Chapter

Cell-Cell and Cell-Matrix Interactions during Axons Guidance

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Abstract

The establishment of neuronal connections during development is a critical process for the correct function of central nervous system and for their regeneration during adult stages. Axon extension and guidance toward their targets are a complex process involving several signals provided by extracellular milieu where secreted factors, other cells, axons, and extracellular matrix proteins are interacting to establish the wiring of the brain. The expression of those signals at specific time and space, and their mechanisms of action during axon projection are the subject of numerous studies. This knowledge had contributed to understand the complex panorama of brain wiring during development and the origin and possible cure of central nervous system diseases. In this chapter, we focus on cell-cell and cell-matrix interactions as two important signals during axon guidance, and how these interactions impact the response to diffusible guidance cues. We emphasize the need and the challenge to understand the complex relations among simultaneous signals to guide axons projections, and how this knowledge could influence approaches to deal with neural regeneration issues.

Keywords: growth cone, guidance cues, fasciculation, extracellular matrix, axonal regeneration

1. Introduction

After neural tube formation, multipotent stem cells migrate and generate precursor cells that will differentiate into neurons and glial cells. Neurons will extend cell projections to become integrated to the brain circuits by a finely regulated process. Through cell extensions, classified as dendrites and axons, neurons are responsible for the perception of the external world, the former are in charge of receiving electric impulses of other cells, and the last transmit the impulses far from the cell body. Axons projections are stereotyped, and the accuracy of reaching their target is fundamental for the correct central nervous system (CNS) functioning. Axons project by specialized and motile structures located at the end of the axons called growth cones. These specialized regions sense the external milieu, detecting signals that originate complex cellular mechanism involved in axon elongation; therefore, neuronal pathfinding is highly regulated by the availability of the external signals, by the expression of cell receptors, and by specific molecular mechanisms that stimulate or inhibit growth cone displacement. Extracellular matrix

components present in axons pathway can be signaling by forming soluble chemotropic protein gradients and/or by direct interaction with membrane receptors at the growth cones. Besides, other axons present in the pathway could be promoting axons-axons interactions or fasciculation, allowing the guidance of projections toward their final targets. In this chapter, we make a rough description of cellular mechanisms of growth cone motility that drives axon elongation, mainly focused in the cell adhesion and cytoskeleton regulation by guidance cues, followed by some of the evidences about cell-extracellular matrix and cell-cell interactions relevance during axons projection; finally, we address the importance of synergic interaction among the signals, and how they can modulate the response of axons during pathfinding toward their targets.

2. Cellular mechanisms of axon projection

The growth cone is a specialized structure located at the end of axons or dendrites, capable to detect extracellular guidance cues and to integrate them into a projection or retraction movement that guides the axon toward their innervation target [1]. During axonal elongation, changes in the growth cone morphology and in the direction of its projection depend on the cytoskeleton dynamics and on the regulation of cell adhesion, inducing the formation of filopodia and lamellipodia at the leading edge and exerting tensile and traction forces that will influence neuron elongation [2, 3]. According to the cytoskeleton distribution, the growth cone can be divided into three structural domains: the central domain (C domain), in which there are stable microtubule (MT) bundles entering the growth cone from the axon axis, organelles, vesicles and actin bundles; the peripheral domain (P domain), located in the distal part of the growth cone, containing actin filaments (F-actin bundles) that form the filopodia and lamellipodia, and dynamic MT that extends from the C domain and invades the P domain following the F-actin bundles. Finally, the intermediate zone between the C and P domain called the transition zone (T zone), which contains actomyosin contractile structures located perpendicular to the F-actin bundles [4, 5].

