Sema6B, Sema6C, and Sema6D Expression and Function during Mammalian Retinal Development

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Abstract

In the vertebrate retina, the formation of neural circuits within discrete laminae is critical for the establishment of retinal visual function. Precise formation of retinal circuits requires the coordinated actions of adhesive and repulsive molecules, including repulsive transmembrane semaphorins (Sema6A, Sema5A, and Sema5B). These semaphorins signal through different Plexin A (PlexA) receptors, thereby regulating distinct aspects of retinal circuit assembly. Here, we investigate the physiological roles of three Class 6 transmembrane semaphorins (Sema6B, Sema6C, and Sema6D), previously identified as PlexA receptor ligands in non-retinal tissues, in mammalian retinal development. We performed expression analysis and also phenotypic analyses of mice that carry null mutations in each of genes encoding these proteins using a broad range of inner and outer retinal markers. We find that these Class 6 semaphorins are uniquely expressed throughout postnatal retinal development in specific domains and cell types of the developing retina. However, we do not observe defects in stereotypical lamina-specific neurite stratification of retinal neuron subtypes in Sema6B−/− or Sema6C−/−; Sema6D−/− retinas. These findings indicate these Class 6 transmembrane semaphorins are unlikely to serve as major PlexA receptor ligands for the assembly of murine retinal circuit laminar organization.

Materials and Methods

Animals

The day of birth in this study is designated as postnatal (P) day 0. The Sema6B−/−, Sema6C−/−, and Sema6D−/− mice were previously described [8,11,12]. For the phenotypic assessment of...
adult wild-type, Sema6B<sup>−/−</sup>, and Sema6C<sup>−/−</sup>; Sema6D<sup>−/−</sup> retinas, 4 independent animals of each genotype were analyzed. For the phenotypic assessment of P17 wild-type, Sema6B<sup>−/−</sup>, and Sema6C<sup>−/−</sup>; Sema6D<sup>−/−</sup> retinas, 2 independent animals of each genotype were analyzed. This study was carried out in strict accordance with the recommendations in the Guide for the Care
and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine (Protocol Number: M011M80). Mice were euthanized prior to tissue harvesting to minimize suffering.

**Immunohistochemistry**

Eyes were fixed in 4% paraformaldehyde for 1 hr at 4°C, equilibrated in 30% sucrose/PBS and embedded in OCT embedding media (Tissue-Tek). Retinal sections (20–40 μm) were blocked in 5% fetal bovine serum in 1 X PBS and 0.4% Triton-X100 for 1 hr at room temperature and then incubated overnight at 4°C with primary antibodies: rabbit anti-tyrosine hydroxylase (Millipore at 1:1000), goat anti-ChAT (Millipore at 1:100), rabbit anti-calretinin (Swant at 1:2500), guinea pig anti-vGlut3 (Millipore at 1:2500), rabbit anti-Dab-1 (generous gift from Dr. Brian Howell at 1:500), rabbit anti-calbindin (Swant at 1:2500), rabbit anti-N-terminal melanopsin (ATS at 1:2000), rabbit anti-PlexA2 (generous gift from Dr. Fumikazu Suto at 1:400) [13], Armenian hamster anti-PlexA4 (generous gift from Dr. Fumikazu Suto at 1:400) [13], mouse anti-PKCα (Millipore at 1:200), mouse anti-synaptotagmin 2 (ZNP-1, Zebrafish International Resource Center at 1:2000), rabbit anti-neurokinin 3 receptor (Calbiochem at 1:3000), rabbit anti-cone arrestin (generous gift from Dr. Cheryl Craft at 1:3000), guinea pig anti-vGlut1 (Millipore at 1:2000), chicken anti-vimentin (Millipore at 1:1000), and mouse anti-glutamine synthetase (Millipore at 1:1000). Sections were washed 6 times for 5 min in 1 X PBS and then incubated with secondary antibodies and TO-PRO-3 (Molecular Probe at 1:400) for 1 hr at room temperature. Sections were washed 6 times for 5 min in PBS and coverslips were mounted using vectashield hard set fluorescence mounting medium (Vector laboratories), and confocal fluorescence images were taken using a Zeiss Axioskop2 Mot Plus, LSM 5 Pascal confocal microscope.

