The Basics of Axon Guidance: Role of Netrin, SHH, BMP and Ephrin

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Declaration:

It is hereby declared that the work presented in this thesis titled "The Basics of Axon Guidance: Role of Netrin, SHH, BMP and Ephrin" has been completed by us (Chowdhury Nusaiba, Noor-A-Afrin and Moumita Dey) as a prerequisite submission for the undergraduate thesis under the course **BTE 450: Biotech Project** in the Biotechnology program of the Department of Mathematics and Natural Sciences of BRAC University, Dhaka. The thesis submitted is my/our own work of review paper while completing a degree at Brac University. It does not contain any materials that have been accepted, submitted, or published for any other degree or diploma at a university or other institution. The thesis does not contain material previously published or written by a third party, and in places where it is, these are appropriately cited through full and accurate referencing. All the primary sources of help have been rightfully acknowledged.

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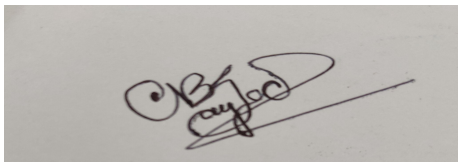

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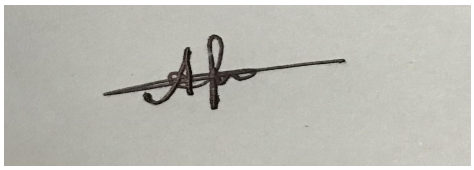

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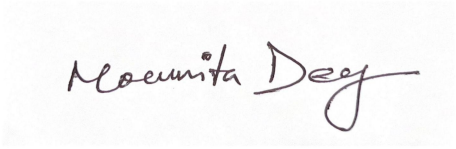

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Dedicated To

Our Parents and Siblings.

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Abstract

Neurons navigate through tissues, over both long and short distances, to locate their target synaptic partners, forming the neural circuit. Specific guidance cues belonging to highly conserved families direct the neurons through their axons along specific pathways by attractive and repulsive actions. These guidance systems create a surprisingly diverse set of neuronal circuits, in the: spinal cord, forebrain, and retinotopic organization. The exact dynamics of the growth cones while navigating through the signaling pathways are not thoroughly understood, only the major mechanisms and proteins commonly found are recognized. The role of several extracellular receptors and ligands in the process has also been identified. Having bifunctional molecules acting as both growth-inductive and inhibitory cues, axon guidance remains a complex process with immediate as well as long-term visible consequences in the form of neural disorders leading to many diseases. The overall mechanism with four of the key molecules that guide this pathfinding in three separate commissures are the topics of this review.

Introduction

The extraordinary feats of the brain of living organisms in information-processing and response-production is largely determined by an extremely complex network of nerve cells or neurons. The human brain alone consists of around 80 billion neurons. The appropriate wiring of the nervous system, hence, depends on the eerily complicated ability of axons and dendrites of the neurons to navigate through pre-existing tissues and pinpoint their correct synaptic partners to form synapses [1]. In the formation of functional neural circuits, it is important that the axons sense and respond to the correct axon guidance cues present in the extracellular environment to ensure that the growing axons, particularly commissural axons, extend from one intermediate target to the next without stalling or recrossing previous targets appositely [2]. Commissures are a primary neuroanatomical feature in living organisms, and a common organizing principle present throughout the central nervous system (CNS). Meanwhile, commissural axons are

neurons connecting two sides of bilateral animals whose axons cross through the floor plate (in case of vertebrates like humans) or through the midline (in case of invertebrates like Drosophila embryos) to change direction and extend longitudinally in the direction of the brain [3]. Commissural axons must initially grow towards the midline, leaving it on the opposite side and never turn back in order to reach the contralateral side of the CNS. The extending axons have a flattened, membranous structure called the growth cone in their tips that are highly motile and sensitive to the presence of the extracellular guidance cues [4,5]. These cues are multifunctional; they can either attract or repel the growth cones, and can function at either close or long range, and can even alter the response or sensitivity of the growth cones to other guidance cues [5]. At long ranges, the gradient diffusion factors are important to guide the growth cones of the commissural axons, whereas at short-range contact-mediated mechanisms involving extracellular matrix (ECM) molecules and non-diffusible cell surfaces are important [6]. In both cases, an adhesive and growth-permissible physical substrate is required for axon growth. Extensive studies on these guidance cues have resulted in the identifications of four major groups: the netrins, slits, semaphorins, and ephrins, along with other classes of molecules that also play a key role in guidance such as morphogens, growth factors, cell adhesion molecules (CAMs), and glycoproteins [2]. Each guidance cue works in its own way, and sometimes they combine or reverse each other's effects. The basic mechanism of axon guidance mediated by the key guidance cues will be discussed here with their role in the three main commissures.

The basic mechanism of Axon Guidance:

Growth cones, the extremely motile structures at the terminals of developing nerve fibers, respond to biochemical stimuli in the environment to direct axons to their destination. The reorganization and dynamics of actin and microtubule cytoskeleton control how the axon advances, retracts, turns, and branches, which are caused by motility and guiding of growth cones. The growth cone's centermost portion is dominated by packed microtubules, while the periphery is characterized by actin filaments, which form a cobweb in veil-like lamellipodia and heaps in finger-like filopodia [7,8] [9] (see Figure-1 below).

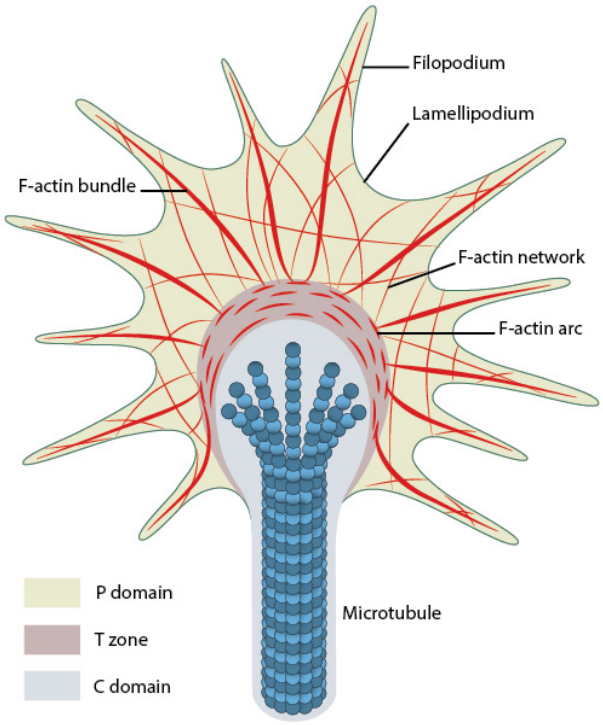

Figure-1: The peripheral (P) domain, the transitional (T) domain, and the central (C) domain are the three main sections of the cytoskeletal elements found in growth cones. The P domain is made up mostly of unipolar actin filament bundles that are inserted in a less polar actin matrix. It has filopodia and lamellipodia that are active. Microtubules can also be detected in this region on a temporary basis. The T domain connects the P and C domains via a narrow interface. The C domain is found in the growth cone's center, closest to the axon. It's mostly made up of microtubules and has a lot of organelles and vesicles [9]. Image sourced from: steve. 2018. "What Is Axon Guidance and the Growth Cone? | MBInfo." MBInfo. 2018.

Filopodia are required for the detection of guiding cues and the control of the growth cone. Actin filaments are essential for cell motility and are well implanted at the forefront of the growth cone, making them accessible targets for guiding cues. Even though multitudes of stable microtubules reside in the growth cone's center, a number of active microtubules can proactively examine the periphery and pierce filopodia. Therefore, they can engage with signaling pathways associated with guidance cue receptors' cytoplasmic domains. Furthermore, because actin

filaments and microtubules are linked, signaling pathways that control one cytoskeletal element's movements will also impact the other. For activities like cell motility and growth cone guiding, regulatory and structural connections between the two systems are essential [8].

The Growth Cone Cytoskeleton Is Composed of Polarized Polymers:

Actin filaments and microtubules are the two key cytoskeletal components in growth cones. Actin filaments and MTs are constantly active. Both polymers must exist in a stable state at times and as dynamic structures at other times in order for the cell to function properly. Neurons must exercise fine control over the dynamics of both MTs and actin filaments in order to develop long axons and dendrites and lead these processes to their final synaptic partners. Neurons include a complicated combination of actin- and MT-associated proteins, as well as a range of isoforms and post-translational modifications, to fulfill these activities [7,10].