The lamellipodia and filopodia are dynamic structures, from which the elongation process starts. Lamellipodia are broader structures, rich in actin filament networks, while the filopodia are thin extensions, about 100–200 nm diameter and 10 μm in length, constituted by a 10–30 very close actin filaments arranged in parallel [5]. The rate of F-actin and MT polymerization and the retrograde flow of F-actin determine the extension and retraction of the growth cone. In a rough description, actin monomers assemble into F-actin filaments at the cell membrane boundary of the P zone, pushing the membrane during the elongation, thus generating tensile forces. By a retrograde flow driven by actomyosin contractility and by the cell-extracellular matrix interaction, filaments push themselves backward to the T region where filaments are severed and recycled [2, 5, 6]. Assembly and disassembly of the F-actin filaments by controlling the polymerization rate of globular monomeric actin (G-actin) are important for the advance or retraction and is influenced by guidance cues [7, 8]. At the same time, MT plus ends point toward axon tips, and their assembly and disassembly at growth cone are regulated by the F-actin bundles and by the traction force exerted by the actomyosin contractility, allowing the capture and guidance of MT extension through the T and P domain, stabilizing the filopodia [5]. MT polymerization and invasion of P domain, coupled to substrate anchor of growth cone projections by cell adhesion sites linked to the cytoskeleton, promotes growth cone, pulling forward using the actomyosin-mediated

force projection; therefore, MT advances while the C domain transforms in an axon segment, consolidating the axon elongation [9]. Cytoskeleton dynamics and cell adhesion regulation therefore are very important during the response to guidance cues, for example, at the side of the growth cone turning toward an attractant cue, a stabilization and a decrease of F-actin retrograde flow and anchoring through adhesion sites is present, while the inhibition of F-actin and MT polymerization occurs at the growth cone side retracted in response to a repulsive guidance cues [10–12].

Adhesion of growth cones to the substrate is finely regulated during axon elongation or retraction. Adhesion sites or "contact points" (CP) are constituted by protein complexes that allow the adhesion and generation of traction force on the substrate [13]; these complexes mediate the anchoring of cells by the transmembrane protein integrins that have a primordial role, coupling the cytoskeleton to the extracellular matrix and by recruiting adaptor and signaling proteins at the cytosolic side [14]. Adhesion complexes include several associated proteins that mediate the interactions with the cytoskeleton, regulate actin polymerization, and participate in the signaling exerted by cell adhesion [2, 14, 15]. During axon projection, assembly-disassembly of CP are involved in response to guidance cues; the inhibition of turnover of CP inhibits axons outgrowth, while localized assembly and turnover of CP promote axon extension in response to guidance cues [13, 16, 17], for example, activation of integrins and subsequent focal adhesion kinase (FAK) phosphorylation are involved in the attraction of dendrites mediated by the chemotrophic protein semaphorin 3A [18], while repellant factors as myelin-associated glycoprotein (MAG) induce growth cone turning by a rapid endocytosis of integrins and loss of cell adhesions [19]. In summary, the projection or retraction of growth cones responds to extracellular signals that guide them to specific targets and trigger a complex network of signal transduction mechanisms that includes the dynamic remodeling of cell adhesion sites and cytoskeleton that together translate into elongation or retraction movements for the redirection of the neuronal projections.

2.1 Cell-extracellular matrix interactions

As mentioned earlier, neurons project their neurites to specific targets, guided by extracellular signals integrated by the growth cone. The mechanisms to direct axons projection are triggered by secreted chemotropic proteins, by proteins anchored to the substrate, or by direct interactions between axons mediated by proteins anchored to the cell membrane, as cell adhesion proteins or even chemotropic protein receptors [20, 21]. Growth cones respond to gradients of diffusible molecules, or of proteins associated with the substrate, that guide them to the innervation target; these molecules can be chemoattractive or chemorepellant, and the extracellular matrix can stabilize the gradients from target cells or intermediate cells, extending them at a greater distance [21]. Although classical chemotropic proteins as ephrins, netrins, slits, and semaphorins are some of the more studied guidance cues, in this chapter, we focus our attention to ECM and cell-anchored proteins as axon guidance cues.

The interaction with extracellular matrix (ECM) components was one of the first proposed axon guidance cues that exert a "contact guidance" effect, improving axon projection [22]. ECM components surround cells and are distributed along the pathways of axon projection [23, 24]; therefore, they are not only part of the support in which neurons are divided and maintained but also has a relevant role in the signaling and the determination of the differentiation and migration of neurons, and in the elongation processes of neurites [25–27]. In addition, the physical

properties of ECM as topography and stiffness have now an increasing interest as factors that influence axon projection [28].

