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How axons see their way – axonal guidance in the visual system

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1. ABSTRACT

In humans up to 80% of the information received from the outside world is processed by the visual pathway. Therefore, understanding the molecular and cellular bases of the formation of the retinofugal projection has been in the focus of research during the last decades. Besides our interest in the development of the visual pathway *per se* this circuit is also an excellent model system to study axon guidance, midline crossing, and formation of topographic neuronal maps in general. The generation of genetic animal models as well as the design of *in vitro* loss- and gain-of-function paradigms have provided insight into transcriptional networks, identified signalling molecules, extracellular matrix components, morphogens, and activity patterns which are involved in the establishment of the visual pathway. To provide a picture as complete as possible, we will summarize molecular mechanisms involved in axon guidance and retinotopic mapping as well as neuronal activity shaping retinal and thalamocortical projections focusing on the mouse as a model system and highlight discoveries made in other organisms that contribute to our understanding.

2. INTRODUCTION

The eye as the sensory organ of the visual system captures information from the outside world which is then processed via the visual pathway producing the image we experience. To accomplish this, incoming information must be processed in an efficient and organized fashion through a circuitry established during embryonic development. The establishment of this circuitry is the focus of this review. We will follow the path of the visual projection starting at the retina passing through the optic chiasm, superior colliculus (SC) and lateral geniculate nucleus (LGN) in the midbrain, and ending at the primary visual cortex. During this rather long journey from the eye to the brain the axons encounter several choice points where the further direction of growth has to be determined. How are these decisions made? Is it the identity of the neuron, the environment the neuron is encountering, neuronal activity, or any combination thereof that guides the way and initiates the map of the outside world? This review will discuss the different choice points in the order the axons encounter them and give an overview of how transcription factors, signaling molecules and their receptors as well as neuronal

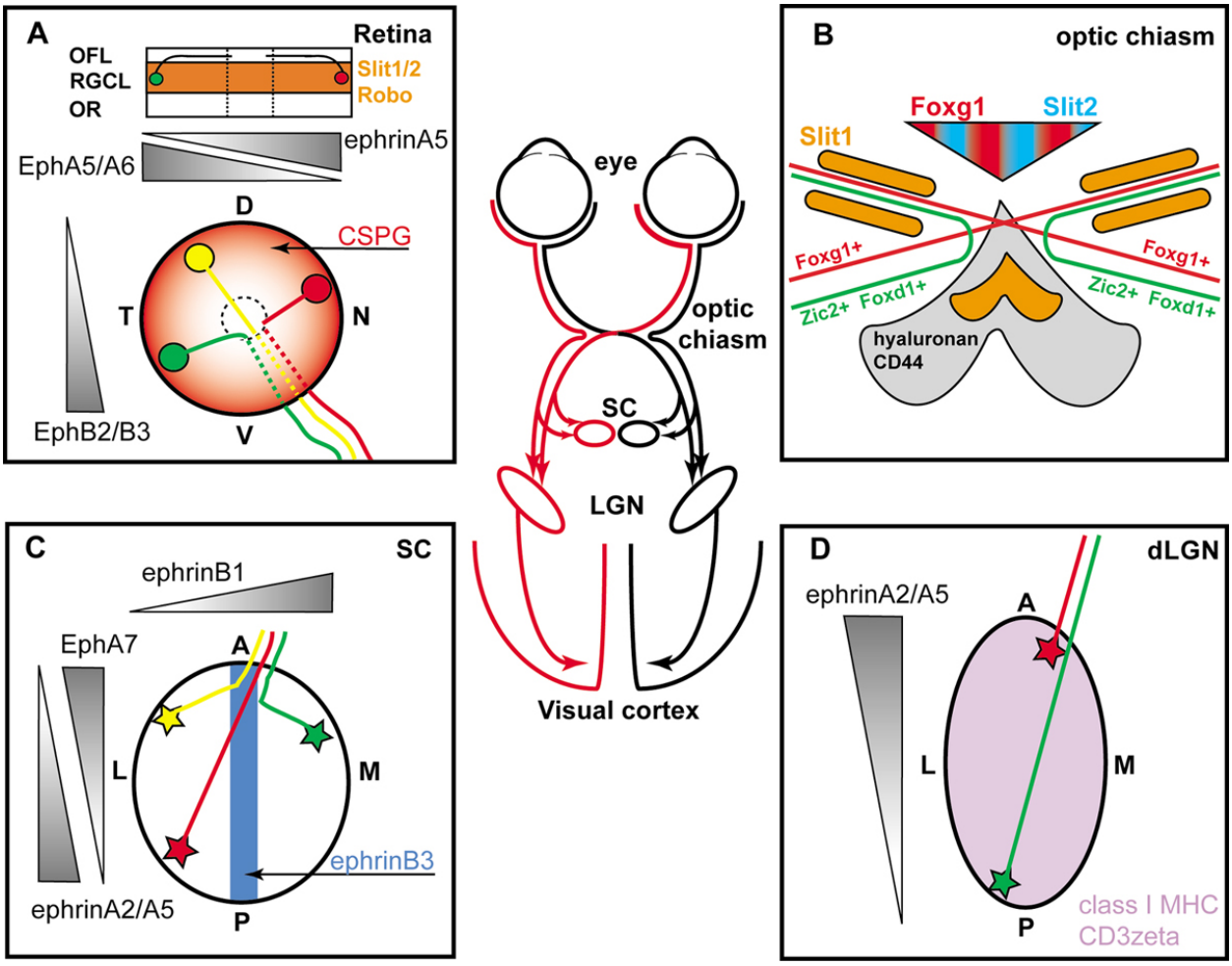

Figure 1. Schematic summary of the visual pathway displaying molecules involved in axon guidance at specific decision points. Center: Retinofugal projections (black and red lines) towards the SC and LGN as well as thalamocortical projections are illustrated here. (A) Retina. Coronal section (top) and frontal view (bottom) of the retina displaying molecules involved in intraretinal guidance. The optic disc is depicted as a dotted-line circle in the center of the retina. (B) Optic chiasm. Midline crossing (red) and ipsilateral projecting (green) axons as well as molecules involved in channeling and guiding RGC axons at the optic chiasm are depicted. (C) Superior colliculus. Retinotopic projection of temporal (green), nasal (red), and dorsal (yellow) RGC axons and molecules involved in the initiation of the retinotopic map in the SC are summarized. The expression pattern of ephrinA2 outlined here holds true for the chicken tectum while ephrinA2 expression in mouse SC displays the highest expression midway through the posterior half of the SC decreasing anteriorly and posteriorly (59) (D) Dorsal lateral geniculate nucleus. Topographic mapping of nasal (red) and temporal (green) RGC axons in the LGN is illustrated. For simplicity and due to the 2D-schematic drawing, the ephrin gradients and RGC projections are shown in a strict anterior-to-posterior axis, even though the gradients are tilted with respect to all anatomical axes of the head from low expression at dorsal-medial-posterior part of LGN to high expression at the ventral-lateral-anterior part. A, anterior; CSPG, chondroitin sulphate proteoglycan; L, lateral; dLGN, dorsal lateral geniculate nucleus; M, medial; N, nasal; OFL, optic fibre layer; OR, outer retina; P, posterior; RGCL, retinal ganglion cell layer; SC, superior colliculus; T, temporal; V, ventral.

