Chemotropic Molecules: Guides for Axonal Pathfinding and Cell Migration During CNS Development

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Different molecules (netrins, semaphorins, slits) with chemotropic functions and their receptors (neogenin, DCC, neuropilins, plexins, robos) have been identified that guide axons during development of the nervous system to establish the complex pattern of connections among a large number of neurons. These molecules have been recently identified to play a role in cell migration of the central nervous system during development.

The neurons and the glial cells of the central nervous system (CNS) derive from a specialized region of the embryonic ectoderm called the neural plate. This region differentiates under the influence of inductive signals emanating from the mesendoderm and axial mesoderm, prechordal plate, and notochord and closes to form the neural tube. The neural tube is composed of a layer of neuroepithelial cells that leave a cavity in the middle (the future ventricular system of the brain). Regionalization mechanisms control differences in the proliferation and differentiation rates of the neuroepithelium. These differences result in the different specialized areas and the diversity of neural cells in the mature CNS (except for microglia). Neuronal progenitors mainly proliferate in the ventricular and subventricular zones of the neural tube and migrate to colonize the superficial zones of the tube (Fig. 1C).

An extremely precise pattern of connections among a large number of neurons (~10^12 neurons and 10^15 of exact synaptic contacts in a human brain) is necessary for a mature nervous system to perform the functions of which it is capable. This precise connectivity is established during the embryonic and the early postnatal development, once postmitotic neurons have accomplished their migration from the proliferative sites (see below; Fig. 1C). In general, the axon is the cell process through which a neuron sends messages to another/s. How can the axon of a callow postmitotic neuron select the correct targets, usually far away from its position in the brain (Fig. 1A)? One piece of this puzzle is the chemotropic molecules. (The terms “chemotropic” and “chemotactic” will be used interchangeably in this review.) Their discovery is, indeed, the corroboration of the first hypothesis launched to explain the formation of the complicated interneuronal connectivity in the CNS: Cajal’s chemotactic hypothesis (11). This hypothesis was based on Pfeffer’s chemotaxis, was observed in leucocytes by researches from Bordet to Metchnikoff, and was formed merely two years after the first description of the axonal growth cones (Fig. 1B; see legend for details) by Ramón y Cajal from experiments in the spinal cord of chicken embryos in 1891.

The chemotactic hypothesis was debunked and forgotten for almost a century, briefly dusted off by Sperry’s chemosensitivity hypothesis (for an historical overview, see Ref. 15), and eventually refuted by Tessier-Lavigne identified the first family of chemotropic molecules secreted by the floor plate of the neural tube, the netrins, which guide commissural axons of the spinal cord to cross the midline (6). (The floor plate is a narrow part of the neural tube in the ventral midline. The roof plate is the corresponding region in the dorsal midline of the tube.) With the theory of Chajal’s hypothesis was confirmed. After the netrins, other molecular families with chemotropic activity were identified in the CNS: semaphorins, slits, and some growth factors. All of these are diffusible molecules that, secreted by intermediate and final targets, give rise to gradients that attract or repel growing axons along their way to their final targets. An orchestra of simultaneous chemotropic signals, in combination with contact-mediated mechanisms (laminin, cell adhesion molecules, etc.), allow axons to establish the proper connectivity of the CNS. Note, however, that chemotropism is a concept merely related to guidance: axons or cells attracted or repelled from one region. It is also related to survival, which is promoted or avoided by trophic factors. To understand the physiological role of each of these molecules, we need to know the spatial distribution of the cells that secrete the molecule; in other words, we need to know the distribution pattern of cells generating the gradient of the chemotropic molecule. All of the data reviewed here show a matching pattern of expression of the cells (that is, all cells secreted the cue in the original studies), which helps to explain the physiological effects of the molecules.

The role of the chemotropic molecules in the guidance of cell migration during CNS development is currently emerging. Postmitotic cells migrate from their sites of origin in the ventricular zone of the neural tube to find their final location (Fig. 1C). Cells (neurons, oligodendrocytes, astrocytes) travel relatively long distances, up to kilometers in some cases, and chemotropic molecules orient them in their migration (8). Cell migration and axonal pathfinding are two events in close relationship during CNS development, sharing molecular mechanisms, which include chemotropic molecules. For a compreh
hensive account of the effects of chemotactic molecules during CNS development known to date, we recommend two excellent reviews (8, 9).

