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Guidance of glial precursor cell migration by secreted cues in the developing optic nerve

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SUMMARY

Oligodendrocyte precursors are produced in restricted foci of the germinative neuroepithelium in embryo brains and migrate to their sites of function, while astrocytes are produced in a wider area in the neuroepithelium. We investigated the guidance mechanisms of glial precursor (GP) cell migration in the optic nerve. GP cell migration in newborn rat optic nerve was monitored by the UV-thymine-dimer (TD) method. A double labeling study using NG2 and TD revealed that many of these in vivo migrating cells were NG2 positive, while some of them with large TD-positive nuclei were NG2 negative. An in vitro cell migration study using optic nerve with chiasma and/or eyeball tissue revealed that the GP cells migrated under the guidance of repulsive cues secreted from the optic chiasma. We detected the expression of netrin 1 and Sema3a in the optic chiasma, and that of Unc5h1 and neuropilin 1 in the optic nerve. Co-culture experiments of the optic nerve with cell clusters expressing guidance cues revealed that the migrating GP cells in the optic nerve were heterogeneous. Netrin 1 repelled a subtype of NG2-positive and PLP-positive GP cells with small nuclei. Sema3a repelled a subtype of GP cells with large nuclei.

Key words: Cell migration, Glial precursor, Netrin 1, O-2A, Optic nerve, Sema3a, Rat

INTRODUCTION

In the mammalian central nervous system (CNS), oligodendrocyte precursors are produced in restricted foci of the germinative neuroepithelium (Ono et al., 1997; Spassky et al., 1998), while astrocyte precursors originate in a wider area of the neuroepithelium. These glial cell precursor (GP) cells are produced in the perinatal period. At the time of their production, the embryo brain has developed and the distance between the site of origin and the site of function has expanded. Therefore, the GP cells, especially oligodendrocyte precursors must traverse a great distance. To guide cell migration, neurons follow radial fibers (Rakic, 1990) or gradients of secreted trophic cues (Yee et al., 1999) and repulsive cues (Wu et al., 1999; Zhu et al., 1999; Tamamaki, 1999). However, the guidance mechanisms underlying GP cell migration are not yet known. Labeled GP cells in slice cultures were seen to migrate in a distinct manner (Kakita and Goldman, 1999). The migrating GP cells had a leading process and a tailing process, which were indistinguishable from those of tangentially migrating neurons in the intermediate zone (Tamamaki et al., 1997). This similarity in morphology of the two cell types during migration, led us to speculate that the GP cells also used trophic and repulsive guidance cues to find proper trajectories of cell migration.

We examined the guidance mechanisms of GP cells in newborn rat optic nerves. O-2A progenitors (Raff, 1989), a GP-cell type in the optic nerve, originate at a focal point on the floor of the third ventricle located just above the optic chiasma (Ono et al., 1997) and then migrate distally in the optic nerve (Small et al., 1987). The optic nerve is the most suitable tissue for the study of GP cell migration because it contains glial cells, but no intrinsic neurons. Moreover, the optic nerve can be regarded as a part of the CNS and glial cell types in the CNS play roles in the maintenance of the optic nerve. The optic nerves were cultured either alone or with connected structures, such as the eyeball and chiasma. To study the guidance mechanisms underlying GP cell migration, we used a new method to detect cell migration both in vivo and in vitro (Tamamaki et al, 1999). We also used a collagen gel culture system to investigate further the effect of guidance cues.

MATERIALS AND METHODS

UV-thymine-dimer labeling

Newborn rats were deeply anesthetized hypothermically. The skull around the bregma was removed and the brain tissue over one side of the optic nerve was aspirated. The proximal parts of the optic nerves

were irradiated with UV light through a fiber optic cable of 200 μm diameter for 5 seconds. Five hours after the irradiation, the optic nerves were processed for thymine-dimer (TD) detection in paraffin wax sections or in cryostat sections as described previously (Tamamaki et al., 1999). For investigation of the cell migration in vitro, the optic nerves with their adherent optic chiasmas and/or eyeballs were immersed in culture medium (DMEM+10% FCS), irradiated with UV light, and cultured for 5 hours.

