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Neuronal growth cone migration

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Summary. The neuronal growth cone is a semi-autonomous portion of the developing neuron that is highly specialized for motile activity. Migrating neurons may share some features with neuronal growth cones. I review some of what has been learned about growth cone initiation, the differentiation of axons and dendrites, the role of the cytoskeleton in motility, the movements of membrane vesicles, the factors regulating the rate and direction of growth cone movement, and the further differentiation of growth cones as they enter the target area and initiate synaptogenesis. Where appropriate, I draw comparisons to what is known about the migration of neurons.
Key words. Growth cones; neural development; cell motility; cytoskeleton; actin; synapse formation.

Introduction

The neuronal growth cone is the tip of an elongating axon or dendrite that is intimately associated with pathway formation and synaptogenesis. While the movement of the cell body determines the position that the neuron will assume, the movements of the growth cones determine the initial morphology of the developing cell. Movements of the growth cones also determine which cells the neuron will have the opportunity to form synapses with.

Growth cones have frequently been compared to motile cells. Ramon y Cajal described the growth cone as 'a concentration of protoplasm of conical form, endowed with ameboid movements'[52]. Speidel, studying the movements of growth cones in vivo, noted the resemblances between growth cones, fibroblasts, and endothelial cells[58]. That the growth cone can indeed be viewed as an independent motile structure is most convincingly demonstrated by severing the growth cone from its cell body in vitro. When this is done, normal movement of the growth cone continues for several hours[55]. Growth cones were recently termed 'leukocytes on a leash', for these reasons, as well as for biochemical similarities in signal transduction pathways between leukocytes and growth cones[51]. An even more felicitous comparison might be made between growth cones and migrating neurons.

I would like to briefly summarize the stages of neuronal migration, highlighting the similarities to growth cone movement. Figure 1 shows the structural similarities be-

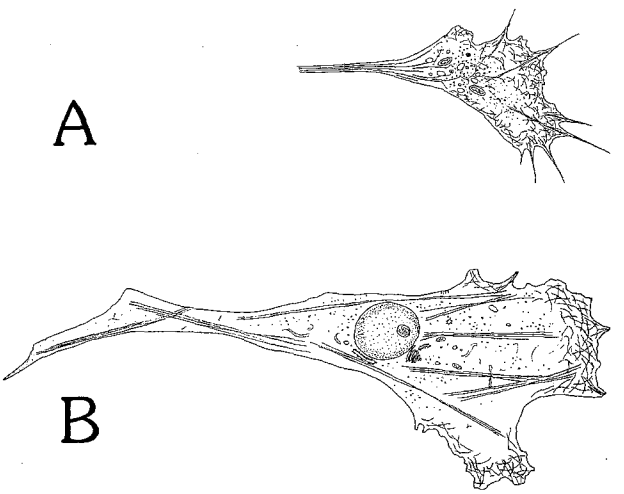

Figure 1. Similarities between growth cones and motile cells.
A Schematic of a growth cone, showing lamellipodia, filopodia, and the distribution of cytoskeleton and vesicles in the growth cone. The lamellipodia are thin, veil-like sheets that extend forward while remaining attached to the substrate. Filopodia are very narrow spikes which extend and retract rapidly, often moving from side to side with no attachment to the substrate, as if probing their environment. Microtubules extend from the neurite only partly into the growth cone, most vesicles are in the growth cone core, and actin filaments fill the peripheral portions of the growth cone. Most of the actin filaments form an apparently random meshwork that fills the lamellipodia, bundles of microfilaments fill the filopodia and extend partly back into the growth cone. This is a composite drawing, reflecting data on the cytoskeleton from mammalian growth cones[41], and data on the vesicles from molluscan growth cones.[24].
B Schematic of a migrating cell. This is of a fibroblast in vitro. Motile fibroblasts have lamellipodia very similar to those in growth cones, also filled with a crosslinked actin meshwork. The fibroblast also has a number of stress fibers, which are bundles of actin filaments that traverse the cell, and usually end before the leading lamellipodia.