**Netrins and axonal guidance**

Netrins are secreted proteins of ~65 kDa related to laminin, one of the most important contact-mediated stimulators of axonal growth. At the COOH-terminal end, netrins show a specific basic domain that allows them to bind extracellular matrix components and thereby modify their ability to diffuse. Four members of this family (netrin-1 through -4) have been identified in vertebrates, but there is no evidence to date to support the existence of a mammalian netrin-2 (6). Netrin-1 and -2 bind similarly to deleted in colorectal cancer (DCC), neogenin, and Unc5H-1, -2, and -3 (the known receptors for netrins in mammals), but netrin-3 shows a preferential binding to the Unc5H receptors (Fig. 2A). The binding properties of netrin-4 are as yet poorly understood.

Netrins are a paradigmatic case of double-function molecules that guide axonal growth cones: they are capable of both attracting and repelling axons (6). When neurons responding to netrins express DCC or neogenin, the result is an attraction of the axon toward the molecular source, but if they express receptors from the Unc5H family, netrins will repel them. In the case of coexpression of both types of receptors, DCC/neogenin and Unc5H, a repellent effect will predominate. Both types of receptors are used by netrin-1 to exert
a survival-promoting (trophic) effect on neurons from the ventricular zone of the brain stem (7).

Netrin-1 has either attractive or repulsive effects, depending on the neuronal population and the expression of the cited receptors. It is attractive for the growth cones of cortical and hippocampal projection neurons, habenular neurons, thalamocortical axons, precerebellar neurons, retinal ganglion cells, and commisural interneurons of the spinal cord (6, 9). However, netrin-1 repels trochlear axons, cranial motoneurons, and the parallel fibers of the cerebellar granule cells (9). In the absence of netrin-1 function, most of the main commissures of the brain (corpus callosum, anterior and hippocampal commissures, optic chiasm) fail to develop (9). In addition to these long-range effects, secreted netrins have a short-range function in both guiding neuritic outgrowth and in the maintenance of neurooligodendrogial interactions.

Semaphorins and axonal pathfinding

The large family of semaphorins (from the Greek semas, “signal for recognition,” i.e., the flag signaling system “semaphores”) comprise ~30 members that can be classified as either secreted or transmembrane semaphorins. The secreted ones are class II (invertebrates), III (vertebrates), and V (viral), whereas the other classes (I and IV–VII) are transmembrane. All of them have a common domain called a “sema” of ~500 amino acids with 12–16 cysteine residues, which confers the binding specificity of each semaphorin (12). The sema domain is necessary and sufficient to execute their activity. For the purpose of this review, class III semaphorins are the most interesting. They bind selectively to the two known members of the neuropilin family, neuropilin-1 and -2 (Fig. 2B), which in mammals are the biological receptors of secreted semaphorins. Neuropilins have a short cytoplasmatic domain (40 amino acids) that is not capable of intracellular transduction of the semaphorin signal. Plexins, transmembrane molecules that bind transmembrane semaphorins, can also combine with neuropilins to form a receptor complex in which neuropilins represent the binding domain and plexins the transduction domain. Receptors for vascular endothelial growth factor (VEGF) have been reported to combine with neuropilins (and maybe with plexins) to form cell membrane complexes (Fig. 2B).

The effects described for secreted semaphorins on axonal pathfinding are almost exclusively repulsive (Fig. 3, A–D). In some cases, these molecules collapse growth cones, an effect directly related to repulsion, even promoting apoptosis of some neuronal populations. An opposite trophic effect (that has been recently observed in oligodendrocyte progenitors see below). Sema 3A, the molecule that gives the rest of the family its name, is the most extensively studied, and in all cases it has been described as a repellent factor for axons, from motor neurons and spinal motoneurons to pyramidal neurons of the cortex (9). Sema 3A exclusively binds neuropilin-1 (2B), and the main effects observed when Sema 3A is knocked out appear in the peripheral nervous system (mainly, lack of many groups of sensory neurons). To date, Sema 3A is the only chemotactic molecule with a demonstrated effect orienting the outgrowth of dendrites; interestingly, it attracts apical dendrites of pyramidal neurons toward the pial surface of the neocortex (10), although it repels axons and attracts dendrites of the pyramidal neurons of the cortex.

