RC2 EXPRESSION IN CENTRAL NERVOUS SYSTEM WHITE MATTER; AN ANALYSIS OF NEURAL STEM CELL DISTRIBUTION DURING DEVELOPMENT

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ABSTRACT

Introduction

Radial glial cells have many pivotal roles in the developing central nervous system. Currently, they are recognised to have four main functions: to direct neuronal migration, to act as neuronal progenitors, to encourage angiogenesis, and to coordinate the formation of axon tracts in the white matter. In this study, we will investigate the fourth of those functions, as we seek to quantify the number of radial processes crossing the white matter in the developing spinal cord. We will then compare the number of processes between wild-type specimens and animals missing genes encoding for proteins essential for normal axon guidance, specifically Semaphorin6A and PlexinA4. This may help to determine whether these signalling pathways are relevant in the developing spinal cord.

Methods and Materials

We examined cryosections from mouse embryos of age E15.5, which were stained to show the traversing radial glial processes. We used a series of 50μm reference lines spaced 25μm apart to quantify the number of processes at each level.

Results

We showed that there were especially large numbers of glial processes in the lateral columns of white matter in all three phenotypes described. However, we found no link between the number of axons and Semaphorin6A or PlexinA4 deficiency.

Discussion

Our results support conclusions made by previous studies, that many radial glial cells can be seen in the developing spinal cord at a time when axon tracts are developing, suggesting a possible role for these processes in their development. The fact that they are seen in especially great numbers in the lateral columns may suggest which axon tracts they have a role in guiding the development of.

Key Words: axonogenesis, development, radial glia, white matter patterning

Abstract Word Count: 278

INTRODUCTION

The History of Radial Glial Cells

Our understanding of radial glial cells has expanded greatly since their discovery in the late 19th century. Giuseppe Magini is the first to have identified and outlined these processes, which he did in two papers in 1887 and 1888, but it was nearly a century until some insight into their function was provided. Wilhelm His, a contemporary of Golgi and Magini, had originally hypothesised that they facilitated the migration of immature neurons in the developing CNS, but this was only confirmed by Pasko Rakic in 1971 after his experiments on monkey cerebella, where he observed that immature neurons bound to radial fibres during migration (Bentivoglio and Mazzarello, 1999). More recent findings, however, have shown that aside from aiding migration, radial glial cells may have a role as stem cells, differentiating into not just glial cells, but neurons as well. This was first reported by a group led by Paolo Malatesta, in 2000, when they used fluorescence-activated cell sorting to demonstrate the neuronal fate of radial glial cells (Malatesta, Hartfuss and Götz, 2000).

The Function of Radial Glial Cells

Radial glial cells seem to have four main roles. Their first function is to aid neuronal migration in the developing CNS. They can thus travel from germinal zones to their target destination, where they can develop into functional neurons. This is a vital role in the cerebrum especially, as which lamina the neuron occupies plays a large part in determining its function (Rakic 1972). Secondly, they have relatively recently been recognised as neuronal progenitors, as they may generate some of the neurons that they aid the migration of. This demonstrates that neuronal and glial lineages are not always separate. Cells performing this role are situated in the ventricular zones, and only a minority of radial glia in the CNS display this function (Malatesta, Hartfuss and Götz, 2000). Another recently discovered function is their role in brain angiogenesis, by both directly interacting with blood vessels and modulating canonical Wnt signalling (Ma et al 2013). Finally, the function that will be further explored in this paper is facilitating the organisation of white matter in the spinal cord and cerebrum. In the spinal cord, they seem to form boundaries between the developing axon tracts, preventing abnormal decussation and allowing them to form along the correct pathways (Barry et al 2013) (Barry, Pakan and McDermott 2014).

The Timing of Axon Tract Formation

In the adult human, the white matter is split into 4 bilateral principal columns. These are the dorsal column, lateral column, ventral column and the ventromedial column. The columns are further divided into several functional axon tracts, which radial glial cells may play a role in the development of. These tracts include the corticospinal tract and the posterior column pathways (Altman and Bayer, 2001). Generally, axons form these tracts by migrating from the ventricular zone of the spinal cord, outwards towards the pial surface, and thus the arrangement of an outer layer of white matter surrounding the inner grey matter is formed. The different functional pathways develop at very different stages of development. In the human embryo, the posterior column pathways develop early, and they are apparent by the 7th gestational week. This can be investigated by immunological staining by proteins such as GAP-43, an indicator of axon growth. Immunoreactivity of GAP-43 is greatest in the dorsal columns between the 10th and 14th weeks, showing that this is the peak period of tract development (Clowry, Moss and Clough, 2005). In contrast, the corticospinal tract forms far later. The axons are still developing and forming synapses until late into prenatal, and even into early postnatal, development (Martin). Myelination of all tract axons continues into postnatal development, until around 82 postconceptional weeks (Gao et al 2009).