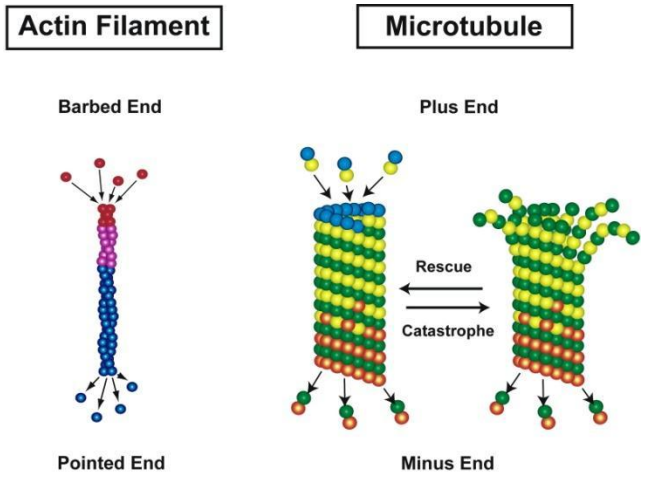

Figure-2: ATP-actin and ADP-actin may be added and removed from both the barbed and pointed ends of actin filaments in vitro. α/GTP-β-tubulin dimers add to the plus or growing end of microtubules, and α/GTP-β-tubulin dimers dissociate from the minus end [10]. Image sourced from: Dent, Erik W., and Frank B. Gertler. "Cytoskeletal dynamics and transport in growth cone motility and axon guidance.", *Cell Press*, October 2003.

Growth cone motility is regulated by Actin Filament dynamics:

During retrograde actin flow, actin filaments also pull the growth cone membrane backward and are essential in growth cone retraction. Both filopodia and lamellipodia have a constant state of

myosin-dependent retrograde flow, which comprises phases of net building of actin filaments at the sharp end, reverse migration of actin networks, and filament breakdown proximally. Retrograde actin flow may control axon extension and prevent microtubules from entering the growth cone's peripheral area [55,57].

The dynamics of Microtubules are critical for growth cone guidance:

Microtubules, which were long thought to have a secondary function in guiding the development cone towards attractive signals and away from repulsive signals to actin filaments, are now viewed as key participants. Microtubules can explore lamellipodia and filopodia because of their dynamic characteristics, or their capacity to expand and shrink under dynamic instability. The fast-growing ends of microtubules face outward towards the edge in growth cones. Specific stabilization and destabilization of microtubules were sufficient to produce attractive and repulsive turning [8].

Retrograde F-Actin Flow:

This constitutive process occurs when ATP-actin is formed into filaments along the membrane in the distal P area of the growth cone, and transported backward into the T region as polymeric F-actin. Several proteins are likely severing and depolymerizing F-actin in the T region, which is now made up of ADP-actin monomers. The cycle is continued as ADP-actin is regenerated into ATP-actin. The backward movement of F-actin in the P region of the growth cone is a myosin motor-driven activity that happens in both filopodia and lamellipodia [10].

A model for Axon Guidance:

When the growth cone is engaged in random movement, there is balanced actin polymerization and depolymerization occurring across the growth cone with time. This would culminate in balanced protrusion and retraction, allowing the growth cone to follow a straight path [10].

Actin polymerization has been demonstrated to be aided by guidance cues such as attractant cues that attract axons. Attractive signals cause axon branching and growth cone filopodia to quickly rise in cortical neurons, along with an increase in actin filaments. As a result, microtubules in the axon shaft and the growth cones play apart. The freshly polymerized actin bundles can then engage with these dynamic microtubules to trigger outgrowth in new directions. It promotes the production of filopodial structures on hippocampal neurons, which necessitates the phosphorylation of Ena/VASP proteins via PKA activation (protein kinase A). By antagonizing capping proteins that typically stop actin filament elongation, Ena/VASP proteins enhance

elongation at the growing end of actin filaments which look barbed. Ena/VASP inactivation lowers the length and quantity of filopodia by reducing the number of bundled actin filaments, whereas boosting Ena/VASP activity enhances filopodia production by increasing the number of actin filament bundles. Thus, through the actin remodeling activities of Ena/VASP proteins, attractant cues like- netrin can alter axon guidance by modulating the development of growth cone filopodia [8].

Repellent guidance cues collapse growth cones by the depolymerization of actin filaments. Loss of actin filament bundles and the preservation of retrograde flow prompted microtubules to discontinue their dynamic exploration of the lamellipodia and collapse backward into the growth cone core. These alterations in cytoskeletal dynamics had no effect on axon elongation, but they did limit axon branching, which resulted in changes in the direction of growth [8] (as can be viewed in Figure-3).

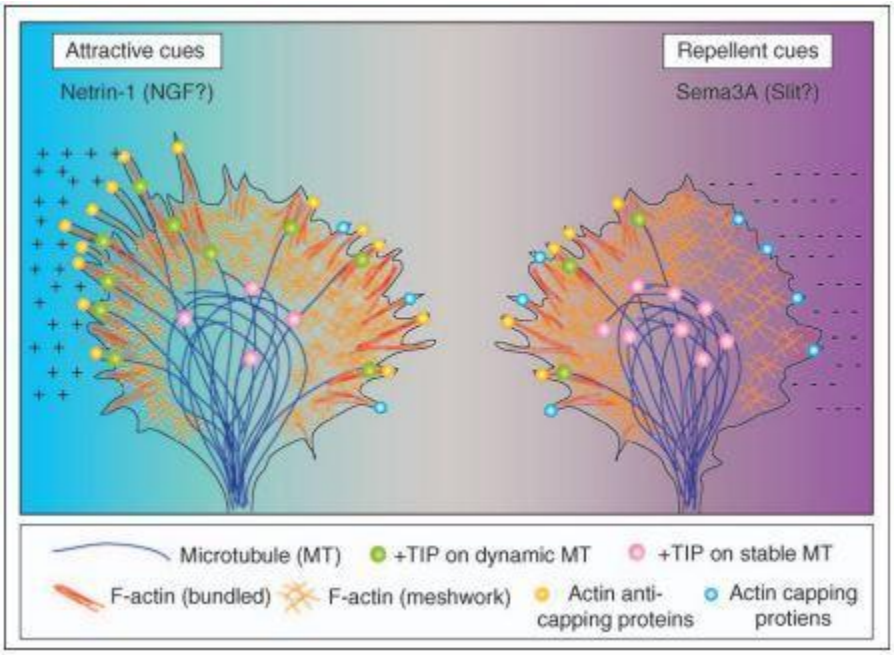

Figure-3: Model of cytoskeletal rearrangement and dynamics in response to attracting and repelling signals in growth cones. The unstimulated side of the development cone is the side facing away from guiding signals. The growth cone is made up of bundled stable microtubules in the core, a lamellipodium meshwork of actin filaments, actin filament bundles that pierce filopodia, and dynamic microtubules that extend towards the periphery and interact with actin filament [8]. Image sourced from: Kalil, Katherine, and Erik W. Dent. "Touch and go: guidance cues signal to the growth cone cytoskeleton." *Current Opinion in Neurobiology,* October 2005.

Molecules involved:

Netrin

Netrins are a family of secreted proteins that work as chemotropic axon guidance cues during development of the neural circuit. First described in 1990, in the nematode *Caenorhabditis elegans,* netrin was named UNC-6 and has been found to be conserved in all species examined since then [11]. In mammals, three secreted netrins have been found (netrin-1, -3 and -4), along with two Glycosylphosphatidylinositol (GPI)-anchored membrane proteins, netrin-G1 and G2 [12]. In broad terms, netrin has been found to have two activities: breaking down neuronal symmetry and guiding the growing axon [13]. Moreover, netrin and its downstream effectors play a crucial role in initiating a left/right directional bias for circumferential guidance. Among all the netrins, netrin-1 is the most extensively studied protein, due to its prominent role in axon-guidance, as well as axon branching, regeneration, synaptogenesis, cell-cell adhesion, as well as cell migration [14,15,16]. Cells expressing netrin-1 in significant amounts have been found to serve a growth boundary for axons. In vertebrates, netrin-1 is an extracellular matrix-protein secreted by the floor plate (FP) cells at the ventral midline of the neural tubes in embryos, where it creates a gradient orienting axons with respect to the ventral midline [17,18]. As netrin diffuses away from the floor plate, it becomes associated with cell membranes or ECM [15]. Netrin-1 then acts locally by enhancing growth cone adhesion of the axons [17]. The neural progenitor cells (NPCs) in the ventricular zone (VZ) locally produce this netrin-1, depositing it on the pial surface like a haptotactic adhesive for the growth cones [20]. The circumferential gradient created by netrin is bifunctional: it attracts some axons to the midline and repels others [21]. For the secreted netrins, the receptor that mediates chemoattraction is DCC (deleted in colorectal cancer), while receptors for repulsion include an UNC5 homologue, and sometimes UNC-40, which primarily disrupts ventral guidance, a member of the DCC superfamily [12]. Axons only secreting DCC will not be able to repulse, but only attract. Conversely, axons able to secret both DCC and/or UNC5 conditionally have the capacity to switch between attraction and repulsion. The attraction-repulsion “switch” can be caused by a number of factors including, but not limited to, (a) relative concentration of netrins on the growth cone surface, (b) extracellular concentrations of netrin-1 along with relative affinities of specific receptors for that, and (c) presence of other signaling molecules (e.g. Ca2+, cGMP, or cAMP)

that favor either attraction or repulsion in the extracellular environment [12,22]. The following table shows how the different components and factors change for the attractive and repulsive behavior of netrin.