ECM comprises about 40% of extracellular space in developing brains as compared with the 20% in adult brains [29]. Some of the most relevant ECM proteins implicated in axonal projection are laminin, fibronectin, collagen, and tenascin. Laminins are a heterotrimeric glycoproteins family, formed of α, β, and γ subunits. During CNS development, laminins have an important role in promoting cell migration and axonal outgrowth [26], and the absence of laminins results in important axon-targeting alterations [30–32]. Fibronectin is a glycoprotein present in the early development at central and peripheral nervous system in the spinal cord and cortex [33, 34] and is involved in cell migration, cell adhesion, and in stimulation of neurite outgrowth during development and after peripheral nervous system injury [35–37]. Both laminins and fibronectin have an important role in modulating the response to chemotropic proteins [38, 39]. Collagen is a family of fibrillar glycoproteins that gives structure and support to cells as well as anchorage for other proteins [40, 41]. Collagens have an important role in neurite outgrowth, axon guidance, and axon targeting, and their absence impacts central and peripheral axons, targeting as a motor axon guidance and retinal ganglion cell projection [42–44]. Tenascin is another ECM glycoprotein family with several functional domains [45]. *In vitro* and *in vivo* experiments have shown an inhibitory effect of tenascin for several kinds of axons as hippocampal and cerebellar neurons [46, 47]; however, specific alternative spliced variants promote neurite outgrowth, as the fibronectin type-III domain of tenascin C that induce cerebellar neurons outgrowth [48]. Chondroitin sulfate proteoglycans (CSPGs) are ECM proteoglycans with both inhibitory and attractant effects on axonal outgrowth. The accumulation of CSPG in scar tissue, after injuries in adult CNS, inhibits axon outgrowth [49]; however, it is also a permissive signal along axonal pathways during the development of retinal projection, or in the cortex [50–53], and their inhibitory effects are attenuated by the presence of laminin-1 [54, 55].

Recent studies have shown that the ECM stiffness determines cellular processes such as differentiation, proliferation, and migration [56–58]. Particularly, the work of Engler et al. demonstrated for the first time that the stiffness of the substrate in which stem cells are grown in vitro can modulate their differentiation into cell types such as bone, muscle, or neurons [59]. Probably, one of the first studied aspects has been the role of stiffness in the elongation of neurites; Flanagan et al. reported that when primary neurons of the mouse spinal cord (E13.5) grew in matrices with less stiffness, close to that found in the brain, the elongation of the neurites was greater [60]. However, there are divergences in the data depending on the model, since it has been reported that on softer substrates, PC12 cells show few neurites, relatively short and unbranched, whereas on stiffer substrates, cortical neurons and astrocytes (E17-E19) turn out to have longer and branched projections [57, 60, 61].

In the case of developing nervous system, variations in stiffness during development stages, and at different regions as cerebral cortex and optic tectum have been reported [62, 63]. Guidance by chemotropic proteins as slits and semaphorins of retinal ganglion cell (RGC) axons projecting from the retina to the optic tectum (OT) has been extensively reported [64, 65]; interestingly, tissue stiffness also determines their projection, since RGC axons project toward softer OT and grow as fascicles while traversing stiffer regions. Once the axons arrive at the OT, the softer tissue slows down the projections and splays apart the fascicles to branch them and to form synapses with their stereotypic targets [63]. On the other hand, the prevention of axon regeneration after injury can be in part due to changes in ECM and tissue stiffness, as shown for glial scar after spinal cord injury, where components as

collagen IV and laminin, and changes in glial intermediate filaments as vimentin and GFAP, soften the tissue at the scar [66].

Besides stiffness, ECM topography is also a factor that determines the orientation and projection of neurites. Since early observation about the alignment and orientation of axons by "contact guidance," and the improving of axon elongation by aligned collagen fibrils [22, 67], advance in micro- and nanofabrication of biocompatible fibrous substrates, with specific topography and orientation, has shown to improve neurite elongation and orientation, promoting nerve regeneration [68]. Fibers alignment and dimensions are important to improve axonal guidance and elongation, for example, the micrometer versus nanometer dimensions of poly(lactic-glycolic acid) PLGA fibers improve the alignment of neurites [69], and aligned versus nonaligned gelatin and chitosan fibers induce a higher formation of filopodia in Schwann cells, improving the orientation of axon projections along the fibers [70].