tween a growth cone and a migrating cell. Following cell division, the neuron extends a process and begins to migrate. This is analogous to the initial development of growth cones. The neuron, like the growth cone, then spends a variable length of time migrating. Cell movement is generally dependent on the dynamic properties of the cytoskeleton, and on the movement of membrane through continuous cycles of exo- and endocytosis. The role of the cytoskeleton and the movements of the plasma membrane and vesicles in growth cones have been studied extensively. Both neuronal migration and growth cone movement are highly regulated processes with multiple extrinsic factors influencing the rate and direction of movement. The extracellular matrix, cell surface cues, and soluble growth factors possibly play a role in this regulation. Finally, when the neuron or growth cone arrives in the appropriate area, movement ceases and further differentiation commences. For technical reasons, many of these processes are currently understood in greater detail for growth cones than for migrating neurons. Thus, it may be productive to view the migrating neuron as 'a growth cone without a leash'. I will review some of what is known about the commitment and migration of neuronal growth cones, with the hope that some of this knowledge can be applied to the study of neuronal migration.

Commitment of the growth cone

Neuronal precursors generally assume a roughly spherical shape during mitosis, elongating processes only after cytokinesis [54]. The leading and trailing processes of migrating cells share some features with the dendrites and axons of the more mature cell [50, 65]. During migration of cerebellar granule cells, the trailing process elongates to form the parallel fibers, and in the cerebral cortex, the trailing process appears to become the axon. However, the axon of spinal cord motor neurons and retinal ganglion cells arises out of what had been the leading edge of the migrating neuron. These diverse examples suggest that there is no uniform, simple relationship between cell migration and the establishment of neuronal polarity.

Migration is clearly not required for the formation of growth cones and the subsequent differentiation of axons and dendrites, as both can take place in the complete absence of cell contact. Embryonic hippocampal neurons in vitro are apparently uncommitted with respect to the location of their axons and dendrites. In the first stage of neurite outgrowth, lamellipodia are elaborated in a ring around the cell body. A few of these lamellipodia become part of growth cones, which extend away from the cell body, leaving neurites behind them. After about a day of outgrowth, one of the neurites begins to grow much faster than the others, and acquires the characteristics of an axon. A few days later, the other processes begin to elongate rapidly, and differentiate into dendrites [22]. This establishment of cell polarity has a significant random component to it. Initially, the entire circumference of the cell seems competent to develop growth cones, and the growth cones that do extend are apparently randomly distributed. After the initial outgrowth, any of the processes are capable of differentiating into the axon. The longest process becomes the axon; however, if it is cut shorter, then the next longest becomes the axon instead [21].

Since the orientation of axons and dendrites in vivo is clearly not random, there must be intercellular signals that establish the 'correct' polarity of process outgrowth. Whether these signals arise from the process of cell migration, or from other factors is unknown. The in vitro results suggest that the establishment of neuronal polarity could develop after an initial random outgrowth. If a process growing in the direction of the future axon were able to elongate more rapidly than processes growing in the other directions, and the cell limited the number of axons to one, this would be sufficient to establish neuronal polarity.

Growth cone migration

Cytoskeleton

The only known cytoskeletal component present in most of the growth cone and its lamelli- and filo-podia is actin. The actin filaments form a few bundles in the growth cone palm, and run the full length of filopodia. The growth cone lamellipodia are filled with a network of individual filaments which are crosslinked to each other and to the inner face of the plasma membrane [41, 67]. The structure and movement of the growth cone actin cytoskeleton is remarkably similar to that utilized in neutrophil, fibroblast, and ameboid locomotion [11]. The cell biology of neuronal cell migration has not been investigated in as much detail. Figure 1 shows schematic views of a neuronal growth cone and a migrating fibroblast. The epitope CDA 1, present specifically in the lamellipodia of growth cones and motile fibroblasts, is on a unique conformation of actin or an actin-associated protein [20] (S. Devoto, K. Goslin, C. Barnstable, and G. Banker, in preparation). This provides further evidence that the molecular structure of the cytoskeleton of the growth cone and of motile fibroblasts is modified in relation to the cytoskeleton of the neuronal cell body and nonmotile cells.

In growth cone whole mounts examined with the electron microscope, actin microfilaments often appear to be attached to tubulin at the base of the growth cone [41]. If the microtubules are relatively immobile to pushing and pulling, this would allow the actin to exert force on the membrane. Recently, a protein was described that has the characteristics of both an actin-associated protein and a tubulin-associated protein. This protein is greatly enriched in growth cones and may serve to crosslink the tubulin of the developing axon or dendrite to the actin of the growth cone [26].