	Attraction	**Repulsion**
Receptor(s) involved	**DCC (only)**	**DCC and UNC5**
Netrin-1 concentration	Decreases	Increases
Cytoplasmic Ca2+ concentration	Increases	Decreases
cGMP and/or cAMP concentration	Increases	Decreases
Surface UNC5	Decreases	Increases
Substrate adhesion to laminin-1	Absent	Present

Table-1: Summary of major factors in the "switches" between attractive and repulsive functions of netrin-1 and the way they differ.

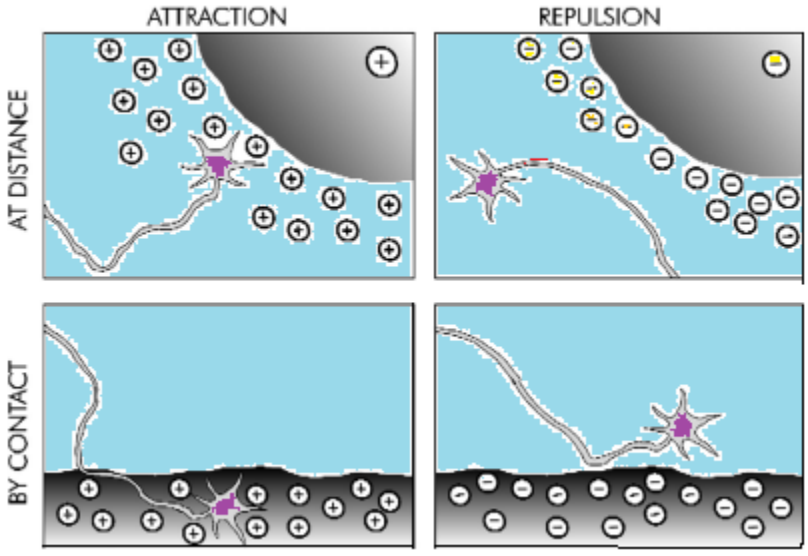

Figure-4: Summarized representation of how the attractive and repulsive actions work

SHH

The Sonic Hedgehog (SHH) protein is encoded by the shh gene and acts as a chemical signal primarily required for embryonic development. It is also involved in cell development, cell specialization, and appropriate body forming (patterning). This protein is necessary for the development of the brain and spinal cord (central nervous system), as well as the eyes, limbs, and many other body components [23]. SHH was discovered first in a search for mutations that affect patterning of the Drosophila larva. The expression of this protein can typically be observed in the prechordal plate, notochord, and floor plate of the vertebrate embryo [24]. According to a study, sonic hedgehog (SHH) may replicate *in vitro* the additional chemoattractant activity of the floor plate and thereby operate as a chemoattractant on isolated axons [25]. Moreover, during direct retinal ganglion axonal growth at the diencephalic ventral midline, SHH can act as a negative factor [26]. Similar to netrin, SHH also works by creating a gradient (see Figure-5).

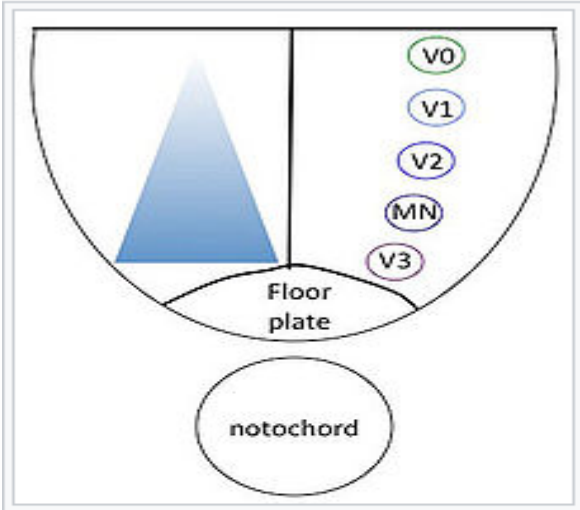

Figure-5: Floor-plate secreted SHH creating a gradient along the ventral neural tube, working in a concentration-dependent way to specify neuronal fates and guiding axons. V0 to V3 indicates different classes of ventral interneurons while MN stands for motor neurons [27].

Image sourced from: Kolpak, Adrianne, Jinhua Zhang, and Zheng Zheng Bao. 2005. "Sonic Hedgehog Has a Dual Effect on the Growth of Retinal Ganglion Axons Depending on Its Concentration." Journal of Neuroscience 25 (13): 3432–41

The mode of action of SHH on axons has been shown by a direct effect of SHH on the growth cones of the targeted neurons[27]. Despite SHH being only transcribed medioventrally in the

floor plate and the underlying notochord, this protein is found across the ventral half of the neural tube, suggesting a more extensive signaling role [28]. SHH has the ability to collaborate with other hormones to stimulate certain cell types. In fact, SHH and retinoic acid often work together to induce interneuron progenitors [29] [30].

BMP

Bone morphogenetic proteins (BMPs) belong to a family of multi-functional growth factors that are also a part of the transforming growth factor beta (TGFβ) superfamily which includes activins, inhibins, Growth Differentiation Factors (GDFs), etc.[31] Starting from the formation of the skeleton, various organs, the retina, lens, and ciliary body, to establishing the correct left-right patterning of the embryo, to directing the pathway of sensory neurons through axon guidance, BMPs contribute to a large range of developmental processes [32]. BMP is also a morphogen, i.e. signaling molecules that are produced in a constricted region of a tissue that diffuses from their source over a distance to provide positional information through a long-range concentration gradient [33]. Morphogens, along with netrin-1, determine the fate of neuronal cell specification and axon guidance. In fact, BMP and SHH work together to pattern neural progenitors in the ventral and dorsal spinal cord, before acting as guidance cues by repelling the axons of the differentiated commissural neurons from the dorsal midline (BMP works alone) and attracting it to the ventral midline (SHH and netrin-1 work together) [34]. BMP7 and BMP6, both expressed by the roof plate of spinal cord, simultaneously function as a chemorepellent. The former was shown to directly cause commissural axon growth cones collapse, producing a rapid change in cytoskeletal organization [35]. BMPs also regulate the rate of growth of dorsal interneurons (dI) axons during their extension through the spinal cord [36]. An important guidance receptor in translating BMP chemorepellent signals into directed motion of the axons away from the dorsal midline is the BMPRIB, transducing *in vitro* reorienting activity of the roof plate [37]. BMPs, hence, act as a classic feed-forward signal.

Ephrin

Expressed typically on the cell surface, ephrin is a part of the eph family receptor interacting protein, and it serves as the ligands of Eph receptor. The eph/ephrin signaling controls a wide spectrum of biological activities such as guidance of axon growth cones, creation of tissue boundary, cell migration, and segmentation, during embryonic development. Not only that, it maintains several processes which are critical during adulthood long-term potentiation, angiogenesis as well as stem cell differentiation. On top of that, the anteroposterior mapping labels for the retinotectal/retinocollicular projection are also believed to be controlled by ephrin-A2 and -A5. This is proved as the focal retinal labeling exhibits moderate map abnormalities if any of the genes for the ephrin proteins mentioned is dysfunctional. In case of double heterozygotes for the genes, the phenotype is influenced by absolute levels. Moreover, ephrins play an essential role as anteroposterior topographic labels in the axon competition process for mapping. Specific ephrins (A2 and A5) are needed for mapping on both axes of the brain [36], working as both attractive and repulsive molecules as shown in Figure-6 below.

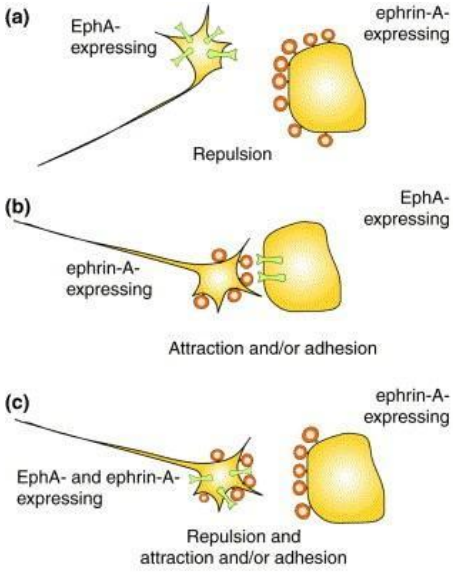

Figure-6: Schematic drawing of the interaction of EphA and ephrin-A expressing cells. EphA receptors and glycosylphosphatidylinositol (GPI)-anchored ephrin-As mediate bi-directional signaling [38]. Image sourced from: Knöll, Bernd, and Uwe Drescher. 2002. "Ephrin-As as receptors in topographic projections." Trends in neurosciences 25, no. 3: 145-149.