**In situ Hybridization**

In situ hybridization was performed on fresh frozen retina sections (20 μm thickness) as described previously [6]. The digoxigenin-labeled antisense riboprobes specific for Sema6B, Sema6C, and Sema6D used in this study were previously described [8,9].

**Results**

*Sema6B, Sema6C, and Sema6D mRNA Expression in the Developing Mouse Retina*

To investigate whether Sema6B, Sema6C, and Sema6D regulate retinal development, we first analyzed mRNA expression...
of Sema6B, Sema6C, and Sema6D during postnatal retinal development by in situ hybridization (Fig. 1). We performed in situ hybridization at the postnatal ages of P7 and P14, developmental time points when inner and outer retinal circuits are established. We found that Sema6B, Sema6C, and Sema6D are all expressed at these stages of postnatal retinal development. Specifically, Sema6B is expressed most prominently in the ganglion cell layer (GCL) and inner nuclear layer (INL) at P7 and P14 (Fig. 1A, 1G). At both ages, a majority of retinal neurons in the GCL and inner INL express Sema6B (Fig. 1A, 1G), whereas Sema6B expression was either low or absent in the outer nuclear layer (ONL) at these postnatal ages. On the other hand, Sema6C was expressed broadly in the INL and sparsely in the GCL at P7 and P14 (Fig. 1C, 1I). A majority of retinal neurons in the INL strongly express Sema6C, but only a subset of neurons in the GCL appear to express Sema6C at both ages. Sema6D expression was strong in neuronal subtypes that reside in the GCL and inner INL at P7 and P14 (Fig. 1E, 1K). The retinal cell types that express Sema6D robustly in the GCL at these ages are retinal ganglion cells and/or displaced amacrine cells, while those expressing Sema6D strongly in the INL close to the OPL are likely horizontal cells, given their morphological features and horizontal process staining (Fig. 1E, 1K). At P7 and P14, Sema6D was also expressed in a subset of neurons in the inner INL (Fig. 1E, 1K). We used the corresponding sense probes as references for detecting background staining and did not observe specific signals with these probes at P7 and P14 (Fig. 1B, 1D, 1F, 1H, 1J, 1L).

These expression patterns of Sema6B, Sema6C, and Sema6D during postnatal retinal development suggest a role for these semaphorins in regulating retinal development in vivo.

Normal Neurite Stratification of Inner Retinal Neurons in Sema6B−/− and Sema6C−/−; Sema6D−/−

To determine whether these class 6 semaphorins regulate retinal development in vivo, we analyzed mice that harbour null mutations in genes encoding Sema6B (Sema6B−/−), or both Sema6C and Sema6D (Sema6C−/−; Sema6D−/−) [8,12]. For each genotype we analyzed eyes from 4 animals. We examined neurite elaboration in distinct retinal subtypes in the IPL and OPL of these mutant mice by immunohistochemistry using a range of retinal cell-type markers. Each of these markers labels neurites belonging to specific subtypes of RGCs, amacrine and bipolar cells within the IPL or photoreceptors, bipolar and horizontal cells within the OPL.