Drugs known to affect actin have a profound affect on growth cone motility. Cytochalasin B, which alters actin filaments, causes the growth cone to round up, the filopodia to be withdrawn, and movement to cease in growth cones of embryonic chick dorsal root ganglion cells[66]. Using video-enhanced differential interference contrast (VEC) microscopy on cultured adult *Aplysia* neurons, fibers were seen under the plasma membrane in the lamellipodia that continuously move in the retrograde direction at a rate of 3.5 mm/min, even while the lamellipodia are moving forward. The moving fibers are co-localized with phalloidin-stained actin filaments that can be found after fixation. The rate of movement of these fibers is consistent with the rates of actin filament polymerization given the growth cone actin concentration. Cytochalasin B reversibly destroys the filaments, blocks the retrograde movement, and eliminates most of the polymerized actin from the lamellipodia[23]. Although individual actin subunits within the filament are moving backwards, the filament could remain apposed to the membrane, and might even push the membrane outwards, through the constant addition of monomers to the membrane end and depolymerization at the cell body end.

The above results suggest that the actin-rich lamelli- and filopodia are moving forward, pulling the axon behind them. However, the importance of actin in axonal elongation is inversely related to the adhesivity of the substrate. Chick embryo dorsal root ganglia cells, when plated on a highly adhesive substrate (polyornithine), elaborate extensive neurites in the presence of cytochalasin B, despite the absence of recognizable lamelli- or filo-podia[44]. The neurites are curiously looped and extensively branched, suggesting that one role of the growth cone in this situation is to steer elongation and that actin filaments are involved in the regulation of neurite branching.

Membrane flux and neurotransmitter release

The membrane of the growth cone must also be in constant flux to support the rapid motility of the growth cone. When lamellipodia and filopodia extend and retract, there is a net flow of membrane into or out of these regions. As the growth cone advances forward, there must be an addition of membrane from outside the growth cone, equal to the increased surface area of the axon. Membrane addition is known to take place at the growth cone[9,10,29]. In aldehyde-fixed growth cones most vesicles are in the palm of the growth cone, suggesting that membrane addition occurs in this region. Very few vesicles are found in lamellipodia and filopodia; either there is no exocytosis or endocytosis in these regions, or vesicles are very rapidly transported or not preserved by fixation.

The ultrastructural morphology of growth cones is significantly different when the tissue is preserved by freeze-substitution. In retinal ganglion cell growth cones in vivo, this technique reveals a multilamellar stack of membrane disks (MLS) in the palm of the growth cone; these are probably transformed into the irregularly shaped vesicles seen following aldehyde fixation. The MLS may be an intermediate pool of membrane between the cell body source and the growth cone plasma membrane[15]. Single disks that appear to derive from the distal portion of the MLS are seen subjacent to filopodia, and may serve as the immediate source of plasma membrane for an extending filopodium[16]. Following incubation with cationized ferritin, coated pits appear in the plasma membrane. The coated pits give rise to coated vesicles that ultimately fuse with the MLS[16]. Continual recycling of membrane within the growth cone would provide a mechanism for retaining growth cone specific surface components in the growth cone.

VEC microscopy of aplysia neurons in vitro confirms that membrane vesicles only rarely move into the lamellipodia, and when they do, they invariably reverse their direction and return to the core of the growth cone[23,25]. Since rapid, directed organelle movement uses a microtubule substrate, the absence of vesicles in lamelli- and filopodia could be a secondary effect of the absence of microtubules[60,61]. Alternatively, the thinness of the lamellipodia may not allow vesicles to enter.

In vitro, the top lamellipodial membrane shows continuous retrograde movement, this may serve to translocate extracellular ligands towards the body of the growth cone. The source of the membrane for this retrograde movement is not clear. It is possible that the membrane of the substrate side of the lamellipodia moves anterogradely, or exocytosis occurs on the distal substrate side of the growth cone and endocytosis on the proximal surface side of the growth cone[14]. In vivo, some situations may resemble the tissue culture situation, as when the growth cone is moving over a basement membrane. In other situations, all surfaces of the growth cone would be roughly equivalent, each contacting, and perhaps adhering to other cells. The plasma membrane movements in these cells might be quite different from what is seen in vitro.