There are 18 genetically distinct viruses that utilize the Eph receptor (Eph), ephrin ligand (ephrin), or ephrin-Eph signaling to their advantage [37]. On a gloomier note, the overexpression of Eph and ephrin can develop a number of human cancers, including gastrointestinal malignancies and, specifically, colorectal malignancies [39].

In studies where chick embryos were injected with truncated EphA3 receptors, clear mapping errors were found in the retinas labeled in the temporal extremes. In 9 out of 15 embryos, there were additional terminations in abnormal locations. As the following Figure-7 shows, part-A portrays a pattern of retroviral vector infection in chick retina. The temporal retinal axons in chick were labeled by DiI and visualized by fluorescence microscopy. Part-B of the image shows the map of wild-type temporal axons, while part-C shows mutant chick temporal axons demonstrating ectopic arbors. The arrows in the *inset* show ectopic arbors [40].

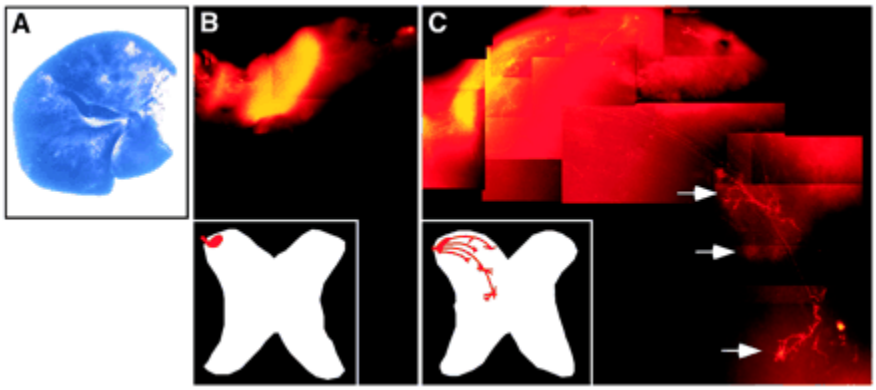

Figure-7: Knockout chick showing mapping and guidance errors in temporal retinal axons due to cytoplasmically truncated EphA3 [51] Image sourced from: Suárez, Rodrigo, Ilan Gobius, and Linda J. Richards. "Evolution and development of interhemispheric connections in the vertebrate forebrain." *Frontiers in human neuroscience* 8 (2014): 497.

Processes:

Spinal Cord commissure

In the developing spinal cord, commissural neurons are formed in the dorsal cord region and grow by extending their axons towards the floor plate (FP) intermediate target [39]. After crossing the midline, most of the commissural axons exit the floor plate on the contralateral side, before turning rostrally towards the brain [41]. However, the particular trajectory of the spinal axons are not homogeneous, and depends on their point and time of origin. Some axons may cross the midline ventrally, while others cross dorsally; some axons cross over to both sides of the spinal cord while some axons cross either ventrally or rostrally. During early developmental stages, such as approximately day 9.5 in a mouse embryo (E9.5), the spinal cord commissural axons face the dilemma of whether to cross the midline or not; around E14, the axons cross the midline ventrally from the floor plate, but at later periods, some axons cross dorsally above the central canal as well [42]. One thing observed is that commissural axons of the spinal cord never cross the roof plate (RP) at the dorsal midline which led to the discovery of a RP-derived chemorepellent, mediated by the bone morphogenetic protein (BMP) family [27]. In the vertebrate spinal cord, BMP, SHH and netrin-1 act along the dorsal-ventral axis together to control the pathway finding of the axons from the dorsal spinal cord to the ventral midline (see Figure-9). As already mentioned previously, the BMP7 protein in the spinal cord RP repels axons of growing commissural neurons, thereby orienting their advance towards the floor plate [27]. These seem to be acting over a distance to aid in cell patterning of the dorsal spinal cord region [43]. While BMPs work to repel the axons of the differentiated commissural neurons from the dorsal midline, netrin-1 and SHH work to attract them to the ventral midline as shown in Figure-8 [34].

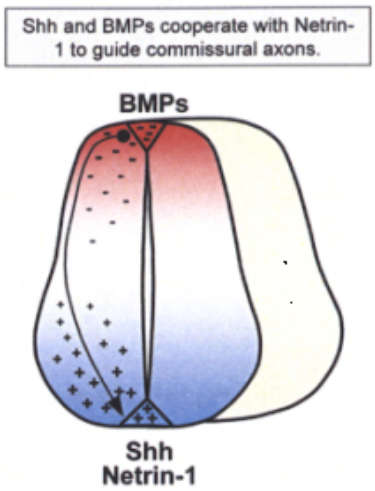

Figure-8: SHH and Netrin-1 act as attractive cues, while BMP acts as a repulsive guidance cue to cooperatively guide commissural axons [23] Image sourced from: Bambakidis, Nicholas C., and Kaine Onwuzulike. 2012. "Sonic Hedgehog Signaling and Potential Therapeutic Indications." In Vitamins and Hormones, 88:379–94. Academic Press Inc. doi:10.1016/B978-0-12-394622-5.00017-1.

From *in situ* hybridization analysis, it has been demonstrated that the cells in the white and gray matter of the adult spinal cord express netrin-1 [44]. It acts as a demarcation of the midline, both for vertebrates and invertebrates. Initially during axogenesis, the commissural axons grow around a boundary created by netrin1-expressing cells in the ventricular zone [20]. The floor plate cells at the ventral midline of the spinal cord secretes netrin-1 and creates a bi-functional gradient, as discussed previously [21]. Although netrin-1 was thought to only function in the long-range like this, it has been found that it acts locally as well when neural progenitor cells (NPCs) in the ventricular zone produce it and deposit it on the pial surface as a haptotactic adhesive substrate, guiding axon growth ventrally in reliance with DCC receptor. As development proceeds, the commissural axons grow around a boundary created by the netrin-1-expressing cells [20]. Through the reorganization of the actin and microtubule networks, netrin-1 changes the entire architecture of the cytoskeleton after binding to specific receptors, stimulating commissural axon guidance [12]. In broad terms, the floor plate cells at the ventral midline of neural tubes in vertebrates' spinal cord secrete netrin-1 which acts at a short range for

commissural axon attraction towards the dorsal midline, while repelling other axons at long range [19]. For repulsion, netrin-1 acts as a negative guidance cue, causing the growing axon to collapse and retract the growth cone. These are released at the midline locally, where it repels motor axons to prevent it from crossing the midline as can be seen in Figure-9 [45]. Overall, through the combined effect of their attractive and repulsive mechanisms, netrin-1 acts as a boundary, directing the formation of spinal neuron circuits.

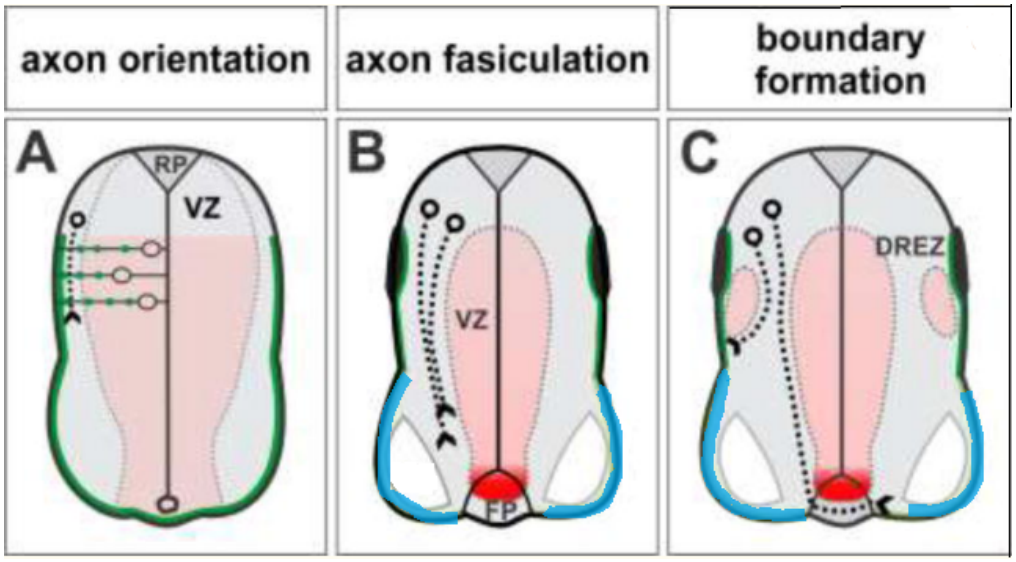

Figure-9: Diagrammatic representation of how netrin-1 acts as a boundary directing spinal cord commissural axon guidance. In (A) the green color shows regions containing netrin-1 secreting cells; these orient ventrally-directed growth of axons. In B) and C) the commissural axons grow adjacent to pial-netrin1 in the dorsal spinal cord (dark green parts), while avoiding the pial-netrin1 in the ventral spinal cord (light blue) [20]. Image sourced from: Varadarajan, Supraja G., and Samantha J. Butler. 2017. "Netrin1 Establishes Multiple Boundaries for Axon Growth in the Developing Spinal Cord." Developmental Biology 430 (1). Elsevier Inc.: 177–87