activity work in concert to establish the visual circuitry (Figure 1). Studies in a variety of organisms have contributed to our knowledge of axonal guidance in the visual system. Typical model organisms such as *Drosophila*, zebrafish, *Xenopus,* chicken, and mouse have been in the focus of axon guidance research in general and the visual system in particular. Even though a monophyletic origin of the eye is strongly supported by genetic experiments, divergent, parallel, and convergent evolution has led to the emergence of different eye types resulting in different strategies to process visual input (reviewed in (1)). For example, eye location, either frontally or laterally directly influences the degree of binocular vision and hence the anatomy of the projection at the chiasm which is established by specific guidance cues. While many signaling molecules and their specific functions have been conserved from *Drosophila* to vertebrates there are also examples of divergence which we

will discuss. In order to provide a picture as comprehensive as possible we will mainly focus on the mouse as a model system and highlight discoveries made in other model organisms that contribute to our understanding of the molecular mechanisms of neuronal circuit formation in the visual system.

3. ANATOMY OF PROJECTION – THE TOURIST MAP

Retinal ganglion cell (RGC) axons exit the eye at the optic disc, travel along the base of the ventral diencephalon toward the midline where they meet axons from the contralateral eye and form the optic chiasm. At the chiasm a portion of axons cross the midline to project to the contralateral midbrain targets, superior colliculus and lateral geniculate nucleus, while a different axonal population grows away form the midline to project to ipsilateral midbrain targets. Projections to the midbrain occur in a topographic manner which appears imprecise at the time of establishment but experiences refinement as the visual system matures before first sensory input. Thalamic axons grow into the subcortical telencephalon during embryonic development while further outgrowth towards the occipital neocortex occurs postnatally and is established in a topographic manner to produce an accurate image of the outside world.

4. DECISION POINTS IN THE VISUAL PATHWAY

4.1. Intraretinal guidance

During retinal development, newly born RGCs need to undergo differentiation and send out there axons towards the optic fiber layer where they fasciculate and navigate towards the optic disc. For differentiation and survival of RGCs, the Brn3 POU-domain transcription factors (in mouse Brn3a-c), specifically Brn3b, seem to play essential roles. Brn3b expression is first detected in RGCs at E11.5, when first RGCs are generated, followed by Brn3a and Brn3c (2, 3). Mice deficient for Brn3b display normal division and migration of RGCs but a subset of RGCs undergoes enhanced apoptosis leading to substantial loss of RGCs before birth (2, 4). Investigating axonal outgrowth at E13 through E15.5, a time when *Brn3b* deficient RGCs are still present, showed disorganized axonal outgrowth with axons failing to coalesce normally into axon bundles *in vitro* which possibly leads to the thinner optic nerves observed *in vivo* (4). Since some RGCs are still present in *Brn3b* deficient mice, Brn3a/c might partially compensate for the loss of *Brn3b*. In fact, in *Brn3b/c* deficient mice, RGC axons were more severely affected than in *Brn3b* single mutants, with the subpopulation of RGCs normally expressing both, Brn3b and Brn3c, failing to send out any processes *in vitro* (3). Comparison of transcriptional profiles of wildtype and Brn3b deficient retinae revealed 87 genes whose expression was changed, among them Brn3a and seven other transcription factors (5). Phenotypic alterations reported for Brn3b deficient mice as well as microarray data indicate an involvement of Brn3b in RGC differentiation by acting upstream of other transcription factors.

After differentiation, RGCs extend their axons into the optic fiber layer of the retina and project towards the optic disc where they exit the retina. Several factors have been identified so far which are involved in intraretinal guidance. Among them are the Slits (Slit1-Slit3 in vertebrates) an evolutionary conserved family of secreted axon guidance cues. Slits act through their receptors, the Roundabouts (Robo, Robo1-Robo4 in vertebrates), to mediate axon pathfinding. Slit1 and Robo2 are expressed in the RGC layer by the time the first RGCs are generated and slightly later in development Slit2 is also expressed (6, 7). In wildtype retina RGC axons are restricted to the optic fiber layer. In *slit2* mutants, and even more pronounced in *slit1/2*-deficient retinae, a subset of RGC axons extended away from the optic fiber layer entering the outer retina. The degree of ectopic projections was higher in the ventral compared to dorsal parts of the retina. Within the outer retina, axons in *Slit2* or *Slit1/2* deficient mice formed highly fasciculated bundles, grew in the gross direction of the optic disc, and were able to exit the retina through the correct passage indicating the presence of other guidance cues (6). Interestingly, *Slit1* mutants do not show a phenotype concerning intraretinal projection. These results imply a role for Slit1 and Slit2 in restricting RGC axons to the optic fiber layer. Data from single and double mutants argue for a compensation of *Slit1* deficiency by Slit2 but not vice versa. Furthermore, within the optic fiber layer of *Slit2* or *Slit1/2* deficient retinae perturbations such as looped and curved trajectories as well as axons crossing between different fascicles were seen, most pronounced in peripheral dorsal retina. Since *slit2* and S*lit1/2* deficient retinae did not vary in the degree of perturbed projections, the optic fiber layer organization seems to be regulated by Slit2 alone (6). In summary, Slit1 and Slit2 play a role in restricting RGC axons to the optic fiber layer in ventral retina while Slit2 alone is involved in regulating the ordered growth within the optic fiber layer in the dorsal peripheral part of the retina (Figure 1A). Semaphorin3E (Sema3E) is another candidate potentially involved in restricting the growth of RGC axons to the optic fiber layer (8). Semaphorins are phylogenetically conserved, secreted, lipid-anchored, or transmembrane proteins which act as repulsive or attractive cues during axon guidance. Sema3E expression in chicken is found in the outer retina where no RGC axons are detected, most pronounced during intraretinal RGC axon growth. Using recombinant Sema3E fused to alkaline phosphatase Steinbach and colleagues visualized Sema3E receptor sites in the RGC axon-rich optic fiber layer. *In vitro,* RGC axon growth cones collapsed when encountering Sema3E. This collapsing effect was mediated by low cGMP levels since cGMP agonists were able to compensate for the collapsing activity of Sema3E.