The role of Radial glia in Axon Tract Formation

In the developing spinal cord, radial glial cells form a consistent scaffold along the entire rostrocaudal axis. They arrange themselves with their cell bodies in the ventricular zone, adjacent to the central canal, and an endfoot extends to the pial surface. They are arranged perpendicular to the axis of the cord. It has been proposed that this morphology and arrangement enables them to assist in the formation of axon tracts in the white matter. Their proposed function is to both guide the axon tracts along the correct pathways and to prevent abnormal decussation. For example, in rats the dorsal midline glial septum stops premature decussation of the corticospinal tract. Evidence for this function comes from numerous sources. Firstly, electron microscopy has revealed that adhesions do form between radial glia and the migrating axons. Also, vimentin staining demonstrates that radial glia are especially well organised between embryological days 14 and 18 in rats, which is when the axon tracts are forming at their greatest rate. The density of vimentin staining decreases after this period, providing evidence that radial glia have started to differentiate, as they are no longer needed for this purpose. (McDermott, Barry and McMahon, 2005) (Barry et al 2013).

Disorders Involving the Malformation of CNS White Matter

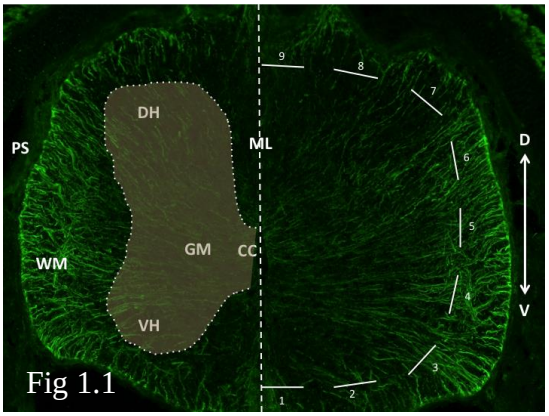
Deficiencies in the development of white matter can lead to a number of debilitating disorders. First, there is the group of disorders known as leukodystrophy characterised by abnormal myelination in the developing child, leading to gradually deteriorating motor and sensory function. Symptoms include gait disturbances, abnormal movement patterns, declines in language and concentration, and finally an early death (Kehrer et al 2014). Another condition to consider is lissencephaly, which is caused by a defect in neuronal migration affecting both the grey and white matter. In the extreme case, this symptom is known as agyria, as the gyri and sulci are completely absent (Perez et al 2013). The defect in migration usually occurs between the 12th and 24th weeks of gestation. Diagnosis is usually during infancy, and symptoms include epilepsy and severely retarded motor and cognitive development (Sasaki et al 2012).

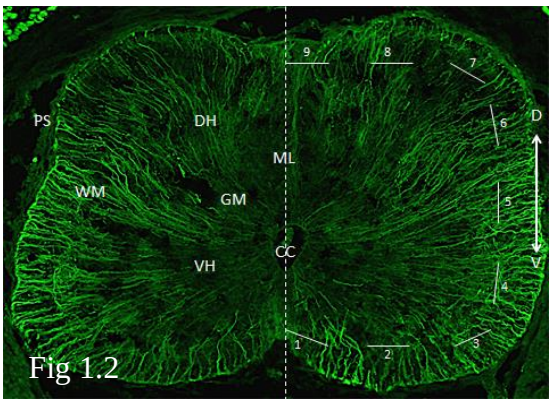
The aim of this study is to quantify the number of radial processes present in different areas of the developing mouse spinal cord in normal specimens, and compare this to mice with mutations in the gene encoding for Semaphorin6A, and the gene encoding for PlexinA4, a Semaphorin receptor. These proteins are usually vital for normal axon tract development, so this may help to reveal whether or not the signalling pathway they form a part of is relevant in the developing mouse spinal cord. Also, the location of certain developing axon tracts in the spinal cord is known, so the differences in number of radial glial cells present between axon tracts can be investigated.