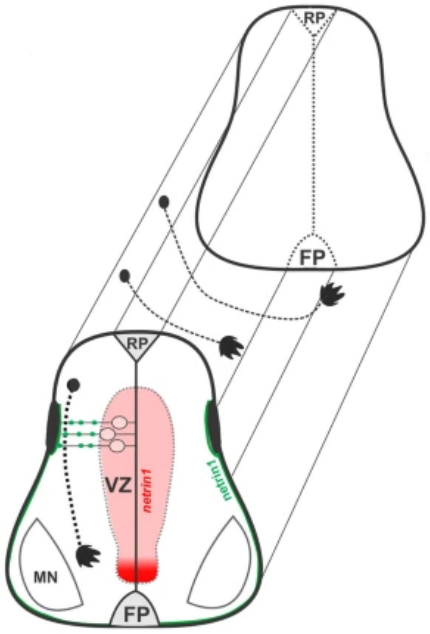

Figure-10: Three-dimensional view of a spinal cord commissure and how commissural axons grow [27] Image sourced from: Kolpak, Adrianne, Jinhua Zhang, and Zheng Zheng Bao. 2005. "Sonic Hedgehog Has a Dual Effect on the Growth of Retinal Ganglion Axons Depending on Its Concentration." Journal of Neuroscience 25 (13): 3432–41.

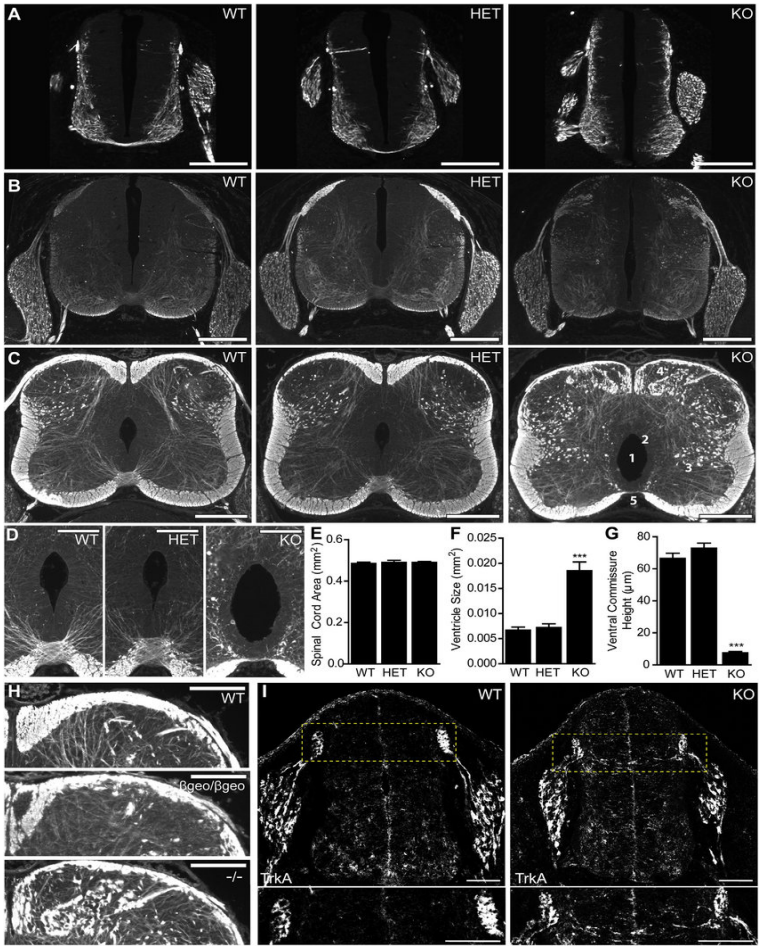

Figure-11: Netrin-1 À/À knockout spinal cords imaging [47] Image sourced from: Bin, Jenea M., Dong Han, Karen Lai Wing Sun, Louis-Philippe Croteau, Emilie Dumontier, Jean-Francois Cloutier, Artur Kania, and Timothy E. Kennedy. "Complete loss of netrin-1 results in embryonic lethality and severe axon guidance defects without increased neural cell death." *Cell reports* 12, no. 7 (2015): 1099-1106.

As can be seen in the diagram above (Figure-11), multiple defects are apparent in the netrin-1 À/À knockout spinal cords, including a larger central canal (as seen by the number 1 in third row KO), thinning of the ventricular zone surrounding the deformed central canal (2 in same picture), (3) mislocalized axon bundles (marked 3 in that image), a disorganized dorsal root entry zone (marked 4), and finally the resultant nearly complete loss of ventral commissure. Image-D shows the magnification of ventral commissure and C shows the central canal in. Overall, a complete

loss of netrin-1 can result in mice embryonic lethality due to serious axon-guidance defects, leading to increased neural cell demise [47].

As for SHH, it is expressed in an anterior–to-posterior increasing gradient in the spinal cord midline (as seen in experiments with chicks) repelling spinal cord commissural axons to grow anteriorly after midline crossing, as shown in Figure-12 [46]. SHH, which is expressed as a gradient from the floor plate, controls commissural axon guidance in the human spinal cord by functioning as a chemoattractive agent in cooperation with Netrin1 [1]. SHH also plays a part in the spinal cord's longitudinal axon guidance and the retinal ganglion cell axon guidance [2,3]. Morphogens like FGFs and Wnts along with SHH interact with heparan sulfate which plays an important role during cortical development [4].

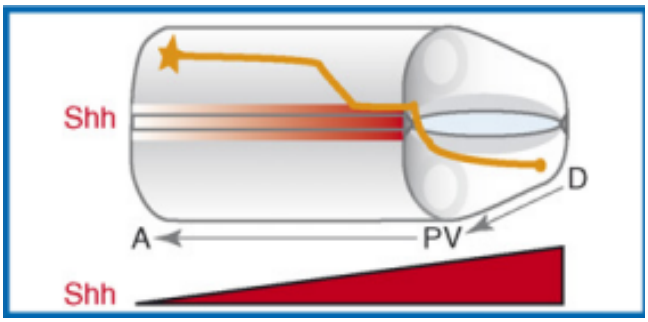

Figure-12: Diagram of SHH repelling spinal cord commissural axons from growing anteriorly [46] Image sourced from: Zou, Yimin, and Anna I. Lyuksyutova. 2007. “Morphogens as Conserved Axon Guidance Cues.” Current Opinion in Neurobiology.

The Ephrin family guidance cues are also required for axon guidance in both the anterior–posterior-(ephrinAs) and medial–lateral dimension (ephrinB). In the embryonic spinal cord of vertebrates, ephrin-B3 is located primarily on the floor plate (FP) at the ventral midline (VM), while ephrin-B1 and -B2 genes are expressed mostly in the dorsal spinal cord. This results in intersecting EphB receptor-bearing commissural axons to navigate between both the ventral and dorsal ephrin-B domains. Commissural axons in the developing spinal cord (as seen in mice) follow a flanking trajectory toward and across the floor plate (FP), lock on a ventral midline (VM)-associated intermediate target, which they never cross again. Studies have also shown that ephrin-B proteins stimulate the collapse of commissural growth cones. The decussated commissural axons, on the other hand, inappropriately enter and occupy the dorsal regions of the

spinal cord when EphB/ephrin-B interactions are disturbed *in vitro*. This facilitates the hypothesis that ephrin-B3 functions as a repulsive barrier at the spinal cord midline may even constrain the orientation of commissural axons on the opposite side of the FP. In summation, Ephin-B3-mediated forward signaling is essential in guiding axons at the midline of the embryonic spinal cord [48]. A summary of the axon-guidance molecules with their key functions in the spinal cord commissure is shown below in Table-2.

