorin expression in the same cell regulates integrin function and thereby the local adhesion of the cell to extracellular matrix, thus affecting cell migration and vascular morphogenesis.

We have not yet examined fetal rat retina, but in mice there is low level PlxA1 and -A3 (but not PlxA2) mRNA expression in embryonic day (E)16.5 embryos. Consistent with the present rat study, expression of these receptors (including PlxA2) was much greater postnatally, and by P7, expression in the mureine retina was found in the GCL and the inner half of the INL where amacrine cells predominantly reside. PlxA2 expression was also present in the outer part of the INL, perhaps associated with bipolar cells. In P14 rat retina we found relatively similar patterns of expression in INL, expression in the inner part of INL being especially obvious for semaphorins-3B and -3E, and for NP2, and PlxA2.

During development, many types of molecules, acting at different times and in different combinations, affect the growth of RGC axons and eventually direct the patterned innervation of, and terminal arborization within, central visual target sites. These molecules include neurotrophins; extracellular matrix molecules such as laminin chondroitin sulfate proteoglycans, and tenascin-R; adhesion molecules such as L1, netrins, slit proteins, and the Eph family of receptor tyrosine kinases and their ligands, the ephrins. We observed mRNA expression for many secreted class-3 semaphorins and their receptors in P0 rat RGCs, suggesting that these proteins could also play a role in central axonal guidance and circuit formation. Most visual system studies to date have focused on semaphorin-3A, which appears to be the member of the semaphorin-3 family that is least expressed in rat retina. There is evidence that semaphorin-3E may influence the guidance of RGC axons within chick retina and semaphorin-3D appears to play a role in RGC axonal guidance in zebrafish. Membrane-associated semaphorins such as semaphorin-5A expressed by oligodendroglia in the optic nerve have also recently been implicated in inhibiting RGC axon growth. Our new data emphasize the need for a more thorough analysis of the impact of the secreted semaphorin-3 family of proteins and their receptors on mammalian RGC axon guidance and plasticity.

The highest level of mRNA expression for class-3 semaphorins and their receptor components was in the GCL. It was notable that, compared to levels at birth, mRNA expression for the proteins under study was much higher in the GCL at P14. For some semaphorins (e.g., semaphorin-3F), the number of hybridized cells appeared less in adult eyes, but in most cases the level of expression in individual cells in the GCL was equally high or only marginally reduced in adulthood. On the basis of retrograde and immunohistochemical labeling, most of these cells were identified as RGCs, but there was also evidence of expression by at least some displaced amacrine cells. Note that not all FG-labeled cells in the GCL were hybridized for a given probe. In sections hybridized for either semaphorin-3A or -3B mRNA, based on measurements of soma diameter, it was concluded that all classes of RGCs expressed these semaphorins. At P14 in particular, there were very few cells in the GCL that were not labeled for either semaphorin-3A or -3B, strongly suggesting that individual neurons can express more than one type of semaphorin-3 mRNA. This is an important possibility that deserves further study.

At P14, naturally occurring RGC death has finished, and RGC axons have reached retinorecipient sites. There has been pruning of axonal branches and maturation of terminal arbors, and formation of synapses with target neurons is underway. Myelination of axons in the superficial SC layers does not begin until about P12 to P14. These various associations are strongly suggestive of a link between the secreted class-3 semaphorins and their receptors and the postnatal maturation of terminal arbors and stabilization of retinal axons and their synaptic contacts in appropriate target areas in the brain. Similar roles for semaphorins have recently been described in hippocampus and in other parts of the limbic system. A recent in vitro study found no evidence that rat RGC growth cones are repelled by either semaphorin-3A, -3B, or -3C; however, these studies were undertaken on cultured P8 RGCs that apparently did not express NP-2, quite unlike our in vivo P14 in situ hybridization data, which showed NP-2 to be the most highly expressed semaphorin-3 receptor in the GCL. At all ages studied, we did not detect an obvious gradient of mRNA expression.
expression for any of members of the semaphorin-3 family across the nasotemporal or dorsoventral axes of the retina. Further quantitative work is necessary here, perhaps measuring protein rather than mRNA levels, especially because recent data indicate a role for semaphorin-3D in guiding RGC axons along the dorsoventral dimension of the zebrafish optic tectum. Furthermore, RGCs express the L1 cell adhesion molecule, a molecule that may be involved in semaphorin-3 signaling, and that is involved in topographic mapping of murine retinotectal connections.