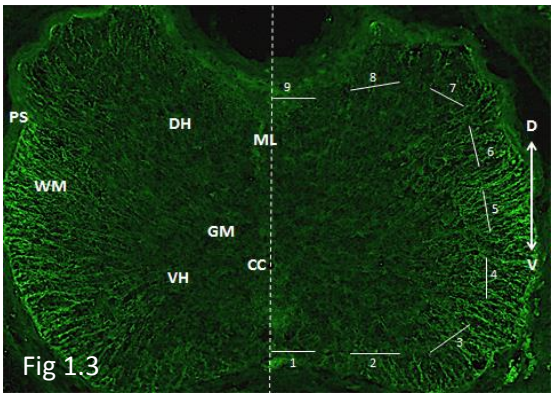
MATERIALS AND METHODS

Animals

Embryonic day E 15.5 (of a 21-day gestation period) mice were obtained from the Bioresource Unit, Trinity College Dublin. Embryos were removed by laparotomy following anaesthesia using halothane, and a terminal overdose of sodium pentobarbital was administered to pregnant dams. Embryos were fixed in 4% paraformaldehyde (PFA) for 24 h at 4 °C. They were then cryoprotected in a 30% sucrose solution and snap-frozen in liquid nitrogen-chilled isopentane and stored at -70 °C.







The images above show our method for quantifying the number of radial glia in each area of the white matter as described above. Fig 1.1 is from a wild-type specimen, Fig 1.2 is from a PlexinA4-deficient sample, and Fig 1.3 is from a Semaphorin6A-deficient sample. PS=Pial Surface, CC=Central Canal, GM=Grey Matter, WM=White Matter, DH=Dorsal Horn, VH=Ventral Horn, D=Dorsal, V=Ventral, ML=Midline.

Immunohistochemistry

15-µm cryosections were used for all analyses, and these were obtained using a Leica cryostat. Sections were blocked in 10 mM PBS containing 20% normal goat serum (NGS) plus 0.05% Triton-X 100 (Sigma, UK),and then incubated in primary antibody in 10 mM PBS containing 1% NGS plus 0.2% Triton-X 100 (Sigma) for 1 h at room temperature. The primary antibody solution consisted of 0.2% RC2, 1% NGS and the remainder PBS. These preperations were then blotted and left overnight at 4°C. Primary antibody binding was detected by incubation with secondary antibodies (1% NGS, 0.2% Alexaphor IgM 488nm, remainder PBS) for 3 h at room temperature. The microscope used was an Olympus Bx51 upright fluorescent microscope, with a mercury lamp attached, and the images were displayed using CellSeus software.

Quantification of process density in the spinal cord WM

For fibre density analysis, 15-µm cryosections were cut from the rostral and caudal regions of the spinal cord, using a Leica cryostat, in a coronal plane. The analysis of radial glial process density in the spinal cord WM was carried out using the line intersect stereological approach (Mayhew, 1992). This involves assessing the number of times immunoreactive processes

intersect with a series of 50-μm reference lines placed centrally in the ventral, lateral and dorsal WM, spaced 25-μm apart, and oriented in planes parallel to the pial surface. To place these reference lines accurately, the each image was printed onto an A4 sheet of paper with a scale factor of 250. This means that 50μm in the spinal cord was equivalent to 20mm on the page, so a series of 20mm reference lines were drawn. These lines were drawn upon an acetate cover placed over the image. The first reference line was placed lateral to the developing ventral floor plate, while the last was placed in the dorsal WM, lateral to the developing dorsal funiculus. Sections were taken from multiple levels within each spinal cord. The number of processes crossing each reference line was noted, and an average was taken for each numbered section for each of the three phenotypes.