In adult wild-type retina sections stained with these markers, we observed that multiple amacrine cell subtypes, including dopami-
nergic, cholinergic, vGlut3+, and AII, all exhibited stereotypical lamina-specific neurite stratifications within the IPL (Fig. 2A, 2D, 2J, 2M). None of these subtypes in Sema6B−/− or Sema6C−/−; Sema6D−/− retinas appeared to differ from those in wild-type retinas (Fig. 2A–2F, 2J–2O). In addition, amacrine cell and RGC subtypes labelled by anti-calbindin or anti-calretinin, both of which define neurite stratification at the borders of S1 and S2, S2 and S3, and S3 and S4 within the IPL of wild-type retinas (Fig. 2G, 2P), exhibit apparently normal neurite stratification in Sema6B−/− or Sema6C−/−; Sema6D−/− retinas as compared to wild-type retinas (Fig. 2G–2I, 2P–2R). To investigate whether RGC dendritic stratification is affected in these mutant retinas, we used an antibody directed against the N-terminus of melanopsin that labels multiple subtypes of intrinsically photosensitive retinal ganglion cells (ipRGCs) [14,15,16]. In wild-type P17 retinas, ipRGC dendritic arbors are found in two distinct domains of the IPL; one stratifies within the S1 sublamina and the other resides within the S4/S5 sublaminae (Fig. 3A). We found that these ipRGC dendritic stratifications within these two different domains of the IPL do not apparently differ in Sema6B−/− or Sema6C−/−; Sema6D−/− retinas compared to wild-type retinas (Fig. 3A–3C). We next investigated whether PlexA-positive neurite stratification within the developing IPL is compromised in these mutant retinas. We previously found that PlexA1 and PlexA3 show broad protein expression across the developing postnatal IPL [4], whereas PlexA2 and PlexA4 are localized in specific, but distinct, sublaminae of the developing IPL.

Figure 5. Neurite stratification of outer retinal neuron cell types in the OPL of Sema6B−/− and Sema6C−/−; Sema6D−/− retinas. Wild-type (A, D, G, J), Sema6B−/− (B, E, H, K), and Sema6C−/−; Sema6D−/− (C, F, I, L) adult retina sections were immunostained with antibodies against cone arrestin (A–C, a marker for cone photoreceptors), calbindin (D–F, a marker for horizontal cells), PKCα (G–I, a marker for rod bipolar cells), and vGlut1 (J–L, a marker for photoreceptor axonal terminals). Neurite stratification of photoreceptors, horizontal cells, and bipolar cells visualized by each of these markers does not show any obvious defects in Sema6B−/− and Sema6C−/−; Sema6D−/− retinas as compared to wild-type retinas. Scale bar: 50 μm in L for A–L.
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Normal Neurite Stratification of Outer Retinal Neurons in Sema6B−/− and Sema6C−/−; Sema6D−/− Retinas

To investigate whether Sema6B, Sema6C, or Sema6D regulate neurite targeting to the OPL, we analyzed neurite stratification of outer retinal neuron types; these include horizontal and bipolar cells, and photoreceptors, within the OPL of Sema6B−/− or Sema6C−/−; Sema6D−/− retinas. We used antibodies directed against calbindin, PKCα, cone arrestin, or vesicular glutamate transport protein 1 (vGlut1) to label horizontal cells, rod bipolar cells, cone photoreceptors, or photoreceptor axonal terminals, respectively [20,21,22]. In wild-type adult retinas, these markers reveal horizontal cell neurites, rod bipolar cell dendrites, and cone and rod photoreceptor axon terminals stratifying within the OPL, (Fig. 5A, 5D, 5G, 5J), and these neuronal OPL stratification events are not apparently different in Sema6B−/− and Sema6C−/−; Sema6D−/− retinas (Fig. 5).

Müller Glia Morphology and Process Extension are Normal in Sema6B−/− and Sema6C−/−; Sema6D−/− Retinas

To assess whether morphology and process extension of Müller glia cells are affected in Sema6B−/− and Sema6C−/−; Sema6D−/− retinas, we used antibodies directed against glutamine synthetase and vimentin to visualize cell bodies and processes of Müller glia cells. Müller glia cell bodies are found in the INL, and their cell processes span across the retina and form the outer and inner limiting membranes in wild-type retinas (Fig. 6A, 6D) [23]. We observed that the morphology of Müller glia cells in adult retinas from Sema6B−/− and Sema6C−/−; Sema6D−/− mice does not apparently differ from that in wild-type retinas (Fig. 6).