In the days after birth, similar to our observations in the GCL, there was an increase in expression of mRNA in the INL for most of the proteins under study. Indeed, in many cases label in INL appeared higher at P14 than in the adult. This was particularly the case for semaphorins-3B, -3C, and -3E, and the NP2 and PlxA2 receptor subunits. This expression was stronger toward the inner margin of INL, where many amacrine cells, and the small number of displaced RGCs, are located. Occasional semaphorin-3B-expressing cells in the INL (E) were immunolabeled with a PKCα antibody (F). Shown are double-labeled rod ON bipolar cells (long arrows) and amacrine cells (short arrows). (F) Composite of two images taken in different focal planes. A small proportion of semaphorin-3B mRNA expressing cells (G, arrows) were immunopositive for parvalbumin (H), a marker for a class of amacrine cell. Scale bar: 25 μm.

**Figure 5.** Pairs of images showing in situ hybridization for semaphorin-3B mRNA (A, C, E, G, bright-field image) combined with fluorescence immunohistochemistry (B, D, F, H). (A) Semaphorin-3B expressing RGCs (arrows) that were immunopositive for βIII tubulin (B, arrows). (C) Semaphorin-3B expressing horizontal cells (long arrows) in the outer part of the INL were immunopositive for calbindin (B, long arrows). Weakly stained calbindin-positive cells in the GCL also expressed semaphorin-3B mRNA (D, short arrows), but weak calbindin immunoreactivity in the inner part of the INL did not colocalize with hybridized cells. Occasional semaphorin-3B-expressing cells in the INL (E) were immunolabeled with a PKCα antibody (F). Shown are double-labeled rod ON bipolar cells (long arrows) and amacrine cells (short arrows). (F) Composite of two images taken in different focal planes. A small proportion of semaphorin-3B mRNA expressing cells (G, arrows) were immunopositive for parvalbumin (H), a marker for a class of amacrine cell. Scale bar: 25 μm.
semaphorin-3 family are localized to similar or complementary sets of interneurons in rat retina.

In rat, the eyes open at ~P12 to P13 and the retinal electroretinogram is first recordable at about this time.⁶⁰ In developing rat IPL, clear amacrine cell synapses are detectable at ~P11 to P12,⁵⁴ synaptophysin immunoreactivity becomes evident at ~P12,⁶¹ and there is in vitro evidence for emerging amacrine-ganglion cell interactions that alter axonal and dendritic growth at approximately this postnatal age.⁶² Taken together with expression in the GCL, the increased mRNA expression of semaphorins and their receptors in a variety of interneurons in the INL of P14 rats may indicate that these molecules play a role in the establishment and stabilization of intraretinal circuitry, particularly between amacrine and ganglion cell processes in the IPL, perhaps also involving the stratification of dendritic and axonal processes.⁵⁴,⁶₃–⁶⁵ A role in the regulation of amacrine cell migration within the developing retina is also possible.¹¹,³⁷,⁶⁴ Last, it is worth noting that at P14 some PKCa-positive rod bipolar cells expressed semaphorin-3B, and bipolar cell synapses in rat IPL are initiated at ~P13.⁵⁴ The continued expression of semaphorin-3 mRNAs and their receptor components by adult RGCs deserves some comment. There is speculation that, throughout normal life, semaphorins play a role in restricting or regulating sprouting and/or synapse formation in specific layers of a neural structure.¹⁹ Expression in mature mammalian RGCs may therefore be essential for maintaining established connections and synaptic contacts in retinorecipient areas in the brain and perhaps also within the retina. In adult spinal cord, injury induces an upregulation of semaphorin-3 that may contribute to the inhibitory nature of the glial scar, preventing regeneration of descending fiber tracts.¹⁸,¹⁹ Furthermore, peripheral nerve injury results in an upregulation in growth-associated proteins such as GAP-43 as well as a downregulation of semaphorin expression in motoneurons,¹⁵ both potentially contributing to regenerative growth.¹⁹ The rodent provides many well-established models for studying degeneration, plasticity, and regeneration in the adult mammalian visual system.²⁰–²⁵,⁶⁶–⁶⁷ The data presented herein provide a baseline for in vivo analysis of the role of secreted semaphorins and their receptors in injury and repair, both within the retina itself and within primary visual pathways.

References

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