RESULTS

Quantification of Radial Glial Processes

As expected, many radial glial processes were seen traversing the spinal cord. They stretched from the central canal to the pial surface but there was an especially large number in the white matter. Figures 1.4, 1.5 and 1.6 below show the average number of glial processes seen crossing the white matter in each section of the spinal cord for all three phenotypes. In each case, the greatest number of processes was seen crossing in the lateral section. In the wild-type specimens, the peak was seen in section four, with an average of 6.44 processes crossing within the 50μm line. In the PlexinA4-deficient samples, the peak was in section 6 at 5.83 while in the Semaphorin6A-deficient specimens the peak was in section 5 at 6.25. The error bars were derived by finding the standard deviation of the numbers used to generate the average for each section of spinal cord. The average of these standard deviations was then found to give a consistent size of error bar. For the wild type samples this came out to ±0.92, for PlexinA4-deficient samples this was ±0.50, and for the Semaphorin6A-deficient specimens this was ±0.84. Our full results can be seen summarised in table 1.1.

Comparison between Phenotypes

The number of processes crossing the white matter in each phenotype was directly compared in figure 1.7. Here, the average number of processes crossing the lateral column of white matter in particular, consisting of sections 4, 5 and 6, are compared. In the wild-type samples, the average was 5.78, in the PlexinA4-deficient samples it was 5.58, and for Semaphorin6A-

deficient samples it was 5.75. As can be seen, each result falls within the margin of uncertainty of the other two, so no statistically significant difference can be found between the three phenotypes. The margin of error was calculated similarly to above, and was found to be ±0.97.

Section Number	Wild Type (n=13) (±0.92)	PlexinA4$^{-/-}$ (n=3) (±0.50)	Semaphorin6A$^{-/-}$ (n=4) (±0.84)
1	3.62	2.67	4.75
2	4.27	3.67	4.50
3	5.83	4.67	5.25
4	6.44	5.17	6.00
5	6.08	5.75	6.25
6	4.81	5.83	5.00
7	3.96	4.58	5.25
8	3.43	4.00	5.00
9	3.69	3.00	3.67

Table 1.1

This table shows the data used to generate figures 1.4, 1.5 and 1.6. It shows the average number of radial processes that were observed crossing each section of the white matter in each samples of each phenotype.

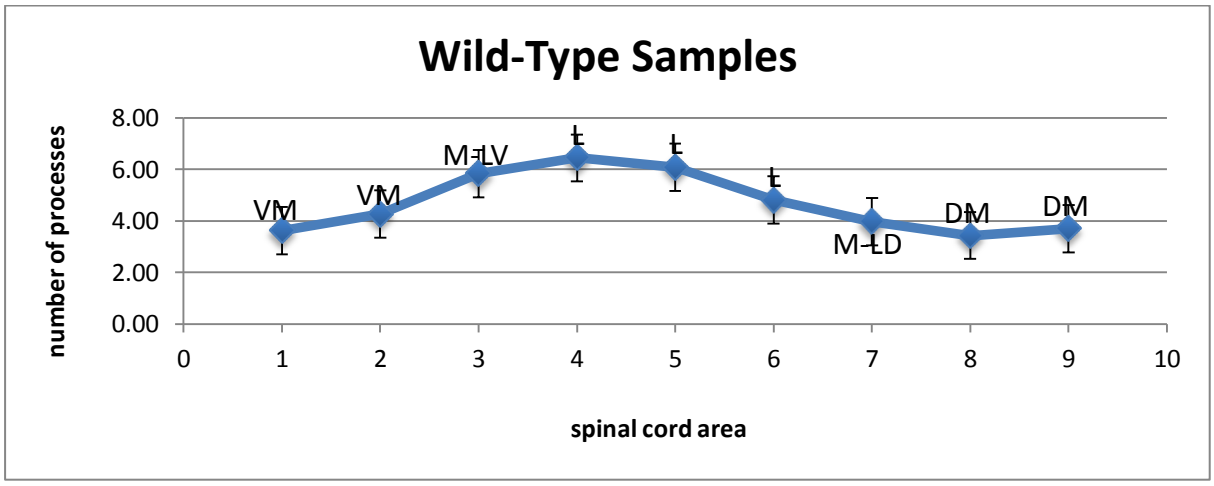


Fig 1.4

This line chart shows the average number of glial processes that were observed to be crossing each section of the wild-type mouse spinal cord. Each numbered area is

labelled with its anatomical position. VM=Ventro-Medial, M-LV=Medio-Lateral Ventral, L=Lateral, M-LD=Medio-Lateral Dorsal, DM=Dorso-Medial.