Axon guidance molecule	**Location of origin**	**Receptors and Co-receptors**	**Functions in the spinal cord circuit development**
Netrin-1	Floor plate	DCC	Commissural axons confined to the ventral spinal cord. Motor axon repelled from midline
		UNC5	Sensory axon repulsion
SHH	Floor plate	BOC/SMO	Commissural axon pre-and post-midline crossing guidance
BMP7	Dorsal spinal cord	BMPR1A	Commissural axon dorsal repulsion
Ephrin(B3)	Spinal cord midline glia	EPHA4	Midline crossing, both dorsal and ventral

Table-2: A summary of the axon guidance molecular described, and their role in the spinal cord commissural axon guidance

Forebrain commissure

Commissural development in mammals is a highly organized procedure and it is regulated by both cell-autonomous transcription factor expression and non-cell autonomous processes, such as the establishment of midline glial structures and their production of particular axon guidance molecules. Axons are directed in the right direction by these systems, allowing distinct brain regions to be linked to their respective objectives [49]. Forebrain commissures have a particularly conserved developmental plan, a comparable place inside the brain, and homogenous patterns of connection among vertebrate species.

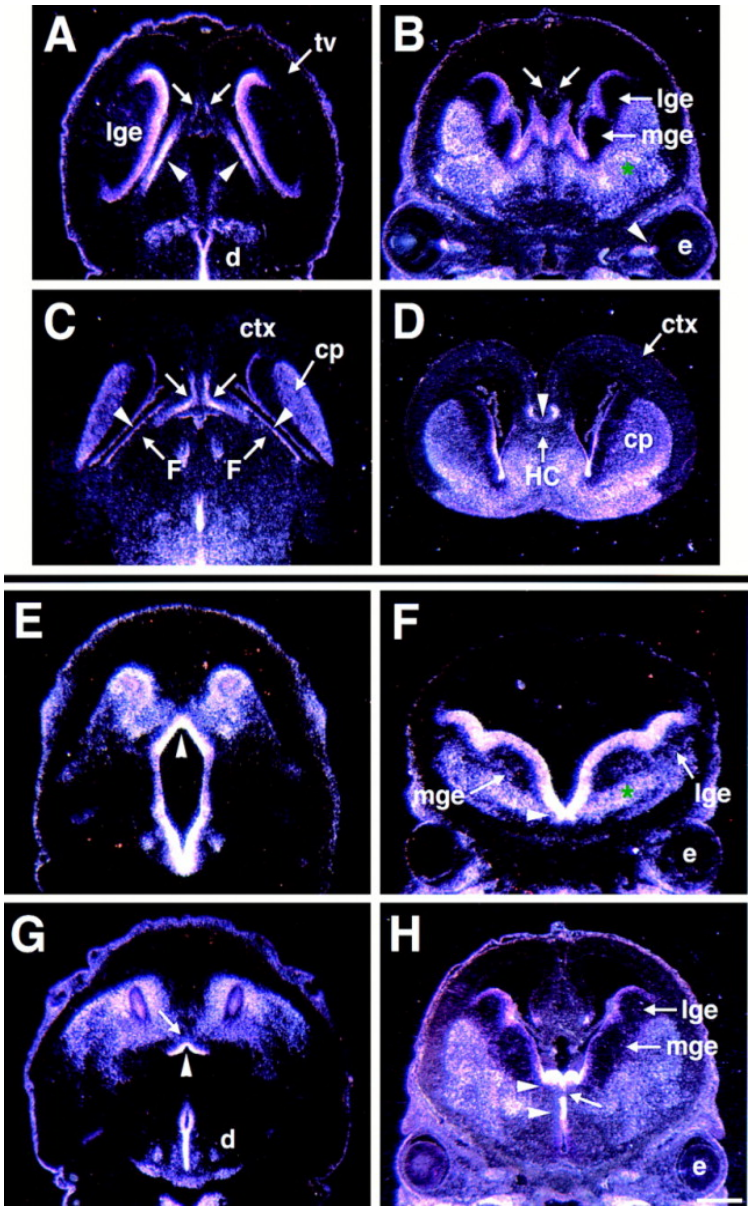

Figure-13: Expression of *Netrin-1* playing a direct role in the formation of the Fimbria, Hippocampal Commissure, Corpus Callosum, and Anterior Commissure [50]. Image sourced from: Serafini, Tito, Sophia A. Colamarino, E. David Leonardo, Hao Wang, Rosa Beddington, William C. Skarnes, and Marc Tessier-Lavigne. 1996. "Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system." Cell 87, no. 6: 1001-1014.

In Figure-13 shown above, the horizontal (A, C, E, and G; rostral is up) and coronal (B, D, F, and H; dorsal is the upwards direction) sections of E12.5 (E and F), E14.5 (A, B, G, and H) embryos and E16.5 brains (C and D) were subjected to *in situ* hybridization with a 35S-labeled *netrin-1*

probe. It was found that the sections in (E) and (G) are more ventral than those in (A) and (C); whereas the sections in (F) and (H) are more anterior than those in (B) and (D). Here, E12.5 means embryonic day 12.5, and so on.

Parts A and B show that Netrin-1 is expressed in the forebrain at E14.5 in the medial walls of the telencephalic vesicles (tv as indicated by the arrowheads) and at the surfaces where the two vesicles meet more rostrally (arrows). Expression is abundant in the ventricular zones of the ganglionic eminences (labeled as medial [mge] and lateral [lge]) as well as ventral forebrain cells that have migrated away from the ventricular surface (shown by green asterisk). Expression is also shown by the diencephalon (d) and epidermis. At the exit point of the retina and along the optic nerve further expression can be seen. The forming fimbria (F) at E16.5 is located along the area of netrin-1 expression at the medial hemisphere walls. The axons are placed between this area and the netrin-1-expressing region: the space where the two hemispheres connect at their rostral end (arrows in [C], with a subsequent higher expression at this age). The observed expression pattern continues across the midline (arrowhead in [D]) in cells touched by axons passing to the opposite hemisphere and creates the hippocampal commissure (HC). At E12.5, netrin-1 is expressed immediately next to where anterior commissural axons would intersect in the developing forebrain, in a tail-like manner (arrowheads in [E] and [F]). Defects in various forebrain commissures are also identified, suggesting that netrin-1 may be playing other guiding functions [59]**.**

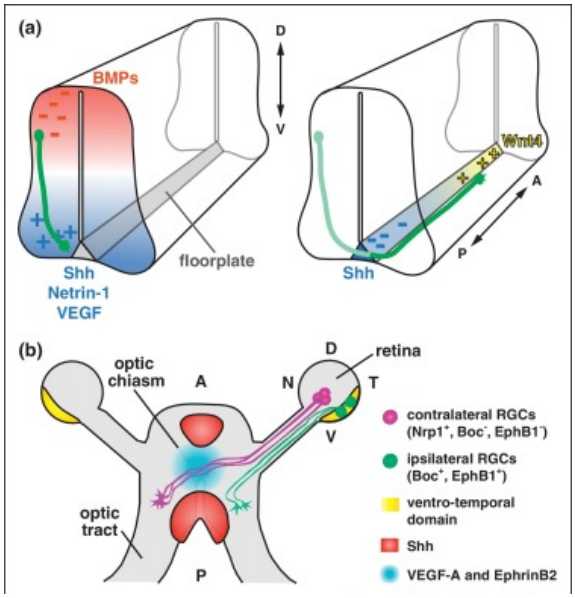

Figure-14: Signaling mechanisms of non-conventional axon guidance cues: the SHH, BMP and Wnt morphogens [52] Image sourced from: Yam, Patricia T., and Frédéric Charron. 2013. "Signaling mechanisms of non-conventional axon guidance cues: the Shh, BMP and Wnt morphogens." Current opinion in neurobiology 23, no. 6: 965-973.

As shown in Figure-14, during the developmental stages in embryos, glial structures are purposefully placed around the forebrain commissural tracts, destined to form mature cellular borders. These glial structures play vital roles in commissural development by providing guidance signals that intercept axons from leaving the determined tract and entering neighboring tissues [53,54]. Moreover, these glia serve as a foundation for the guiding of commissural axon development, being precisely positioned by SHH and Slit proteins' expression. During brain development, aside from netrin, SHH also offers signals for axon guidance and regionalization [55]. SHH and Slits were together shown to regulate the proper placement of glia in the zebrafish midline post-optic and optic commissures. In that system, SHH does not play a direct role in axon guidance, but rather governs the proper patterning of Slit isoforms, which in turn directs axon guidance [56]

Studies have shown that if the midline glial structures are disrupted in any way, it may lead to defects in the development of mammalian forebrain commissures. The role of fibroblast growth factors (FGFs) in midline glial development and commissure formation is critical [49]. Despite

the absence of this much-important fibroblast growth factors, Fgf1, there were no impairments in the expression of Slit2, Slit3, Robo1, Bone morphogenetic protein 4 (Bmp4), or Growth-associated protein 43 (GAP43) in the telencephalic midline, and only a modest drop in Netrin1 expression [57]. Infact, Bmp signaling was shown to be involved in growth and patterning in the dorsal telencephalon. This shows the importance of the axon guidance proteins in the forebrain development,

Furthermore, Ephrins and Eph receptors were found to be a kind of short-range guidance molecule in the developing mouse forebrain. These molecules have the ability to begin bidirectional signaling as well as offer repulsive cell-to-cell interaction activities [58]. A thorough investigation of mice containing defects in genes encoding several Eph receptors and B-class Ephrins provided evidence for the necessity of EphrinB3 and EphB1 in the corpus callosum development. Knockout mice lacking Ephb1 or Efnb3 (the gene encoding EphrinB3) showed abnormal callosal projections and Probst bundle development. Several investigations, however, have found gradients of Eph receptors, and Ephrins that may have given regional signals for afferent connections. The hypothesis is that these chemicals govern axon guidance to the correct cortical location, and may also be involved directly in directing callosal axons across the midline, as well as commissural axon targeting in the contralateral hemisphere [59,60].