Most of the class III semaphorins strongly repel axons of sympathetic neurons, as well as other populations in the neocortex (for example the corticothalamic, entorhinal, hippocampal, and mitral cells; see Fig. 3C), the spinal cord, and, as it was adapted for semaphorins by an ad hoc committee (Cell 97: 551–552, 1999).
olfactory axons. Interestingly, Sema 3C attracts axons of cortical neurons and Sema 3B attracts axons from the mitral cell axons of the olfactory bulb (4); these are the only two known exceptions in which secreted semaphorins attract growing axons, and both neuronal populations are repelled by other secreted semaphorins, which implies a complex battery of physiological signals contributing to proper axonal guidance, some of these signals being closely related to each other (i.e., the axons of mitral cells are attracted by Sema 3B and repelled by Sema 3F and slit-2 but do not respond to the rest of secreted semaphorins nor to netrin-1, even when they express corresponding biological receptors; see Fig. 3C (4)).

**Slits: the third big family of molecules guiding outgrowing axons**

The third big family of chemotropic molecules is slit. In mammals known members of this family are slit-1, -2, and -3 (1, 9). These secreted molecules are 200 amino acids in length with an NH$_2$-terminal leucine-rich region that is crucial for their biological functionality. Although not yet demonstrated in vertebrates, slits may be less diffusible than other secreted molecules because of the low diffusibility of their NH$_2$-terminal fragments. In contrast to semaphorins, the biological receptors of slits, called robos (derived from “roundabout,” the *Drosophila* mutation from which the cue was originally isolated, called this because commissural axons remain at the midline, crossing and recrossing, instead of leaving midline and projecting to the contralateral side) have a big cytoplasmic domain that allows intracellular transduction. But direct proof of the involvement of robos as receptors for slit functions in mammals is still missing. In vertebrates, Robo-1, Robo-2, and Rig-1 are the three known receptors of the robo family. As happens in *Drosophila*, in mammals a promiscuity in the binding of slits to robos has been reported (every slit binds every robo with similar affinity; Fig. 2C), with the exception of Rig-1, whose relationship to slits is not completely clear. Remarkably, in invertebrates the interaction slit-2/netrin-1...
Other chemotropic cues that guide axonal pathfinding

In addition to the three families of secreted cues reviewed above, diverse molecules have been described to have chemotropic effects on axonal pathfinding (for details about these molecules and their effects, see Refs. 3 and 9).

The hepatocytic growth factor/scatter factor (HGF/SF, originally described as a mitogen for adult hepatocytes and as a scatter factor for epithelial cells) attracts axons from spinal and cranial motoneurons and neocortical neurons; moreover, it promotes motility on these cells. Reelin (the protein responsible for the mutation “reeler,” which results in a dramatic disorganization of the cytoarchitecture of the brain) is a protein secreted from Cajal-Retzius cells that is essential for corticogenesis (neuronal migration and development of cortical laminae). Nonetheless, it is still controversial whether reelin is directly involved in the formation of neuronal connections or whether its effects are indirect, due to cytoarchitectonic changes. The bone morphogenetic proteins (BMPs) were originally identified as morphogens for the bone tissue, but they also have important roles in the survival, induction, and differentiation of many neuron types. BMP-7 seems to be the repellent factor secreted by the roof plate of the neural tube that, in coordination with the attraction exerted by netrins secreted by the floor plate, orients growth cones of commissural axons in the spinal cord. BMP-7 also promotes a certain degree of growth cone collapse in these commissural neurons. Another interesting possibility is that, in coordination with BMP signals, another strong morphogen, called Sonic Hedgehog (a secreted protein related to Hedgehog, a gene that controls many events during embryonic development in Drosophila and was named after a character in a video game (Sonic), that plays a central role in different events of vertebrate development, such as specifying the identity of different cell types in the ventral neural tube, regulating cell proliferation (mainly in the CNS), and controlling finger formation in the limbs), guides axons of retinal ganglion cells during development and specifically regulates the collapse of these growth cones in their centripetal outgrowth, preventing them from leaving by way of the contralateral side through the optic chiasm. Finally, anosmin-1, a molecule defective in Kallmann syndrome (which is an inherited disease of anosmia (lack of olfaction) and hypogonadotropic hypogonadism; other satellite neurological symptoms can also appear), has been described as an attractant for olfactory bulb axons and, more importantly, to be a positive regulator for the formation of axon collaterals. Anosmin-1 and slit-2 are the two chemotropic cues demonstrated to play a role in this phase of innervation (16).