Chondroitin sulphate (CS), a sulfated glycosaminoglycan composed of a chain of alternating sugars usually found attached to proteins as part of a proteoglycan, mediates RGC differentiation and polarization and aids in proper axon orientation within the retina (9). CS is expressed in a receding centrifugal gradient in the rat retina early in development and is progressively lost from the central retina leading to

expression restricted to the dorsal and ventral poles by E16.5 (Figure 1A) (9). Enzymatic removal of CS leads to ectopic differentiation of RGCs and undirected orientation of their axons. *In vitro,* chondroitin sulphate proteoglycan (CSPG) inhibits axon initiation and elongation which was shown to depend on the concentration as well as on the ratio of CSPG to laminin in the substratum (10, 11). This ratio-dependency with laminin or other growth-promoting molecules may provide a mechanism for timing axon outgrowth in the retina.

The neural cell adhesion molecule L1, a transmembrane glycoprotein belonging to the immunoglobulin superfamily, is involved in directed axon pathfinding and fasciculation of axons in the retina. Application of Fab fragments neutralizing L1 function lead to disrupted RGC growth cone orientation and rate of outgrowth in rat retina (12). Interestingly, in L1 deficient mice no signs of defasciculation or altered migration of RGC axons towards the optic disc were reported (13) implicating that other molecules are involved in the process of fasciculation and growth cone orientation. The immunoglobulin superfamily adhesion molecule BEN (also called SC1, DM-GRASP, neurolin, and CD166) might aid L1 in axon fasciculation as *BEN* deficient mice displayed defasciculation of RGC and intercostal nerve motor axons (14).

The Eph family of receptor tyrosine kinases governs the formation of many axon pathways including the retinofugal projection. The ligands for the Eph receptors are membrane attached and called ephrins: ephrinAs are GPI-anchored and ephrinBs are transmembrane proteins, hence the interaction between ephrins and Ephs requires cell-cell contact. Crucial to an understanding of ephrinB/EphB signalling is their capacity to transduce signals bi-directionally opening the possibility that EphBs and ephrinBs may serve as ligand and receptor within the same cell (reviewed in (15) and (16)): While RGC mapping in the midbrain later in development is dependent on EphBs acting as receptors, during axon navigation within the retina, EphBs act as ligands in a kinase-independent manner. In mice lacking both, *EphB2* and *EphB3* receptors, RGC axons growing towards the optic disc aberrantly split away from fascicles and bypassed the optic disc with some axons growing into the opposite site of the retina (17). This effect was most pronounced in axons emerging from dorsal retina despite the uniform expression of EphB2 and EphB3 in early development. The guidance function of EphBs is independent of the kinase activity since *in vitro* RGC growth cone collapse was induced by application of only the extracellular domains of EphB receptors (17). It is conceivable that during early intraretinal pathfinding dorsal axons are independent of EphBs and later depend on the high-ventral-low-dorsal EphB2 gradient which prevents overshooting into the ventral retina (Figure 1A). The bone morphogenic protein receptor 1b (Bmpr1b) seems to be involved in guiding ventral RGC axons to the optic disc. Bmpr1b is exclusively expressed in the ventral retina and in *Bmpr1b* deficient mice many ventral RGC axons fail to enter the optic disc instead making sharp turns away from the optic disc (18). However, since in *EphB2, EphB3*, and *Bmpr1b* deficient mice the rate of failure is not 100%, additional, yet unidentified, cues have to be involved as well.

In summary, several cues fulfil different functions to accomplish the correct guidance of RGC axons within the retina towards the optic disc. While the cues responsible to keep the axons within the RGC layer or those directing growth cones towards the optic disc have been identified, it is unclear how these known factors interact with each other. Besides identifying the missing links, discovering new roles for known factors appears as a challenging task for further investigations.

4.2. Exit from the retina and formation of the optic nerve

After reaching the optic disc, RGC axons have to exit the retina, enter the optic nerve, and project toward the midline. Early in development the optic cup is formed as an extension of the central nervous system which stays connected to the "early eye" via the optic stalk. RGC axons exiting the retina at the optic disc grow into the optic stalk which is then called the optic nerve. The axon guidance molecule netrin-1 and its receptor deleted in colorectal cancer (DCC) are mediating the exit of RGC axons from the retina. Netrin-1 is expressed in glial cells at the optic disc surrounding RGC axons exiting the retina while its receptor DCC is expressed by RGC axons (19). Anti-DCC antibodies reduced RGC axon outgrowth in response to netrin-1 *in vitro*. No defects in intraretinal guidance towards the optic disc were observed in absence of *netrin-1* and *DCC in vivo*, however, axons failed to enter the optic stalk, resulting in optic nerve hypoplasia thereby suggesting a local function of netrin-1/DCC signaling at the optic disc to guide axons into the optic stalk (19). The fact that *netrin-1* or *DCC* deficiency leads to optic nerve hypoplasia but not complete aplasia suggests that additional mechanisms are involved in guiding axons to exit the retina. An unsolved question is how RGC axons manage to leave the attractive environment of netrin-1 expression in the optic disc and project further into the optic nerve. *In vitro*, laminin can switch netrin-1 mediated axon attraction to inhibition in *Xenopus* RGCs (20). However, this mechanism might not apply for the mammalian visual system: *In vitro* studies have shown that embryonic mouse RGC axons were able to grow on laminin in response to netrin-1 (21). If laminin was able to switch responsiveness of RGC axons to netrin-1, RGC axons would not be able to enter the optic nerve head since both molecules are expressed there (19, 22). Therefore, the cues aiding RGC axons to leave the optic disc still need to be identified in the mouse visual system.