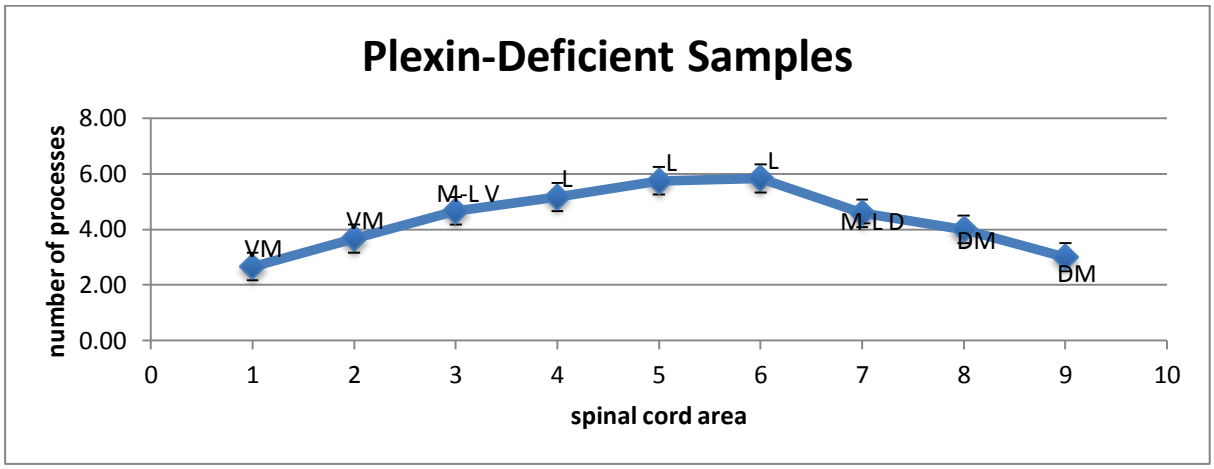


Fig 1.5

This line chart shows the average number of glial processes that were observed to be crossing each section of the PlexinA4-deficient mouse spinal cord. For labels, see above.

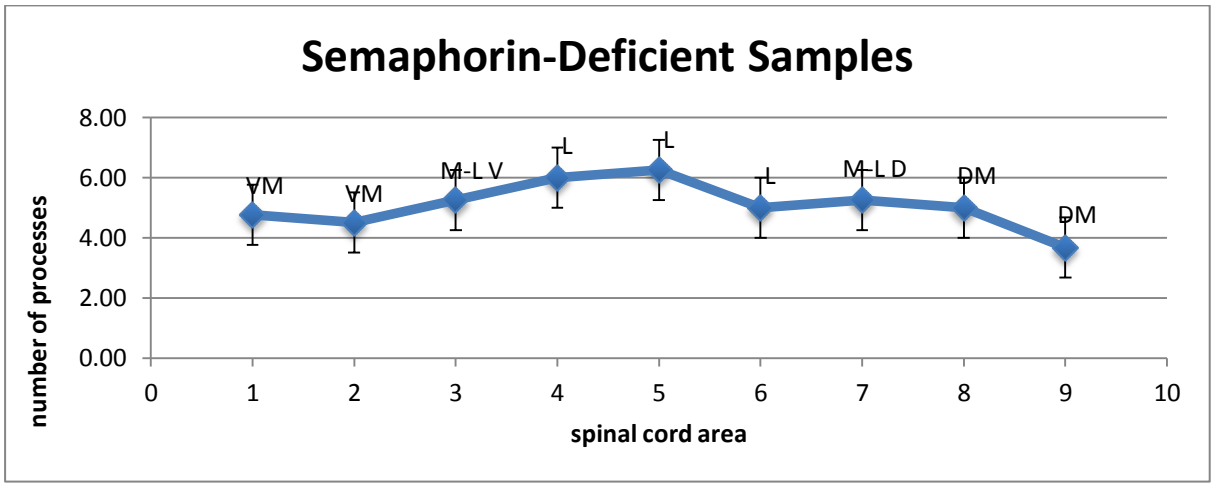


Fig 1.6

This line chart shows the average number of glial processes that were observed to be crossing each section of the Semaphorin6A-deficient mouse spinal cord. For labels, see above.