Of course, many other molecules that play a chemotropic role may remain unidentified, including the cases in which one structure or organ has been identified as capable of attracting or repelling growing axons. To better focus the review, we did not cover this important field, which remains open for current and future research.

Organization and modulation of chemotropic effects on growing axons

As we have seen above, the growth cone of a growing axon is exposed to a great variety of different guiding molecules during its navigation toward the proper target. Many of these signals are due to secreted molecules, the different chemotropic molecules. These act by either attracting axons or repelling them. We can talk of a kind of orchestra of signals, so harmonious that the effect of one molecule cannot be achieved until the neuron has been previously exposed to other molecules. It has been demonstrated that these signals establish a kind of hierarchical organization of the receptors for the different guidance cues: at the same time that one repellent factor activates, the attractants that could be present are silenced through a combination of intracytoplasmic domains of their respective receptors, to prevent a tug-of-war of signals simultaneously presented to the growth cone (18).

Regardless of this complex hierarchy, the history of physicochemical stimuli experienced by a growth cone is crucial for it to reach a target or not: two sequential stimuli would have very different results depending on the order of exposure (5). This is the case cited above: spinal commissural axons respond to Sema 3B, Sema 3F and slit-2 but only after crossing the midline, toward which they have been attracted by netrin-1. Therefore, to cross or not to cross the midline is much more
than a Hamlet-like rhetorical question for growing axons.

But one chemotropic molecule can be modulated in some other ways, not only by the order that it occupies in the history of stimuli of a growth cone. The addition or presence of other molecules modifies the biological effects of exposure to some chemotropic molecules: neurotrophins modulate the response of sensory axons to secreted semaphorins; some secreted semaphorins and VEGF can antagonize each other by blocking the binding sites of their receptors in the growth cone; molecules from extracellular matrix can dramatically modulate the effects of chemotropic molecules (i.e., laminin turns one secreted semaphorin from attractive to repulsive; we have previously described the effects of heparan sulfate on slit/robo interactions); even chemical inhibitors of metalloproteases potentiate chemoattractant effects of netrin-1. Interestingly, a netrin-synergizing protein (25–35 kDa in size) has been characterized that opens the door to putative synergizing activities for the other chemotropic molecules, which could explain differences in activity even when similar expression of ligands and receptors happen.

The metabolic state of the navigating growth cone is also very important. Either electrical stimulation or changes in the cytoplasmic levels of calcium or cyclic nucleotides such as cAMP and cGMP modulate the effects of secreted cues, even turning netrins and semaphorins from attractive to repulsive or vice versa (14). Of course, down- and upregulation of receptors would also modulate the final effect of one of these cues. All of these facts give us an important perspective: effects of different cues important for axonal pathfinding can be actively and passively modulated by many other factors. This could explain how, with a relatively low number of molecules (we have reviewed here fewer than 20 chemotropic molecules), a huge number of neurons can establish a proper pattern of interconnections and result in a normal-functioning nervous system, capable of processing sensorial information and elaborating motor answers and thoughts.

Chemotropic molecules and cell migration

During CNS development, cell migration is a very important process. In general, cells generate in proliferative areas from the neural tube and migrate to occupy their final position (Fig. 1C). Most of the cells migrate in a radial way, along radial glia, and the mechanisms involved in this gliophilic or radial migration are mainly contact mediated. Contrary to the classical idea, radial migration is not the only significant way for neurons to occupy the entire nervous system. A considerable number of cells migrate independently from the radial glia, in a neurophilic or tangential migration (8). This second form of cell migration in the CNS suggests a parallelism with axonal pathfinding (Fig. 1D). The growth cone (more frequently referred to as the “leading process” in migration) suffers cytoskeleton changes resulting in cell motility, which reflects the most important difference between cell migration and axonal pathfinding: the movement of the soma in the migration process, which usually remains still in axonal guidance. In general, the radially migrating neurons are projection neurons, whereas tangentially migrating ones are interneurons.