After having entered the optic nerve, RGC axons need to travel in a fasciculated manner towards the brain. The transmembrane protein Sema5A is expressed by neuroepithelial cells surrounding retinal axons at the optic disc as well as along the optic nerve and was shown to inhibit axon growth and induce growth cone collapse when applied *in vitro* (21). Antibodies against Sema5A applied to optic nerve preparations allowed retinal axons to escape from the optic nerve bundle (21). It is therefore possible that Sema5A serves as an inhibitory sheath around the optic

nerve to keep axons tightly fasciculated and channel RGC axon growth within the nerve. The homeodomain transcription factor Vax1 is expressed in the optic disc and optic stalk by E10.5 and later by glial cells of the optic nerve and rostral diencephalon (23). *Vax1* deficiency leads to perturbations in RGC axonogenesis and axon-glia interaction in the optic nerve with axons growing within a restricted cross-segment devoid of astrocytes, which are displaced to the periphery of the nerve. Therefore, Vax1 seems to be involved in axon-glia interaction within the optic nerve possibly by regulating the expression of the attractant molecule netrin-1 which was downregulated at the optic disc and nearly undetectable in the optic stalk in mutant mice.

So far both locally-acting attractive as well as repulsive mechanisms controlling the exit of RGC axons from the retina and formation of the optic nerve have been described. While it is well conceivable that additional factors are involved in these processes, their molecular nature still needs to be determined.

4.3. Optic chiasm

The optic chiasm, where RGC axons cross the ventral midline, is formed at an invariant position along the antero-posterior axis of the forebrain. In animals with binocular vision, the centre of the visual field is perceived by both eyes and visual information is processed bilaterally. RGCs perceiving the same visual hemifield project to the same side of the brain meaning that nasal RGC axons cross the midline at the chiasm to project to the contralateral side while temporal RGCs project ipsilaterally (Figure 1). In mice, which have poor binocular vision, only a small proportion of RGCs residing in the ventrotemporal retina project to the ipsilateral hemisphere (24). For the correct development of the optic chiasm, different mechanisms must exist that (1) control formation of the optic chiasm at the appropriate point and (2) supervise divergence (crossing or not crossing) of projections (Figure 1B).

Early in development (E9.5) the paired box gene 2 (Pax2) is expressed by glial cells in the optic stalk sharply ending at the basis of the diencephalon where the expression of sonic-hedgehog (Shh) starts (25). Shortly before axons start to leave the retina, at E11.5, the Shh expressing area splits in two, leaving a gap where Pax2 expression is extending. In *Pax2* deficient mice, no optic chiasm is formed with all axons projecting ipsilaterally without approaching the midline. In these mice, Shh expression does not display the above-mentioned gap where Pax2 is expressed in wild type mice at E11.5. This suggests that Pax2 is involved in forming the pathway for RGC axons towards the midline and that Shh exerts an inhibitory action on RGC axons at the optic chiasm. In addition to the above described phenotype of *Vax1* deficient optic nerve, RGC axons in these mice do not form an optic chiasm. Instead of penetrating the brain at the base of the hypothalamus most RGC axons terminate there in encapsulated bundles, while a small subset of axons escapes these bundles and projects to ipsilateral visual areas (23). Since, in these mutants, axons in commissural tracts of the anterior CNS fail to cross the midline, Vax1 seems to regulate general mechanisms involved in axon guidance at the CNS midline.

The above discussed Slits have a great impact on the location of the optic chiasm in mice. Unlike in the *Drosophila* ventral nerve cord, where Slit prevents ipsilaterally projecting fibers from crossing the midline and contralateral projecting fibers from re-crossing (26, 27), Slit1 and Slit2 collaborate to channel retinal axons along their appropriate pathway and determine the position of optic chiasm formation in mice (28). In contrast to *Slit1*- or *Slit2* single deficient mice, where the development of the optic chiasm appeared normal, in *Slit1/2* double mutant mice the optic chiasm displayed distinct abnormalities consisting of a variety of pathfinding defects and most strikingly the development of an ectopic commissure in the pre-optic area, anterior to the normal optic chiasm (28).

The accurate crossing of RGC projections at the optic chiasm is a prerequisite for proper binocular vision. Cell bodies of crossing and non-crossing axons are spatially segregated within the retina and several factors are involved in establishing the correct routing. In adult mice, ispilaterally projecting RGCs are located in the ventrotemporal retina making up only 2-3% (depending on the strain) of total projections arising from the retina therefore leading to rather poor binocular vision in this species (24, 29). The vertebrate homolog of the *Drosophila* gene *odd-paired,* Zic2, is a zinc finger transcription factor exclusively expressed in uncrossed RGCs and initiates the "ipsilateral pathfinding" program in this subpopulation of RGCs (Figure 1B) (30). The significance of Zic2 for proper development of binocular vision is underlined by the degree of its expression among species displaying different degrees of binocularity. In mice expressing significantly reduced levels of Zic2 (Zic2 knockdown mice, (31)) heterozygous animals showed reduced ipsilateral projection while in most homozygous animals the ipsilateral projection was absent and the expression of ephrinB2 was altered, making Zic2 a potential candidate acting upstream of ephrinB2 (30). In wildtype mice, ephrinB2 is expressed at the mouse chiasm midline as the ipsilateral projection is generated (32) and is selectively repelling a subset of RGC axons emerging from the ventrotemporal retina, where ipsilaterally projecting RGCs reside. Exclusively in this particular region of the retina RGCs express EphB1, a receptor for ephrinB2. Blocking ephrinB2 function eliminates ipsilateral projections and *EphB1* deficient mice show a dramatic reduction in ipsilateral projections. Therefore, Zic2 and ephrinB2/EphB1 contribute to the initiation of ipsilateral projection of RGCs (30, 32). In contrast to Zic2, Isl2, a LIM homeodomain transcription factor, is expressed by a distinct subset of cells exclusively projecting to the contralateral hemisphere (33). Interestingly, *Isl2* deficient mice show increased ipsilateral projections and increased expression of Zic2 and EphB1 thereby suggesting that Isl2 is involved in Zic2 and EphB1 regulation (33).

Embryonic neurons arranged in an inverted V-shaped array at the future site of chiasm formation express