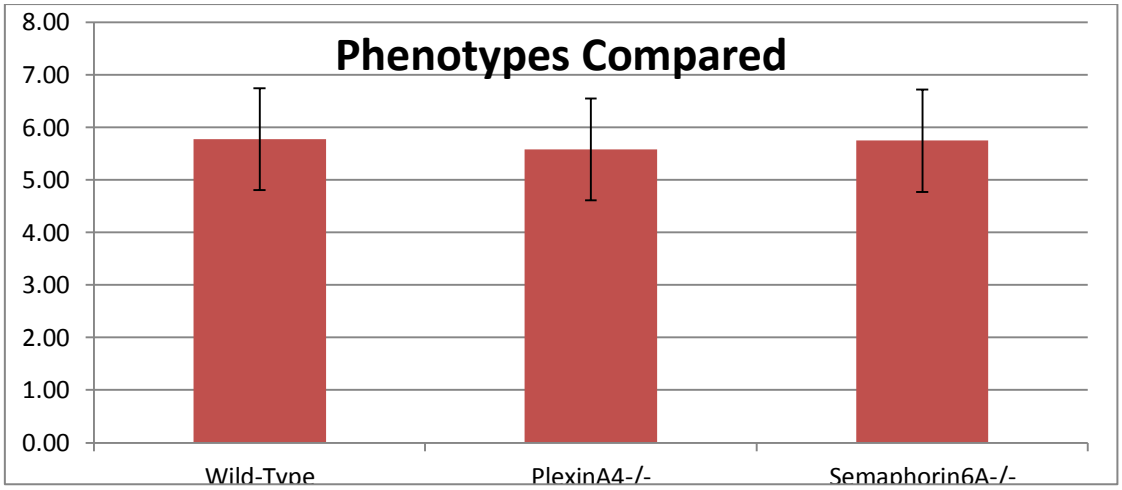


Fig 1.7

This bar chart compares the average number of glial processes seen crossing the lateral sections of the three phenotypic categories of spinal cord. The lateral sections were numbers 4, 5 and 6.

DISCUSSION

Context of our Findings

During the last 15 years, many advances have been made in the understanding of radial glial cells, and their dual role as neuronal progenitors and aids to neuronal migration. Yet they remain poorly characterised in the spinal cord when compared to the cerebral cortex. It had been ascertained that they lack stem cell capabilities here, but it is only recently that some insight has been gained into their potential function. It has been shown that the radial glial scaffold, seen stretching from the ventricular zone to the pial surface, forms during the period of development when the white matter tracts are forming, and then disappears as their development completes. This was shown by vimentin and nestin staining. It therefore seems possible that they could help to direct the development of these tracts, and in fact glial scaffolds have been observed playing a similar role in other parts of the central nervous system. For example, in the rostral migratory stream, the developing optic nerve and the corpus callosum. It should be noted, however, that the scaffold in the spinal cord does not express GLAST or BLBP, two markers typically expressed by radial glial cells, so these may be of a slightly different phenotype (Barry et al 2013).

Discussing our Results

In our study, we examined the number of these processes crossing each column of white matter in the developing mouse spinal cord at E15.5. As expected, we observed the processes traversing the entire cord, from the ventricular zone to the pial surface, and there were especially large numbers while crossing each region of the white matter, supporting the previous conclusions. Our results show that the greatest number of processes was consistently observed in the lateral column of the white matter. If their proposed function was confirmed, this may suggest that axon tracts are developing particularly quickly in that column at this stage of development. In humans, the lateral corticospinal tract and the spinocerebellar tracts are known to occupy this area, so perhaps these glial processes are contributing towards the formation of the mouse's equivalent. Also, we compared the number of processes in normal mice to numbers in both Semaphorin6A-deficient and PlexinA4-deficient specimens. Semaphorins are proteins that have been shown to act as guides for the development of axons in various contexts, but their effects on radial glia in the developing spinal cord have not yet been shown conclusively. Plexins are the receptors which Semaphorins act on. Our results did not show a significant difference between the three phenotypes. This shows that the role of these proteins in the developing spinal cord remains uncertain, and is certainly an area that warrants further study.

CONCLUSION

The many functions of radial glia continues to be an area of considerable research, and we hope to have contributed a useful quantification of the glial processes present at one stage of development to the discussion. Therefore, it is hoped that the full and precise role of these glia in white matter patterning can be elucidated in the near future. Also, we have shown a small glimpse at the possible involvement of Semaphorins and Plexins in the guiding of glial formation.

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