Although tangentially migrating neurons have been described as closely related to corticofugal axons (the name neurophilic and axonophilic migration derive from this fact), it is not clear whether a direct interaction between the migrating cells and the preexisting axonal tracts exists. The pattern of tangential cell migration suggests that, besides contact-mediated mechanisms, long-range mechanisms such as chemotropic molecules could have a relevant role in this migration process. In fact, and leaving Reelin aside (the role of Reelin in neuronal migration has been the focus of many complete reviews; given the controversial role of reelin as chemotropic molecule, we will not review its effects here; see above), in the first half of 1999 slit-2 was the first chemotropic molecule that was shown to have roles in cell migration in the mammalian CNS (8). It repels migrating motoneurons, GABAergic interneurons migrating from the ganglionic eminence into the neocortex, and those migrating along the rostral migratory stream from the subventricular zone of the forebrain toward the olfactory bulb [GABAergic interneurons (granular and periglomerular cells)]. This last type of cells has been recently demonstrated to be induced to migrate by astrocyte-derived, migration-induced activity that seems to be less strong than slit-2. It is very remarkable that the same functional domain of slit proteins is involved in guiding growth cone navigation and in neuronal migration, which confirms the idea of the degree of conservation and sharing of molecular mechanisms in both developmental events.

Netrins were identified to play a role in neuronal migration, again attracting or repelling cells depending on the population studied (8). Midline netrin-1 attracts tangentially migrating neurons that will form the inferior oliva, being this antagonized by slit (2). Netrin-1 repels migrating hypothalamic neurons, postnatally generated cerebellar granule cells, and, surprisingly, late-born striatal projection neurons, which migrate in radial paths from the ventricular surface within the striatal primordium. The relevance of DCC (and thus maybe netrins) in the migration of luteinizing hormone-releasing hormone neurons from the nasal pit, where they are generated, to their final destination in the hypothalamus has also been described (8).

Although the first clue about the role of semaphorins in cell migration was described in the migration of the neural crest cells toward their peripheral targets in the chicken embryo, recently it has been demonstrated (8) that a differential response to secreted semaphorins via neuropilins sorts migrating cells from the ganglionic eminences to invade the striatum, the paleocortex, and the neocortex (Fig. 1C). This population of migrating cells responds to the three largest known families of chemotropic molecules (slits, netrins, and semaphorins), even to HGF/SF.
Interestingly, a similar case of multiple response in migrating cells in the CNS has been observed for oligodendrocyte progenitors that colonize the optic nerve: they are selectively repelled by Sema 3A and attracted by Sema 3F (Fig. 3, E–F) and netrin-1 (17, 19). All of these effects are produced via the same receptors that have been described for axonal migration and that we have reviewed above (Fig. 2, A–B). Sema 3F exerts a double effect; in fact, it is also a chemotrophic (survival-promoting) factor for the same population (17). Early postnatal upregulation of Unc5H receptors reverses netrin-1 attraction into repulsion for these cells.

In summary, chemotropic molecules involved in axonal guidance are also useful in orienting cell migration in the CNS, a closely related event. Furthermore, such a system is not only valid for migrating neurons but also for cells of oligodendroglial lineage and, maybe, for other types of CNS cells. The fact that one of these molecules present in one structure could serve at the same time for growth cone navigation of one cell population and for migration of a different one could be due to the specific localization of molecular isoforms. Nevertheless, differences in the isoforms involved in the intracellular transduction and effector machinery may also participate.

Summary

One century after Ramón y Cajal raised his chemotactic hypothesis to explain how interconnections between $10^{12}$ neurons (the estimated figure for the brain of a primate) can be established accurately, chemotropic molecules (netrins, semaphorins, and slits) surfaced as the fulfillment of this prescient hypothesis. Together with contact-mediated mechanisms (laminin, cell adhesion molecules, etc.), a relatively low number of molecules result in a combination of spatially and temporally ordered signals that determine every single neuron’s connections along the CNS. Axonal growth cones read and interpret this symphony of cues present in the tissue in a moment, growing toward or away from structures to finally follow a very particular path and reach their targets. Interactions between different molecules modulate their activity. A second important point of modulation is the intracellular transductional and effector machineries, as well as the metabolic state of the cell.

These chemotropic molecules also orient postmitotic cells in their migration within the CNS. In this case, the “leading process” acts as a growth cone. Both events, cell migration and formation of connections between them, share molecular mechanisms. These overlappings and interweavings offer us a fascinating field of work for the future. The relevance of chemotropic molecules in neuropathology is increasingly recognized and an important subject of research (13). Their role in neuroregeneration might also be manipulated (up- or downregulation of factors and their receptors, pharmacological blocking or modulation, etc.) to design future successful therapies.

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