CD44, a molecule involved in cell-cell interaction in the immune system which was shown to inhibit embryonic retinal axon outgrowth *in vitro* (Figure 1B) (34). *In vivo* ablation of CD44 expressing cells by application of monoclonal antibodies against CD44 led to stalling of RGC axons at the approximate entry site into the optic chiasm region (35). However, this study did not address the fate of the stalling axons leaving unsolved whether RGC axons remain at the entry of the chiasm, are eliminated due to the failure of reaching the target, or take an alternate route e.g. via the ipsilateral projection. Application of anti-CD44 antibodies in slice preparations led to dramatically reduced crossing when applied on E13 and E14 slices while later treatment did not influence crossing at the chiasm indicating a responsiveness of early but not late crossing axons (36). Surprisingly, the late treatment led to a reduction in the uncrossed axon population. Therefore, CD44 is essential for axon crossing and axon divergence at the optic chiasm causing highly selective responses in different RGC axon populations. Very recently, the major CD44 ligand, hyaluronan was shown to co-localize with CD44 at the midline of the optic chiasm (Figure 1B) (37). Exogenous hyaluronan caused a dose-dependent reduction in midline crossing which was reflected in stalling of axons 150-200µm away from the midline in E13 slice cultures (38). Treatment of E15 slice preparations led to a reduction in the uncrossed pathway emerging from the nasal retina. Surprisingly, matrix bound or soluble hyaluronan did not interfere with RGC axon outgrowth *in vitro*. Taken together, hyaluronan fulfils a function in regulating midline crossing and axon divergence at the chiasm possibly due to a balanced interaction with CD44: By providing a permissive substrate for early crossing axons and a repulsive cue, either mediated directly by hyaluronan or other, yet unidentified factors binding to hyaluronan, for late uncrossed axons. The cell adhesion molecule Nr-CAM is most highly expressed by RGCs crossing the midline at the chiasm late in development (E18.5), while radial glia at the chiasm midline display a high anterior to low posterior gradient with overall levels declining with time (39). When Nr-CAM function was blocked by antibodies in E14.5 whole mount *in vitro* preparations, the proportion of ipsilateral projecting RGCs was increased. *Nr-CAM* deficient mice displayed an age dependent increase in the population of ipsilateral projecting RGCs with late born RGCs making more guidance errors. Therefore, Nr-CAM plays a critical role in guiding the late-crossing population of RGC axons at the chiasm, independent of Nr-CAM expression on radial glia cells as shown in chiasm cell/retina explant co-cultures.

The winged helix transcription factor Foxg1 is expressed in most nasal RGCs, a few temporal RGCs and at the optic chiasm (Figure 1B) (40). *Foxg1*-deficient mice show an eightfold increase in ipsilateral projections, arising from both nasal and temporal RGCs, indicating that Foxg1 is required to establish correct ipsilateral projections (40). The expression pattern of Foxg1 and abnormal development of RGC axons from nasal and temporal retina do not support a completely cell-autonomous role for the transcription factor. It rather indicates that Foxg1 controls the proportion of crossing and uncrossing of both nasal and temporal axons, directly at the chiasm. Another winged helix transcription factor, Foxd1 is expressed in ventrotemporal retina as well as the ventral diencephalon during optic chiasm formation. *Foxd1* deficiency leads to Foxg1 expression expanding from nasal into the ventrotemporal retina while the above discussed proteins involved in ipsilateral projection, Zic2 and EphB1, are completely missing in the retina (41). While many ventrotemporal RGCs in *Foxd1* deficient mice project aberrantly to the contralateral side the population of axons projecting ipsilateral is increased, even though the ipsilateral programm controlled by Zic2 and EphB1 is not functional in these animals. In *Foxd1* deficient ventral diencephalon Foxg1 expands into Foxd1-areas while Zic2 expression is minimized. These results indicate a dual function of Foxd1: it is involved in patterning of the retina by acting upstream of proteins involved in ipsilateral projection and plays a role in patterning the ventral diencephalon where the chiasm is formed (41).

Interestingly, in the zebrafish mutant *belladonna,* which encodes *Lhx2*, a LIM homeodomain transcription factor, RGC axons fail to cross the midline, therefore, no optic chiasm is formed and the anterior as well as the post-optic commisures are absent (42). This stands in contrast to the mouse, where *Lhx2* deficiency leads to a more dramatic phenotype including the absence of eyes and forebrain hypoplasia (43). In wildtype mice Lhx2 is expressed in the optic vesicle at E8.5 indicating a role in early eye formation concurrent with the fatal effects of *Lhx2* deficiency. Nevertheless, later in embryonic development Lhx2 is expressed throughout the neural retina, forebrain, midbrain, and hindbrain (43, 44). It is therefore possible that Lhx2 plays a role early in nervous system development as seen in *Lhx2* mutants and has an additional function later which needs to be examined using approaches where timed disruption of Lhx2 function is possible.

Chondroitin sulfate proteoglycans (CSPGs) are extracellular matrix molecules regulating cell-cell and cell-matrix interactions and are expressed in the ventral diencephalon at the time of chiasm formation (45). Enzymatic removal of CSPGs at E13 when the first RGC axons reach the chiasm leads to stalling of growth cones, misrouting of axons and failure to cross the midline. The same treatment at later embryonic stages had no effect on crossing axons but reduced the ipsilateral projections (46). Therefore, CSPGs fulfil two different functions in the course of optic chiasm formation: they are involved in patterning the early phase of RGC axonal growth across the midline and later control axon divergence at the chiasm.

Heparan sulfate (HS) is a linear polysaccharide occuring as a proteoglycan in which two or three HS chains are attached in close proximity to cell surface or extracellular matrix proteins (47). In this form HS can bind to a variety of protein ligands and regulate a wide variety of biological activities, including developmental processes. The developmentally regulated expression pattern in the CNS suggests a functional role for HS in brain development (48). To define this role of HS, the HS-polymerizing enzyme EXT1 was removed in neurons using

a conditional approach (49). In these mice, retinal axons projected ectopically into the contralateral optic nerve thereby reproducing this aspect of the defects observed in *Slit1/2* double mutants.

While so far Slit1/2 are the only molecules known to be involved in positioning the optic chiasm, the divergence of projections at the chiasm is controlled by multiple factors. Some of them, including the transcription factor Zic2 and ephrinB2/EphB1 signalling, initiate the ipsilateral pathfinding program while others, like Isl2 are involved in the formation of the contralteral projection. Data from *Isl2* deficient mice support a possible role for Isl2 in acting upstream of factors involved in the ipsilateral pathfinding program. Transcription factors like Foxd1 and Foxg1 seem to have multiple roles, including regulation of proteins involved in ipsilateral projection and patterning the ventral diencephalon or control divergence of projection in a more global way, respectively.

After crossing the midline at the chiasm RGC axons have to find their path into the optic tract leading towards midbrain targets. The highly abundant peripheral membrane protein GAP-43 which is expressed in growth cones seems to be involved in guiding this way (50). Mice deficient for GAP-43 show an enlarged chiasm due to stalling of axons and a temporal delay of axon outgrowth towards the optic tract with RGC axons displaying abnormal randomized projection into ipsilateral and contralateral optic tracts. Axon growth out of the chiasm into the optic tract was shown to require cell autonomous GAP-43 function to overcome inhibitory signals at the chiasm-optic tract transition zone (51). In addition to axon guidance in the retina and the positioning of the chiasm, Slits also play a role in guiding RGC axons within the optic tract (52). In mice deficient for *Slit1/2*, a subset of RGC axons extends into the telencephalon and grows along the pial surface and does not project deeper into the tissue. The precise level of Slits is critical for the correct projection as *Slit1* deficient, *Slit2* heterozygotes and *Slit1/2* deficient mice display distinct guidance errors. Therefore, after crossing the midline RGC axons are guided by attractive as well as repulsive cues mediated by GAP-43 and Slits, respectively.