2.2 Cell-cell interactions

During the first stages of CNS formation, neuron clusters projects pioneering axons to form longitudinal, transversal, and commissural tracts [71], functioning as scaffolds for latter or follower axons. Early axon scaffolds are well conserved in vertebrates, and common tracts had been described in zebrafish, chick, mouse, sea lamprey, and others. Among common tracts, the ventral longitudinal tract (VLT) formed by the medial longitudinal fascicle (MLF) and the tracts of the post-optic commissure (TPOC) are present in all the studied vertebrates. In amniotes, there are five early axon scaffolds: the MLF, the TPOC, the mammillo-tegmental tract (MTG), the tract of the posterior commissure (TPC), and the tract of the mesencephalic nucleus of the trigeminal nerve (DTmesV) [72]. Axons scaffolds are established as early as embryonic day (E) 8.5 for the DTmesV or E9.5 for MLF in mouse, soon after neural tube closure [73]. Axon-axon interactions are regulated during axon projection, and fasciculation and de-fasciculation could be present along the neural pathfinding, as reported early in insect embryos as grasshopper [74] or fruit fly *Drosophila* [75]. Fasciculation is a regulated process since growth cones can distinguish among different fascicles, and this behavior is driven by the recognition of cell adhesion molecules, as will be mentioned ahead, mediating a stereotyped targeting. Axons fasciculation can be a permissive or a repulsive cue, promoting or inhibiting axon projection by guiding axons through previously established "routes" by pioneering axons, or by limiting axon projections away of the previously established fascicles. Pioneering axons therefore become an important guidance cue that can determine the routes and the correct pattern of tracts [76]; moreover, their growth cones exhibit different morphology as compared with growth cones of follower axons, and a different speed while approaching the midline at the post-optic commissure in zebrafish embryos, indicating that the response to guidance cues as extracellular matrix or chemotropic proteins is different in pioneering and follower axons, probably by modifying their accessibility or sensibility to the guidance cues [77]; however, if the pioneering axons are eliminated, follower axons can convert to pioneering to establish normal tracts [77, 78].

Axons fasciculation is mediated by cell adhesion proteins (CAMs). CAMs are proteins linked to cell membrane as transmembrane proteins or as GPI-anchored proteins, with homophilic or heterophilic interactions [79]. Among the most relevant CAMs are the members of the calcium-independent cell adhesion immunoglobulin superfamily, like neural cell adhesion proteins (NCAM), several proteins of L1 family as L1, CHL1, neurofascin and NrCAM, and a member of the classic

calcium-dependent cadherins family, N-cadherin [20, 79]. The regulation of axons fasciculation could be exerted by modifying CAM expression or by modifying CAM interactions by post-translational modifications, as the addition of polysialic acid to NCAM (PSA-NCAM) [80]. Enhancement of axonal outgrowth has been previously shown by culturing neurons over transfected fibroblast expressing NCAM, N-cadherin, or L1 [81–83], allowing homophilic interactions of CAM expressed in fibroblasts and axon. CAMs also establish heterophilic interactions among proteins as integrin β1 [84], and receptors of chemotropic proteins as the ephrin receptors, EphA3, EphA4 [85], or semaphorins receptor, neuropilin-1 (Npn-1) with L1 [86], or EphA7 receptor with CHL1 [85], modulating the response to chemotropic proteins but also their adhesion. It has been shown that Npn-1, a receptor for class 3 semaphorins, is involved in the fasciculation of motor and sensory axons during limb innervation, and the selective depletion of Npn-1 in dorsal root ganglion neurons leads to defasciculation of motor projections, even when motor neurons still express Npn1, resulting in dorso-ventral incorrect targeting of motor neurons [87]. Npn-1 depletion also affects fasciculation and targeting of cranial nerves and Schwann cells migration [87]. Interestingly, altered projections of descending GAD65-positive fascicles from the MTG tract, present in double knockout mice for Slit chemotropic protein receptors Robo1/2, modify the nigrostriatal projection (NP) of dopaminergic neurons, impairing both tracts interactions, probably by the absence of homophilic Robo-Robo interactions and heterophilic interaction with NCAM proteins [88], explaining some of the Slit1/2 independent role of Robo-expressing axons during NP projection [89]. These results show that besides their role of mediating attraction or repulsive responses, some receptors for chemotropic proteins also mediate axons fasciculation by homo- and heterophilic interactions, and this role could be concealed or dismissed by the more characterized chemotropic response. Moreover, the absence of the expression of receptors Npn-1 and 2 in some of the DA axons projecting to the striatum and driven by semaphorins during NP formation suggests that fasciculation could be a relevant mechanism of guidance for these axons, and a complementary strategy for the projection of DA axons in addition to the chemotropic response [90].