During neurite extension of *Aplysia* buccal ganglion neurons in vitro, there is a characteristic sequence of membrane movements that occur as the growth cone is converted into a neurite. Numerous filopodia extend from the growth cone, usually adherent to the substrate. A lamellipodium then rolls out, bounded by two of the filopodia. Vesicles moving randomly and by directed transport enter the lamellipodium from the body of the growth cone. The body of the growth cone then assumes the cylindrical shape of the axon, with bidirectional organelle transport[25]. This model of neurite extension suggests that one function of the filopodia is to guide lamellipodial extension, which in turn is responsible for neurite elongation.

Other evidence for exocytosis in the growth cone has come from the development of techniques to assay for

the release of the contents of growth cone vesicles. Embryonic frog spinal cord neuronal growth cones synthesize and release acetylcholine in tissue culture[35,68]. This release often occurs in a burst, suggesting that it may be due to the exocytosis of an acetylcholine containing vesicle. However, elevated external calcium decreases burst release from growth cones, whereas the frequency of exocytosis at a mature synapse is increased[68]. The release of neurotransmitters and growth factors by dendritic and axonal growth cones could in principle have a significant effect on the differentiation of neighboring neurons, this is discussed below.

Regulation of growth cone migration

Soluble factors

The presence and potentially regulated release of soluble factors (neurotransmitters and growth factors) provides a cellular mechanism for the regulation of neuronal form by extrinsic signals from other cells. Some neural structures release an as yet unidentified diffusible chemotropic signal(s) in vitro, and presumably in vivo, that causes appropriate axons to sprout towards the structure[34,42]. In identified neurons of adult *Helisoma* in vitro, serotonin causes the lamellipodia and filopodia to collapse and the growth cone to cease its advance[32,33]. These effects are due to a local action of serotonin, as it has the same effect on growth cones severed from their cell body. Other identified neurons do not show this response to serotonin.

One possible mediator of the effect of serotonin on growth cones is calcium, as growth cones have particularly abundant calcium channels[2,27]. Serotonin increases intracellular calcium only in the affected growth cones, while other agents such as membrane depolarization and the Ca^{++} ionophore A23187, which elevate intracellular Ca^{++}, have the same effect as serotonin; Ca^{++} channel blockers suppress the response of the growth cone to serotonin. Removal of all extracellular Ca^{++}, however, also suppresses growth cone activity[17,46]. These data suggest that the level of intracellular calcium modulates growth cone motility: a moving growth cone can be stopped by either an increase or a decrease in intracellular calcium. Mammalian central nervous system neurons also show a correlation between growth cone calcium levels and neurite extension[19]. In dissociated embryonic hippocampal cells, the application of glutamate causes the regression of dendritic growth cones while not affecting the axons. Local application of glutamate to the growth cone is sufficient for this effect. The calcium channel blocker cobalt suppresses the effects of glutamate, suggesting that a glutamate stimulated rise in cytosolic calcium is responsible for dendritic retraction[45]. Calcium influx profoundly reduces the number of actin filaments in the growth cone, this may be the mechanism by which extracellular factors influence growth cone form and movement[38].

Other intracellular second messengers also modulate neurite extension in vitro. Increases in cyclic AMP suppress growth cone activity in a subset of *Helisoma* neurons; within a subset of these, the effect is mediated by cyclic AMP stimulated calcium influx[45]. In *Aplysia* bag cell neurons, increases in cyclic AMP cause the redistribution of cytoplasmic organelles from the central core of the growth cone into what were peripheral lamellipodia[23]. This would lead to the more rapid growth of the neurite. The retrograde waves in the lamellipodial plasma membrane are also suppressed by cyclic AMP in these cells. Calcium affects the cyclic AMP induced spread of organelles in a subtle way: in the presence of calcium the vesicles move out in a saltatory manner, in the absence of calcium the spread appears to occur on a linear track. Cyclic AMP is probably promoting the polymerization of microtubules into the previously microtubule-free lamella[36,62]. This would provide a substrate on which the organelles move peripherally.