4.4. Superior Colliculus

RGC axons project to the SC in a topographic manner along two orthogonally oriented axes, whereby the nasal-to-temporal axis of the retina is mapped along the posterior-to-anterior axis of the SC and the dorsal-to-ventral retinal axis maps along the lateral-to-medial axis of the SC (Figure 1A, C).

During the initiation of the retinotopic map, ephrinA/EphA signaling governs the mapping along the posterior-to-anterior axis in the SC while ephrinB/EphB signaling is involved in medial-to-lateral axis mapping (53, 54). A decreasing gradient of expression of the receptors EphA5 and EphA6 is found from temporal to nasal axons in mouse retina while the ligand ephrinA5 is expressed in an increasing gradient from anterior to posterior SC and the ligand ephrinA2 displays the highest expression midway through the posterior half of the SC decreasing anteriorly and posteriorly (Figure 1A, C) (reviewed in (53)). This expression pattern predicts a model where high receptor expressing RGC axons are prohibited from terminating in the ligand rich posterior part of the SC while low receptor expressing axons are permitted to invade this part of the SC. However, this simplified model is not comprehensive enough to explain the retinocollicular mapping as studies in transgenic animals teach us. Mice deficient for *ephrinA5* display temporal axons projecting more posteriorly in the SC and a proportion of axons transiently overshooting into the inferior colliculus (55). In *ephrinA2* deficient mice more posterior terminations of temporal axons are seen without overshooting into the inferior colliculus (56). Surprisingly, in both mutants, many axons still terminate in their appropriate region rather than displaying a posterior shift leaving the anterior SC vacant. Double homozygotes for *ephrinA2/ephrinA5* display striking mapping defects with temporal axons terminating more posteriorly and nasal axons more anteriorly. Despite these mapping defects, projections of the RGC axons still fill out the complete SC. Interestingly, double heterozygotes also display a phenotype, indicating that absolute levels of ephrinA2 and ephrinA5 play a role in axon giudance. These findings in single as well as double mutants seemed to be contrary to the idea that mapping is accomplished due to the arrest of RGC growth cones once they arrive at a threshold level of the appropriate ligand. Two main hypotheses (reviewed in (53)) could explain these mutant phenotypes and give a more veridical insight into anterior-to-posterior map formation in SC. The mutant phenotypes can be partly explained by a model of desensitisation of EphA receptors via interactions with their ligands in the retina. It has been shown that the ligand ephrinA5 is expressed in a decreasing nasal-to-temporal gradient in mouse retina (Figure 1A) (57). It is therefore possible that an overlapping expression of ephrinA/EphA in the nasal retina decreases the sensitivity of nasal axons to ephrinAs expressed in SC thereby creating a decreasing temporal-to-nasal gradient of "sensitive" EphA receptors (53). The model of combined repulsion and competition of nasal and temporal axons for termination zones in the SC might explain the difference in the degree of more anterior projections in *ephrinA2/A5* double and *ephrinA5* single mutants as well as the fact that RGC projections still fill up the SC when ephrin gradients are disturbed (53, 56). According to this model, temporal axons are unable to terminate in posterior SC because of repelling ephrins and are forced to terminate in the anterior part of the SC. On the other hand, nasal RGC axons are less sensitive to repulsing ephrin signalling and therefore can project to the posterior part of the SC. Finally, nasal axons encounter greater competition for limiting amounts of permissive factors and tend to avoid this in terminating in posterior SC. Recently it was shown that EphA7 is expressed in a reversely oriented gradient to the ephrinAs in the mouse SC (Figure 1C) (58). *In vitro*, retinal axons are repelled from EphA7 containing stripes and mutant mice display a disrupted retinocollicular map with nasal and temporal axons forming additional or extended temination zones, respectively (58). In summary, the graded repulsion mediated by ephrin/Eph signalling is essential, but not sufficient for correct topographic mapping

in the SC. Therefore, the identification of other repulsive factors or attractive molecules which could balance repulsion or induce competition between RGC axons will add to the understanding of topographic mapping in the SC. The recent finding that ephrinA2 *in vitro* can either inhibit or promote retinal axon growth proposes a mapping model where ephrins act as topographic labels that either favour or repress axonal growth depending on the concentration and the retinal position the axon originates from (59).

The transmembrane adhesion molecule L1, already introduced in the intraretinal guidance section, is also involved in correct RGC projection along the posterior-to-anterior axis of the SC (13). In *L1*-deficient mice, temporal axons projected ectopically to more posterior targets in the SC or inferior colliculus (13). It is likely that RGC axons need L1 in addition to ephrinA/EphA mediated axons guidance to initiate the projection along the posterior-to-anterior axis of the SC. The homeodomain transcription factor engrailed-2 (En-2) was shown to have a dual function in the formation of the retinotectal projection in *Xenopus* and chicken. It is expressed in a caudal-to-rostral gradient in the tectum and ectopical expression led to EphA ligand expression (60). Besides this gene-regulatory function, the transcription factor En-2 has a direct repulsive activity on *Xenopus* temporal axons while attracting axons originating from nasal retina *in vitro* (61). This rises the question whether there are more factors involved in retinal axon guidance displaying yet unknown dual functions. The data discussed here show that the simple model of repulsive gradients cannot account for the initiation of the retinocollicular map along the posterior-to-anterior axis of the SC. The models and data reviewed here imply the existence of more factors including "desensitizing" molecules and permissive or attractive factors such as adhesion molecules, growth factors, or neurotrophic factors which axons compete for (62-64).