Discussion

We show here that the formation of neural circuitry in the inner and outer retina of Sema6B−/− and Sema6C−/−; Sema6D−/− mice is comparable to that observed in WT mice. Previous studies show that these class 6 transmembrane semaphorins signal through the PlexA receptors to regulate neural and non-neural development [7,8,9,10]. We recently found that Sema6A and several PlexA receptors play critical roles in regulating the development of retinal circuits [4,5,6], and thus we hypothesized that these class 6 transmembrane semaphorins might function through PlexA receptors to regulate retinal development.

Our results show that these class 6 transmembrane semaphorins are all expressed in specific and unique retinal cell types during postnatal development. To investigate the possibility that these transmembrane proteins regulate retinal development, we ana-

### Figure 6. Müller glia cell morphology in Sema6B−/− and Sema6C−/−; Sema6D−/− retinas.

Wild-type (A, D), Sema6B−/− (B, E), and Sema6C−/−; Sema6D−/− (C, F) adult retina sections were immunostained with antibodies against glutamine synthetase (GS, A–C) or vimentin (D–F). Overall morphology, process extension, and cell body position of Müller glia cells visualized by these two markers do not differ among wild-type, Sema6B−/−, and Sema6C−/−; Sema6D−/− retinas. Scale bar: 50 μm in C for A–G, and in F for D–F.

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alyzed Sema6B-/- and Sema6c-/-; Sema6D-/- mutant retinas by immunohistochemistry using a range of markers that label distinct retinal neuron subtypes in the inner and outer retina. However, our extensive phenotypic analyses of these mutant retinas did not reveal clear deficits in stereotypical neurite stratification of distinct subtypes in either the inner or outer retina. Although we used many immunological markers that label different subtypes of retinal cells, our analysis did not include several additional retinal neuron subtypes present in the retina. We do not exclude the possibility that more specific retinal neuronal or glial subtypes, which were not visualized by the markers used here, show defects in neurite elaboration or sublaminal targeting. It is also possible that penetrance of retinal phenotypes, if any, in these mutant mice is not high enough to be revealed by the number of the mutant mice we examined (n = 4 mice/genotype analyzed). We also did not assess the cell number and cell migration of each retinal subtype, nor did we investigate mosaic patterns of cell body distribution of these subtypes in the horizontal retinal plane. Finally, our analysis did not include a detailed analysis of retinal neuronal or glial process morphology in the plane of individual laminae. Thus, our present study does not exclude the possibility of phenotypes in Sema6B-/- and Sema6c-/-; Sema6D-/- retinas that affect these aspects of retinal organization. Nevertheless, given the important roles played by PlexA receptors in sublaminal targeting of multiple retinal neuron subtypes [4,5,6], our work shows that these class 6 semaphorins do not serve major functions in retinal sublaminar targeting. Since the transmembrane semaphorins Sema3A and Sema5B together constrain neurites from inner retinal neuron subtypes to the IPL [4], it is possible that class 6 semaphorins are functionally redundant with respect to regulation of retinal development. It was shown that Sema6A and Sema6B function additively to regulate murine hippocampal mossy fiber projections in vivo [8], and thus Sema5B might also cooperate with Sema6A to control specific events of retinal development, such as horizontal cell OPL neurite stratification.

We expect that a variety of membrane-bound and secreted guidance cues will be investigated further to identify the complete spectrum of molecules required for the establishment of laminar organization of the vertebrate retina. Recent studies show that secreted guidance cues, including netrins and slits, direct laminaspecific photoreceptor and RGC axon targeting in the fly and zebrafish visual system [24,25]. It will be of interest to determine how membrane-bound and secreted cues cooperate to build the laminar structure of vertebrate retinal circuits, advancing our understanding of how segregated processing of visual information in the retina comes to be established during development.

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Author Contributions

Conceived and designed the experiments: RLM ALK. Performed the experiments: RLM LOS. Analyzed the data: RLM LOS. Contributed reagents/materials/analysis tools: KK YY. Wrote the paper: RLM ALK.

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