The cell surface and extracellular matrix

Growth cone adhesion to other cells and the extracellular matrix (ECM) plays an important role in neuronal morphogenesis. Axons from the temporal retina show a clear preference for growth on plasma membranes from the anterior tectum as compared to the posterior tectum, indicating that the growth cone is able to sense an as yet unidentified molecular difference in the membranes from the two regions[6,64]. Growth cones express on their surface the cell adhesion molecule NCAM, and one or more of the integrin receptors for ECM constituents[8,63]. The ECM molecules collagen, laminin, and fibronectin each promote neurite outgrowth from neurons in vitro, when the purified form is coated on the substrate[1,3,31,37,43]. A complementary approach has been to analyze neurite elongation over cell surfaces in which many molecules are present and to use antibodies to determine which are required for neurite growth. This approach has suggested that the calcium-dependent adhesion molecule N-cadherin, the calcium-independent cell adhesion molecule NCAM, as well as the ECM components mentioned above, all play a role in neurite extension[4]. Retinal ganglion cell neurites lose their responsiveness to both laminin and NCAM as they mature[18,28,47].

When a growth cone approaches a border between two different substrates, the filopodia continue to palpate randomly, but those that contact the more adhesive substrate remain adherant for longer times and seem to 'pull' the growth cone along this substrate[39,40]. In cultured adult *Aplysia* buccal ganglion neurons, if growth cones contact a border between poly-lysine and untreated glass, they extend filopodia and lamellipodia onto both substrates. However, the membrane that has advanced onto the less adhesive substrate is resorbed, suggesting that selective 'pruning' within the growth cone might play a role in determining the direction of neurite elongation[13].

In the developing chick optic tract, the first axonal

growth cones appear to follow a pathway delimited by the endfeet of optic stalk neuroepithelial cells. NCAM is expressed at high levels on these endfeet, and the intraocular injection of antibodies to NCAM perturbs the adhesion of growth cones to these endfeet [56]. This experiment demonstrates that NCAM is necessary for the normal progression of the pioneer growth cones through the optic stalk. Structural features along developing nerve pathways also appear to provide guidance cues to growth cones. Loosely packed mesenchyme appears to provide channels that guide growth cones into certain pathways in the developing chick hindlimb [59]. At the diencephalic/telencephalic border, there is a knot-like structure composed of very tightly packed nonneuronal cells. This knot expresses relatively low levels of NCAM, and its position suggests that it serves to prevent the shuffling of the optic and olfactory projections [57].

Further differentiation

The structure of the growth cone is not constant. In general, growth cones assume simple, bullet-like shapes when they are migrating in a defined pathway such as a nerve, and more complex, fan-like shapes when they are in decision or turning regions [7, 59]. The shape of the growth cone changes again when it enters the target region. The growth cone begins to branch into an immature axon arbor, and the classical conical shape of the growth cone is replaced by minute swellings at the tips of the axonal branches [7].

The transformation from growth cone to presynaptic terminal occurs over a period of several days and is illustrated in figure 2. Usually a filopodium of the growth cone forms the first contact with the potential postsynaptic cell. In some contacts, the filopodium moves on the surface of the cell, then withdraws and the growth cone continues. Following other contacts, the filopodium remains, and the filopodia cease to palpate and withdraw into the growth cone. These results indicate that while it is the filopodium which makes the first contact with a postsynaptic cell, the synapse arises from the body of the growth cone. Following the stabilization of this initial contact, the growth cone may extend new filopodia and continue to grow while maintaining the initial contact. During approximately the next 48 h, a postsynaptic density appears, clear vesicles the size of synaptic vesicles begin to cluster near the postsynaptic density, the cleft between the cells widens, and the presynaptic dense projections and synaptic cleft material appear. In the following several days, synaptic vesicles increase dramatically in number, cluster near the active zone, and the pre- and post-synaptic membrane specializations become more pronounced [53].

In the inner plexiform layer of the rhesus monkey retina, the sequence of events in synapse formation has been proposed to be a little different. Before the growth cone contacts potential postsynaptic cells, there are electron-dense membrane thickenings. Serial sections confirmed that these were not contacted by growth cones. The growth cones then contact the cells, form presynaptic thickenings, accumulate vesicles, and the synaptic cleft widens [48, 49]. The same sequence is seen in the rat visual cortex [5]. It is not known whether these pre-existing postsynaptic membrane specializations are more than just membrane thickenings, for example whether they contain appropriate neutrotransmitter or growth factor receptors.