3. Synergic effects of guidance cues

As mentioned before, chemotropic proteins, extracellular matrices, and axon fasciculation are the main guidance cues during axon projection, their effects and mechanisms of action had been mainly studied as a separated stimulus by in vitro assays in explants or cell cultures, or in knockout animals; however, the panorama of axon projection during CNS formation implies simultaneous guidance cues, and projecting neurons should be responding and adapting according to all of them. Interactions among ECM with secreted chemotropic factors can modify their effects on axon projection, for example, it has been shown that the attraction response of RGC to chemotropic protein netrin-1 can be modified to repulsion after neurons interact with laminin-1 or with a laminin-soluble peptide fragment [38]; a similar substrate-dependent response was observed for the membrane-bound chemotropic protein ephrin A-5 in RGC of *Xenopus*; a repulsive response was observed when cells were grown on fibronectin, while a response of attraction was exerted when cells grew over laminin [91]. The induction of neurite outgrowth in DRG neurons by nerve growth factor (NGF) and neurotrophin-3 (NT3) is inhibited when aggrecan or aggregates of aggrecan and hyaluronan are present [92], indicating that ECM component can also modify neurite response to secreted trophic factors. Cross-talk among ECM receptors, chemotropic proteins, and neurotrophin receptors has been well documented, for example, the proper innervation of sensory DRG seems to be

dependent on the integrin expression, since a differential expression of α7 integrin in the subpopulations of DRG determines their response to NGF and NT-3 neurotrophin [93]. Semaphorin 3D, a repulsive chemotropic protein, can regulate MLF axons fasciculation by regulating the expression of L1 CAM in zebrafish, suggesting that besides the reported repulsive effects of semaphorins, their action could be exerted by the regulating expression of cell adhesion molecules [32]. Moreover, a complex interrelation among ECM, response to neurotropic factors, and cell-cell interactions has been reported for chick embryo DRG axons from *in vitro* explants cultured over a bioactive substrate; the NGF-induced outgrowth of DRG axons and Schwann cells from tissue explants was dependent on the density of the Arg-Gly-Asp (RGD) integrin-binding domain of fibronectin, and this effect was mediated by the upregulation of L1 and NCAM proteins by NGF that allowed the interactions among DRG neurons and Schwann cells [94].

4. Guidance cues during regeneration

The generated knowledge about guidance cues as chemotropic proteins and ECM in axon guidance has led to multiple approaches to use them into the regeneration of CNS [95–97]. When the axonal continuity is interrupted by an injury or a disease, a correct axonal regeneration is required to effectively restore the nerve; in this process, cells and ECM interactions, chemotropic proteins, and factors as substrate stiffness are important. In vitro use of ECM as fibronectin has shown to support mouse cortical and hippocampal neurons axonal outgrowth mediated by α5β1 integrin [98]; in vivo application of fibrin/fibronectin gel at the rat spinal cord injury site is permissive to axonal outgrowth [99], and when fibrin glue is applied as a microsurgery suture at a sciatic nerve transplantation model in mouse, axons were more branched and travel longer distances reducing the regeneration time [100]. In the area of biomaterials engineering for axonal regeneration, several approaches promote neural outgrowth, combining ECM components and neurotrophic factors as laminin plus microspheres with neural growth factor and neurotrophin-3 for the repair of sciatic nerve in rats [101], or carbon-coated microfibers plus basic fibroblast growth factor and fibronectin for spinal cord injury [102]; moreover, the integration of stiffness, porosity, and adhesion promotion shows that an approach considering multiple factors can help to promote and orient axon outgrowth [103], and a soft and aligned fibrillary fibrin hydrogel promotes and directs axonal projection in a spinal cord injury in mouse [104]. The big challenge therefore is to integrate several cues to obtain a better and controlled growth cone response; the desired response could be obtained by developing biocompatible materials that allow an adequate scaffold containing both the chemical and physical cues, to allow an effective neural regeneration.

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Conflict of interest

The authors declare no conflict of interest.

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