As mentioned above, ephrinB's in the SC and their EphB receptors in the retina are required for mapping along the lateral-to-medial axis in the SC. EphB2 and EphB3 are expressed in an increasing dorsal-to-ventral gradient in the retina while ephrinB1 is expressed in an increasing lateral-to-medial gradient in the SC and ephrinB3 is strongly expressed at the midline of the SC (Figure 1A, C) (65). As expected, *EphB2/B3* double mutants show abnormal projections along the lateral-to-medial axis of the SC. More interestingly, the replacement of the kinase domain and C-terminus of EphB2 with LacZ lead to an equivalent or even more severe phenotype than the double knock out of *EphB2/B3*, indicating that forward signaling outranks reverse signaling (65). The transcription factor ventral anterior homeobox encoded gene 2 (Vax2) is expressed in the developing mouse retina in a steep high-ventral-to-low dorsal gradient and a shallower high-nasal-to-low-temporal gradient (66). In *Vax2* mutants, the expression of EphB2/B3 is reduced in ventral retina and RGCs projecting to the ventral thalamus display a shift in their termination zones from medial to lateral which is more severe than the defects in *EphB2/B3* double mutants, making Vax2 a presumably upstream regulator of EphB expression (65-67). EphrinB1 signaling in medial to lateral topographic mapping seems to be counterbalanced by the action of Wnt3, originally known as a morphogen but apparently displaying guidance molecule character in the SC (68). *In situ* hybridization studies revealed a high medial to low lateral expression of *Wnt3* in mouse SC, similar to the expression pattern of ephrinB1. In a cell-culture assay, Wnt3 was shown to repel dorsal and ventral RGC axons at higher concentrations but acted growth-stimulating on dorsal but not ventral RGC axons at lower concentrations. Ectopic Wnt3 expression in chick tectum repelled ventral axon termination zones thereby confirming the repulsive action of Wnt3 demonstrated in culture. This Wnt3 induced repulsion of axonal growth involved signaling via the Ryk receptor, as demonstrated in a tissue culture assay where repulsion was inhibited after application of antibodies preventing Wnt3 binding to Ryk. Interestingly, the expression of *Ryk* was found in a high ventral to low dorsal gradient in mouse RGCs. Furthermore, the expression of dominant-negative Ryk in dorsal chick RGCs led to a medial shift of RGC termination zones in the tectum. On the other hand, the Wnt3 induced stimulation of axon growth was shown to be mediated by Frizzled receptors, as this action could be blocked by application of secreted frizzle-related protein 2 (sFRP2). Therefore Schmitt and colleagues suggested a model for medial to lateral mapping in the SC whereby the repulsive Wnt-Ryk pathway competes with the attractive Wnt-Frizzled interaction to actively titrate the response to graded Wnt3 expression which in turn counterbalances the ephrinB1/EphB signaling (68).

In addition to the strict division between the lateral-to-medial and anterior-to-posterior axis in the SC, ephrinA/EphA as well as ephrinB/EphB signaling are also involved in dorso-ventral and temporal-nasal mapping, respectively (56, 65). Therefore, it is rather the correct interplay of a pool of interacting partners accomplishing the retinotopic map in the SC than one signaling system controlling a single aspect.

During development, the retinotopic map experiences refinement where exuberant projections are pruned to more restricted areas. The calcium-sensitive adenylate cyclase 1 (AC1) is necessary to enact retraction response in retinal axons to ephrinA5 during the refinement of the retinotopic map (69). In a retinotectal co-culture preparation, retinal explants from *AC1* deficient mice maintained their exuberant branches and lost their regional selectivity of branching when confronted with wildtype SC while wildtype retinal axons find their correct targets in *AC1* deficient SC, indicating a presynaptic function of AC1. Blocking ephrinA signaling in wildtype co-cultures could mimic the effect of *AC1* deficiency, indicating direct interactions between AC1 and ephrinAs. Interestingly, *AC1* deficient axons display altered axonal retraction when encountering ephrinA5. The response of retinal axons to ephrinA5 was shown to depend on cAMP signaling, probably mediated by AC1 (69). Since it is known that AC1 can be activated by neuronal depolarization and calcium entry (70), neuronal activity might be a suitable regulator of AC1 mediated response to ephrinA5. Indeed,

neuronal activity, independent of synaptic activity, acts synergistically with ephrinA repellent signals to mediate growth cone retraction response. Interestingly, cAMP oscillations rather than absolute levels were necessary for the growth cone response (71).

These results demonstrate that neuronal activity and signalling via guidance cues work together in axonal pathfinding and target recognition. Graded repulsion governed by ephrin/Eph signalling in combination with adhesion molecules and yet to be identified attractive molecules initiate an immature topographic map in the SC which experiences refinement guided by neuronal activity during ongoing embryogenesis. To accomplish the correct retinotopic mapping these factors have to work in concert rather than one factor accomplishing a single aspect of projection.

4.5. Lateral Geniculate Nucleus

As discussed for the SC, electrical activity of a developing neuron in the visual system has effects on the number and location of synapses it forms whereby timing and activity-pattern are of importance. Before the first sensory experiences are made, the initially imprecise RGC projections to LGN segregate into eye-specific domains. This is accomplished by spontaneous waves of activity propagating across the retina leading to synchronous firing of neighboring RGCs (reviewed in detail in (72)). Pattern and segregation of these waves are important parameters to achieve and maintain eye-specific retinogeniculate input segregation. This was for instance demonstrated in mice lacking the *beta2 subunit of the nicotinic acetylcholine receptor* where correlated firing of neighboring RGCs is diminished (73). A recent study examining "no b-wave" mutant mice (*nob*), a spontaneous mutation in the x-linked gene coding for the proteoglycan nyctalopin resulting in no apparent b-wave in electroretinograms, supports the importance of pattern and timing of retinal waves to maintain eye-specific retinogeniculate segregation (74). *Nob* mice show normal spontaneous retinal activity and normal eye-specific segregation in LGN early in development. Around postnatal day 15, when mice open their eyes, spontaneously and visually evoked retinal activities in mutants become abnormal while at the same time axons in the LGN start to desegregate.

Neurons in the dorsal lateral geniculate nucleus (dLGN) express elevated levels of class I major histocompatiblity complex (class I MHC) molecules and the class I MHC receptor component CD3zeta during the first two postnatal weeks when RGC axons sort into eye-specific layers in mice (Figure 1D) (75). Absence of class I MHC or CD3zeta results in a significantly altered retinogeniculate projection similar to abnormalities seen in the cat and ferret visual system when spontaneous retinal activity was blocked. In these mice spontaneous retinal waves were shown to be normal. Therefore the authors concluded that the aberrant retinogeniculate projection was directly due to the loss of class I MHC signaling which is downstream of neuronal activity (75).

As in the SC, ephrin/Eph gradients contribute to RGC target recognition in the dLGN and control patterning of eye-specific retinogeniculate layers (54, 76). EphrinA2 and ephrinA5 are expressed in decreasing gradients from the ventral-lateral-anterior part to dorsal-medial-posterior part of the dLGN (Figure 1D) while ephrinA3 is expressed at low levels in the dLGN displaying no gradient (76, 77). In *ephrinA2/A5* double mutants as well as *ephrinA2/A3/A5* triple mutants the eye-specific layers are formed but their shape and location are strikingly disrupted (76).