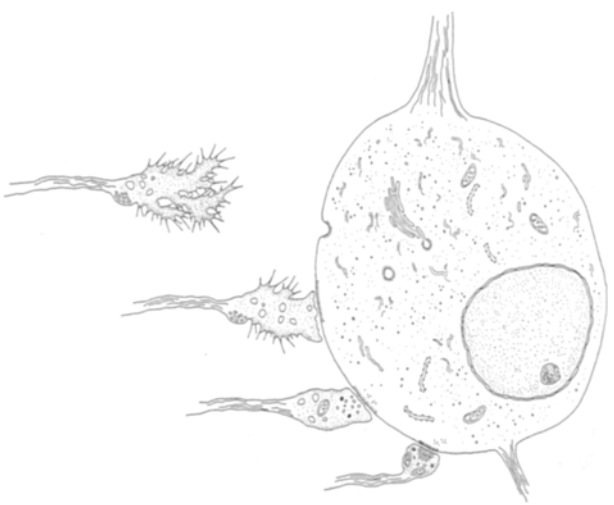

Figure 2. Schematic drawing of the events in synapse formation [53]. From top to bottom: a growth cone approaches the cell and makes contact, all the filopodia withdraw into the growth cone, and the core of the growth cone is gradually transformed into a mature presynaptic terminal. At the same time, there are changes in the structure of the postsynaptic cell. Details are discussed in the text.

Perspectives and summary

There is at least one striking difference between a growth cone and a migrating neutron: the presence of the nucleus, endoplasmic reticulum, and Golgi apparatus in the neuron. This means that the migrating neuron is able to introduce new proteins more rapidly into the membrane and cytoskeleton. The regulation of neuronal migration can therefore more easily take place through the regulation of gene expression. Whether this is an important factor in neuronal migration remains to be determined. The development of tissue culture techniques by Harrison to study growth cones led directly to most of our knowledge about growth cones [29]. Studies of living growth cones, motile behavior, the transition from growth cone to synapse, the role of the ECM, the effect of soluble factors on outgrowth, the activity of the cytoskeleton, the establishment of neuronal polarity, and the release of neurotransmitter were all made possible by tissue culture. Future work on growth cones will continue to use tissue culture to probe the molecular bases for these functions. Improvements in light microscopy and the development of new labeling dyes will lead to further

advances using in vivo systems. Until recently, the analysis of neuronal migration has been hampered by the lack of suitable in vitro model systems. Tissue culture analyses of neuronal migration of neural crest cells and cerebellar granule neurons promise to yield new insights into the mechanism of neuronal migration [12, 30].

Many aspects of growth cone movement are directly relevant to neuronal migration. The establishment of neuronal polarity, the response to ECM components, and aspects of cytoskeletal and membrane dynamics probably are very similar in growth cones and migrating neurons. Other aspects of growth cone movement are not now known to have counterparts in migrating neurons, but I have included them because they may be important for migrating neurons as well. These include the response to soluble factors, the structural changes that occur during growth cone movement and during synapse formation, the release of neurotransmitters, and the role of intracellular second messengers in regulating motility. Further analysis of migrating neurons will reveal how closely the migration of neurons resembles the migration of growth cones.

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The specification of neuronal identity in the mammalian cerebral cortex

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Summary. The determination of neuronal fate in the developing cerebral cortex has been studied by tracking normal cell lineages in the cortex, and by testing the commitment of young cortical neurons to their normal fates. These studies together suggest that neuronal progenitors are multipotent during development and have the potential to produce neurons destined for many or all of the cortical layers. However, the laminar identity of an individual neuron appears to be specified through environmental interactions at the time of the cell's terminal mitotic division, prior to its migration into the cortical plate.
Key words. Neurogenesis; determination; central nervous system; lineage; migration.

During the development of the nervous system, the generation of neuronal diversity stands as a tremendous challenge, both to the proliferating cells that create this diversity, and to developmental neurobiologists who try to understand it. The last decade has seen several breakthroughs in our ability to explore these processes in the central nervous system of mammals. In several regions of the developing CNS, the processes by which a progenitor