Retinotectal Axon Guidance:

The retina is a layer of light sensitive cells found at the back of the eye which receives light and triggers nerve impulses that pass via the optic nerve to the brain, forming a visual image. Axons from the retina, particularly retinal ganglion cells (RGC) axons, navigate out of the eye, travel across the optic chiasm, and dorsally enter through the optic tract to reach the optic tectum [61]. Found at the back of the cerebral cortex, the optic tectum is a major part in the midbrain of vertebrates that functions as the main visual processor of the brain. See Figure-15 below for visual representation of the way the retina and the brain is connected.

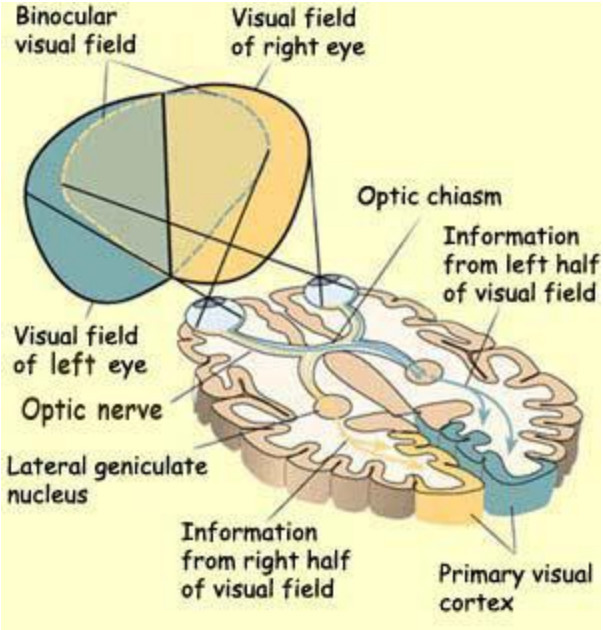

Figure-15: Formation of visual field by the retina and the brain. The left eye's visual field (in blue) is analyzed in the right visual cortex, and the right eye's visual field (in yellow) in the left cortex. The binocular visual field is the central area where the two eyes' visual fields overlap [62] Image sourced from: Pittman, Andrew J., Mei Yee Law, and Chi Bin Chien. 2008. "Pathfinding in a Large Vertebrate Axon Tract: Isotypic Interactions Guide Retinotectal Axons at Multiple Choice Points." Development 135 (17): 2865–71.

Generally, in the vertebrate eye, all the RGC axons project towards the optic disc, and on arrival they change their course to project out of the eye along the axis of optic nerve and go toward the brain [63]. This is somewhat similar to the behavior of commissural axons in the developing spinal cord projecting to the ventral midline floor plate to turn and grow along the axis of the spinal cord toward the brain. The dorsal RGC axons, which are about 10% of total emerging axons, find their targets in the lateral tectum (also known as superior colliculus) while the ventral RGC axons travel towards the medial tectum, forming a smooth topographic representation of the visual world from the retinal layer to the optic tectum/superior colliculus (see Figure-16) [46]. Axons from some other RGCs project to a part of the midbrain called the pretectum that controls the opening of the pupil and certain eye movements. The RGC axons grow in a directed manner towards the optic nerve head (ONH) found just outside the optic nerve. They must pass through this before entering the optic nerve to leave the eye [62].

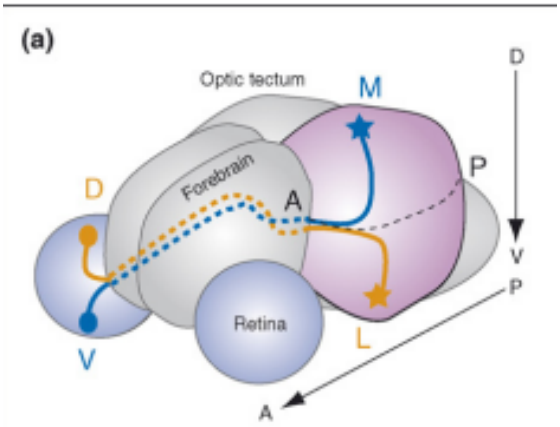

Figure-16: A representation of the dorsal–ventral topographic organization of visual map in vertebrates [46]. Image sourced from: Zou, Yimin, and Anna I. Lyuksyutova. 2007. "Morphogens as Conserved Axon Guidance Cues." Current Opinion in Neurobiology. doi:10.1016/j.conb.2007.01.006.

Guidance molecules including netrin, ephrins, BMPs and SHH are expressed in several places along with pathway, in order to direct the growth of RGC growth cones. At first, the retinal axons meet netrin-1 in the ONH, just before the beginning of the optic pathway. Netrin-1 then proceeds to attract the axons towards this region. It is, in fact, localized to surfaces of the cells that form the optic fissure and early optic disc, further proving that this guidance molecule is involved in RGC axon exit from the retina into the optic nerve [63]. After an extension of 400 μm further along the pathway, the growing axons meet netrin-1 again, but this time they ignore it, demonstrating a loss in attraction due to change in location [64]. Moreover, netrin-1 causes the activation of a 'destructive' molecule, caspase-3, in retinal growth cones in a proteasomal-dependent manner, which is involved in chemotropic guidance of retinal axons. The inhibition of this apoptotic enzyme was also found to block netrin-induced chemorepulsion that is required for growth cone guidance *in vitro* [65]. An *in vivo* study showing how metalloproteinases regulate the growth and guidance of RGC axons by cleaving the DCC receptors necessary for netrin-1 action (previously discussed) underscores the role played by netrin here [66]. In addition, at the optical disk surrounding the exiting RGC axons in mouse eye, immunoreactivity for netrin-1 was measured at significant levels, showing how this guidance molecule assists in axon guidance as the RGC axons leave the retina into the optic nerve. Consequently, in netrin-1- and DCC-deficient mice, retinal axons were found to extend normally to the vicinity of the optic disc but then were unable to exit the eye properly [63]. It has been

postulated that substrate-bound netrin-1 protein may be present in a short-range gradient, directly guiding the RGC axonal growth toward the ONH or its direct vicinity. Subsequent axons are guided into the optic nerve by selective fasciculation [67]. The entire process, with netrin-1 and Ephrin-B gradients along with axon projection direction is visually summarized in Figure-17 below.

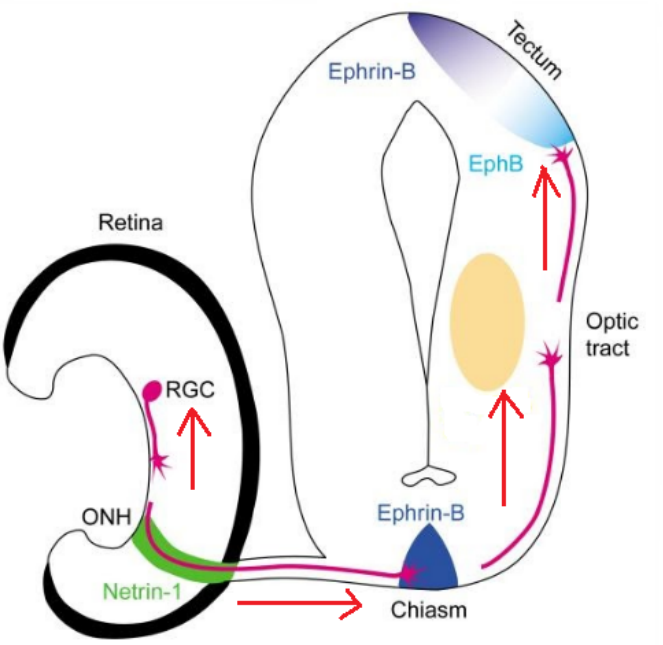

Figure-17: Diagram of the embryonic visual pathway showing the projection direction of the axons (red arrows), and the primary site at which netrin and ephrin molecules work locally in guidance[62] Image sourced from: Pittman, Andrew J., Mei Yee Law, and Chi Bin Chien. 2008. "Pathfinding in a Large Vertebrate Axon Tract: Isotypic Interactions Guide Retinotectal Axons at Multiple Choice Points." Development 135 (17): 2865–71.