The examples discussed here demonstrate the importance of the correct interplay between signaling during pathfinding and first target contact as well as neuronal activity during finetuning of the eye-specific retinogeniculate layers to generate a functional network of connections.

4.6. Thalamo-cortical projection

During embryonic development thalamocortical axons grow into the subcortical telencephalon while further outgrowth towards the cerebral cortex occurs postnatally (78). The initial topography of thalamic projections to visual neocortical areas appears to be controlled by molecules expressed in the subcortical telencephalon, for example the helix-loop-helix transcription factor early B-cell factor 1 (Ebf1) which is present in dorsal thalamus and basal ganglia of the subcortical telencephalon along the path of thalamocortical axons (79). In *Ebf1* mutant mice, thalamic axons fail to make a sharp turn in the direction to the neocortex while growing into the ganglionic eminences leading to dLGN axons projecting abnormally into the amygdala and thalamic axons which grow into the neocortex showing a caudal shift in projection (79). A similar topographic shift in thalamocortical projections was seen in homeodomain transcription factor *distal-less homeobox1* (Dlx1) and *Dlx2* double deficient mice (79). In these animals some thalamic axons failed to grow beyond the basal ganglia while those growing into the neocortex displayed a caudal shift. The internal capsule was highly disorganized and thalamic axons which make a sharp turn within the internal capsule to project into the ganglionic eminences in wildtype failed to do so in mutants. Thus, the apparent misprojection of thalamic axons in both, *Ebf1* and *Dlx1/2* mutant mice might be due to misrouting of thalamic axons before entering the ganglionic eminences therefore rendering these transcription factors important cues in the control of thalamocortical projection and the internal capsule a choice point on their way.

The relative contributions of neuronal activity and directed ingrowth of thalamic axons mediated by axon guidance cues in the formation of retinotopic maps in the neocortex are still intensely debated. Many studies have shown that spontaneous retinal activity is required for the refinement of retinal projections to subcortical targets as discussed above. The controversy of a role of retinal activity in mapping thalamocortical projections might have arisen due to differences in interpretation by various researchers and due to technical issues. Recent studies in mice lacking early spontaneous retinal activity due to the deletion of the *beta2 subunit of the nicotinic acetylcholine receptor* (80) showed that geniculocortical projections fail to refine into a precise retinotopic map (81). Support for a

function of spontaneous retinal activity in thalamocortical mapping comes from studies in ferret and cat (82, 83). In both species the pharmacological blocking of spontaneous retinal activity at the time when LGN axons reach the subcortical plate and innervate layer 4 in the visual cortex completely blocked the formation of ocular dominance columns (ODC) (82). These data from mouse, ferret, and cat strongly support an involvement of spontaneous retinal activity in retinotopic mapping in the visual cortex.

Subplate neurons, which lay in the developing white matter are the first to receive functional synaptic input from the thalamus. These neurons project into the cortical plate before thalamic axons invade cortical layer 4 and are gradually eliminated during ODC formation (84, 85). Ablation of subplate neurons during the first postnatal week blocks the formation of ODC (86) and prevents maturation of the GABAergic synaptic transmission in cat (87) which seems to be necessary for proper ODC segregation.

Neurotrophins, like brain derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3), which are both expressed in cat cerebral cortex at the time when thalamic axons reach the cortical plate might be involved in the establishment of correct thalamocortical connections as well (88). Formation of ODC was inhibited by excessive infusion of BDNF into the visual cortex in cat (89). On the other hand, when the BDNF receptor Trk-B, fused to a human Fc domain was infused, OCD formation was also inhibited (90). These results implicate a dose dependent involvement of BDNF in OCD formation. Surprisingly, in *BDNF* mutant mice or mice with neuron specific ablation of *BDNF*, eye specific segregation to the SC and LGN appeared normal, however, thalamocortical projections have not been investigated in detail in these mice (91). Therefore, it is still possible that BDNF indeed influences thalamocortical projections in mice while another possibility is the compensation of BDNF deficiency by other neurotrophic factors such as NT-3 which is expressed in the cerebral cortex at the time when thalamocortical axons establish layer-specific connections with cortical neurons (88, 92). *NT-3* deficient mice display a reduction in thalamocortical projections and show impaired visual function (93).

Transcription factors like Ebf1, neurotrophins, retinal activity as well as GABAergic inhibitory circuits play a role in the establishment of thalamocortical projections including retinotopic mapping in the visual cortex. However, the precise mechanisms and the interactions of these factors are still unclear. Whether guidance cues known from other choice points in the visual system are involved in guiding thalamic axons to the correct target in the visual cortex as well needs to be investigated.

5. CONCLUSION

The tremendous work in the last decade using transgenic mice as well as loss-and gain-of-function *in vitro* assays revealed a large variety of transcription factors, signalling, and cell adhesion molecules which are involved in axon guidance during the initiation and maturation of the visual pathway. Unique sets of molecules making up the identity of a RGC and its axon have been identified, yet the regulatory mechanisms including initiation of transcription of these molecules are just being elucidated. As illustrated in Figure 1, many molecules playing a role in axon guidance in the retina, at the optic chiasm, and in the SC have been identified while considerably less is known about cues involved in axonal pathfinding towards the LGN and primary visual cortex. Experimental data supporting a role for spontaneous retinal activity working together with guidance cues in the establishment and refinement of the visual pathway changed the debate about visual circuit formation. The growing body of experimental findings concerning molecular and cellular mechanisms as well as patterned neuronal activity begin to provide a comprehensive picture of the intricate mechanisms leading to the complex circuitry necessary for visual input processing.

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Abbreviations: A, anterior; AC1, adenylate cyclase 1; BDNF, brain derived neurotrophic factor; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; DCC, deleted in colorectal cancer; dLGN, dorsal lateral geniculate nucleus; Dlx1/2, distal-less homeobox 1/2; Ebf1, early B-cell factor 1; En-2, engrailed-2; HS, heparan sulfate; L, lateral; LGN, lateral geniculate nucleus; M, medial; MHC, major histocompatibility complex; N, nasal; nob, "no b-wave"; OFL, optic fibre layer; OR, outer retina; P, posterior; RGC, retinal ganglion cell; RGCL, retinal ganglion cell layer; Robo, roundabout; Sema, semaphorin; SC, superior colliculus; T, temporal; V, ventral; Vax2, ventral anterior homeobox encoded gene 2;

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