Another important family of guidance cues in the retinotectal pathways is the ephrin molecules. This family of guidance cues are required for axonal guidance in both the anterior–posterior- (ephrinAs) and medial–lateral dimension (ephrinB1) [46]. Experiments in chick have found that ephrin-A2 and ephrin-A5 are expressed in overlapping posterior > anterior gradients across the tectum while the receptor EphA3 is expressed in a corresponding temporal > nasal gradient across the retina [68]. It has been shown (see Figure-18) that both ephrin-A2 and-A5 act in the retinotectal mapping as chemo-repellents: in case of overexpression, they repel temporal retinal

axons *in vivo*, and are expressed in the target in counter gradients with respect to retinal EphA receptors [36]. This helps to show both the additive and distinctive working mode of ephrins.

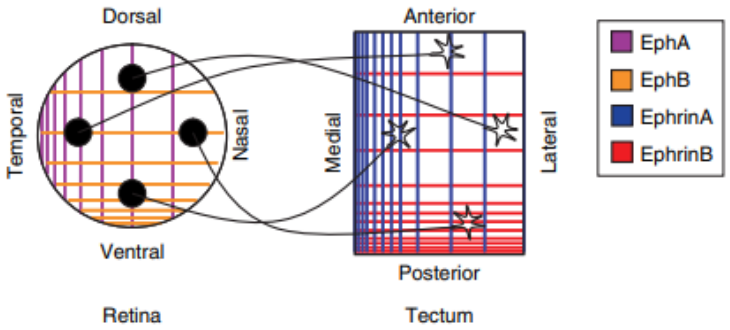

Figure-18: A visual map showing the establishment of retinotectal projections by gradients of Eph receptors and ephrins [72] Image sourced from: Avilés, Evelyn C., Nicole H. Wilson, and Esther T. Stoeckli. 2013. "Sonic Hedgehog and Wnt: Antagonists in Morphogenesis but Collaborators in Axon Guidance." Frontiers in Cellular Neuroscience.

Finally, while the guidance molecule SHH acts as an attractant for pre-crossing commissural axons in the spinal cord, it inhibits the growth of chick RGC axons [46]. It directly regulates the growth of RGC axons and is important in dorsal–ventral patterning of the retina [69]. SHH repression at the midline establishes the accurate navigation of the visual axons. This has been proved by experiments in which addition of SHH to retinal neurons extending over a laminin substrate induced a rapid, reversible and re-inducible retraction of the axon fibers and the growth cones [70]. Interestingly,in the retina, SHH has been found to be bifunctional: low concentrations promote RGC axon outgrowth toward the optic disc, whereas high concentrations push axons into the optic nerve [71]. Along with netrin-1 and EphBs. SHH secreted from the central retina at first works to influence RGC axon projection inside the retina [32]. Alternatively, after leaving the eye through the optic disc, RGC axons approach the optic chiasm to enter the contralateral side of the brain, at which point SHH is expressed along the border of the optic chiasm, forming a barrier at the ventral midline, ultimately guiding the projection of RGC axons. Hence, ectopic expression of SHH at the midline prevents RGC axons from crossing as shown in Figure-19 [70].

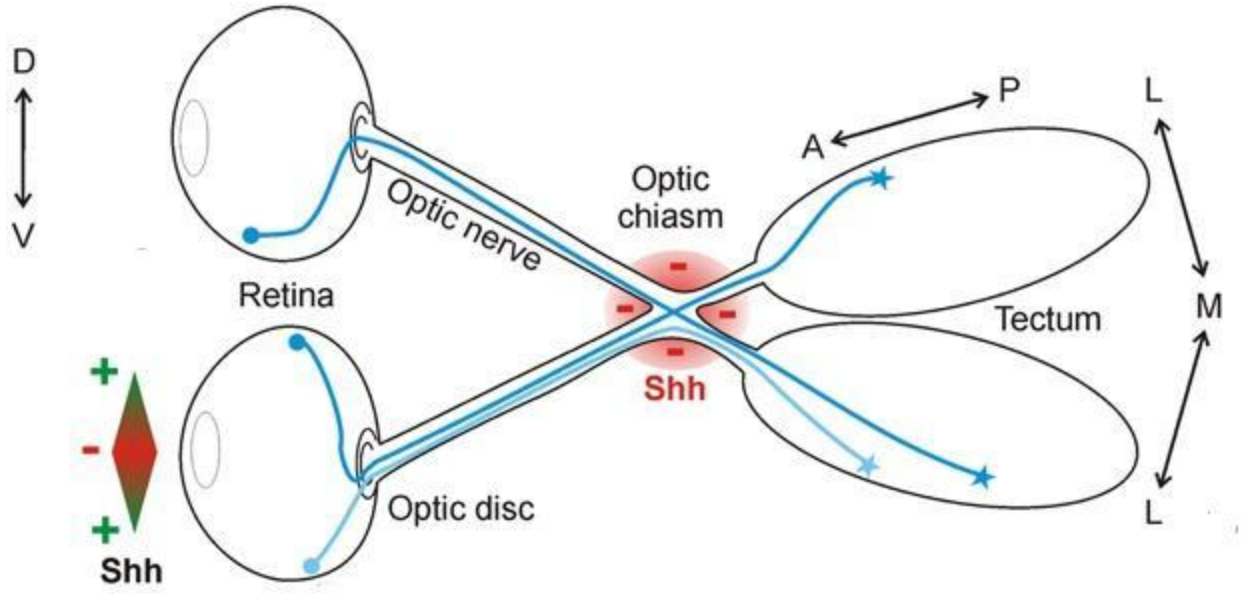

Figure-19: SHH expression along the border of the optic chiasm (red), where it acts as a repellent for RGC axons in the optic nerve [71] Image sourced from: Trousse, F., E. Martí, P. Gruss, M. Torres, and P. Bovolenta. 2001. "Control of Retinal Ganglion Cell Axon Growth: A New Role ForSonic Hedgehog." Development 128 (20): 3927–36.

Similarly, BMPs are repellents too, with no reports of any attractive functions. The BMP receptors Ia, Ib, and II are expressed in the developing retina, where BmprIb has the most striking gradient of expression. With high ventral expression and much lower dorsal expression during embryonic development, this receptor then shifts to a more uniform expression along this axis in the first postnatal week. Moreover, BmprIb-deficient mice have been found to have defects in pathfinding of RGC axons that originate from ventral cells, though the targeting of the axons from the dorsal RGC to the ONH is normal, showing that BMP is needed in localized repulsion and axon guidance only [72]. In fact, the distinct BmprIb gradient of expression along the dorsal–ventral axis of the retina firmly supports a role for BMP signaling in dorsal–ventral retinal mapping.

Conclusion

The nervous system is an amazing organization of numerous complex identities, together which work to create a functional living being. Our current knowledge of the intricacies of the neural circuit can still be said to be at its infancy. As portrayed in this paper, neurones navigate, often over long distances, along very specific pathways to find and reach their targets. They do this by extending the growth cones of their axons. Specific axon guidance cues regulate the cytoskeletal dynamics in the growth cone, controlling signaling pathways, to ensure the right response from the right neuron at the right time. Disorders of the nervous system may arise from a defective assembly or functioning of the guidance cues. Several families of extracellular receptors and their ligands work in conjunction with these guidance molecules. In the presence of elaborate regulatory mechanisms, only a small number of guidance molecules can create such an intricate pattern of neuronal circuit. The four main ways– contact attraction, chemoattraction, contact repulsion, chemorepulsion– help to guide axons. Depending on the cellular context, one guidance molecule can work as bi-functional, either repelling or attracting axons, and the growth cone responses to these are very plastic as well. Moreover, molecules previously thought to only control patterning have been found in axon guidance too. This explains the diversity in growth cone behaviors, mostly attributed to the multifunctionality of the guidance molecules. With a brief overview of the axon guidance mechanism, four different guidance molecules (netrin, SHH, BMP, and Ephrin) are the topics of this paper here along with their role in the three different commissural processes. These sophisticated and dynamic sets of guidance molecules help the neurons to respond to changing environmental cues and successfully reach their destination. Each molecule has its unique mode of action in each of the commissures, yet have certain core similarities in the way they are concentration-dependent or contact-mediated with specific receptors. More insight is needed into the axon-guidance process, from a multidisciplinary approach, to determine how the billions of neurons find their targets. This would facilitate better understanding and treatment of the neuronal disorders resulting from the axon-guidance defects: a central goal of molecular studies of axon guidance.

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