Double labeling for TD and NG2

Cryostat sections were incubated with a 1/2000 anti-NG2 rabbit antibody (Stallcup and Beasley, 1987) and 1/300 anti-TD mouse monoclonal antibody (Kyowa Medics, Co. Japan). The sections were further incubated with biotinylated anti-mouse IgG goat serum and avidin-biotinylated peroxidase complex (ABC; Vector, USA), and the TD-positive sites were colored black by a Ni-DAB reaction. In addition, the sections were incubated with anti-rabbit IgG goat serum and an ABC complex, and processed for a simple DAB reaction to reveal NG2-positive cells in brown.

In situ hybridization and RT-PCR

cDNA fragments of *netrin 1*, *Sema3a*, *Unc5h1* and *neuropilin 1* (*Nrp*) were amplified by polymerase chain reaction (PCR) from a cDNA obtained from E16 rat brains. For the PCR amplifications, the following primers were used: CTTCGTCAACTCGGCCTTCG and GCCTCCTGCTCGTTCTGCTT for *netrin 1*; AGCACAGCTTCC-TCTACACC and TCTCTGTGACTTCGGACTGC for *Sema3a*; GCCCTTGGACTCATTTACTG and GAAGTTGAAGGTCCC-GTAGG for *Unc5h1*; TGGAGGGAACAAGGGAGGAG and CGTTGGCGTCCCCTGAAATG for *Nrp*. Each PCR product was cloned into a pCRII plasmid (Invitrogen). Sense and antisense cRNA probes labeled with ^{35}S were made and used for in situ hybridization as described previously (Simmons et al., 1989).

Optic nerves were collected from ten newborn rats after decapitation. Selectively, mRNA in the optic nerves was purified (Invitrogen, USA) and reverse-transcribed into cDNA with Superscript (Gibco, USA). For detection of *Unc5h1* and *Nrp* cDNAs, the same primer pairs shown above were used in PCRs.

Coculture of an optic nerve and COS1 cells

Newborn rats anesthetized by hypothermia were decapitated. Brains were removed leaving the optic chiasmas and optic nerves on the scale bases. The middle part of the optic nerve, visible on the scale base, was irradiated with UV light, removed leaving a short stump of the optic nerve attached to the chiasma, and embedded in collagen gel with COS1 cell clusters secreting netrin 1 and/or Sema3a. The optic nerves were cultured for 5 hours, in DMEM+10% FCS. After the culture period, the optic nerves were fixed and processed as for the in vivo labeling study. The expression vectors for netrin 1 (pGNET1-myc) and Sema3a (pCOS-H-semaIII-myc), the anti-Nrp antibody and the netrin mutant mice were kind gifts from Dr Marc Tessier-Lavigne. Green Lantan plasmid (GibcoBRL) for the GFP expression vector was used as a control. The expression vectors were transiently transfected into COS1 cells using Fugene-6 (Boehringer-Mannheim, Germany). The expression of netrin 1 and Sema3a in the COS1 cells was confirmed by western blot analysis or immunohistochemistry for the myc tag.

Guidance of dispersed-GP-cell migration

Optic nerves were obtained as described above, cut into short pieces and embedded in collagen gel with BHK cell clusters. One cluster was placed in contact with a cut end of the optic nerve. The other was placed several hundred micrometers away from the opposite cut end. The BHK cells were infected with recombinant Sindbis virus (Invitrogen, USA) for the expression of netrin 1, Sema3a, or GFP. Full length cDNAs of *netrin 1-myc*, *Sema3a-myc* and GFP were cloned in a shuttle vector, pSinRep5. Following the recommended protocol, a viral solution with a titer of 1×10^9 PFU/ml was obtained. 10 μl of the viral solution were added to the medium (MEM+5% FCS) of BHK cells in 3 cm diameter culture dishes. One hour after infection, BHK cells were treated with 0.1% trypsin in PBS and collected in fresh medium. The BHK cells were cultured in a hanging drop of the medium, overnight, to obtain BHK cell clusters. Medium containing 10% FCS was added on top of the collagen gel. After culturing for 3 days, the cocultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) overnight. Whole collagen gel cultures were incubated with an anit-NG2 (1/2000; Stallcup and Beasley, 1987), an anti-O4 (5 μg/ml; Boehringer Mannheim, Germany), an anti-PLP (1/50; Chemicon, USA), or an anti-GFAP (diluted DAKO, Denmark) antibody for 3 days. The whole gels were further incubated with an ABC reaction solution and stained using a Ni-DAB reaction.

RESULTS

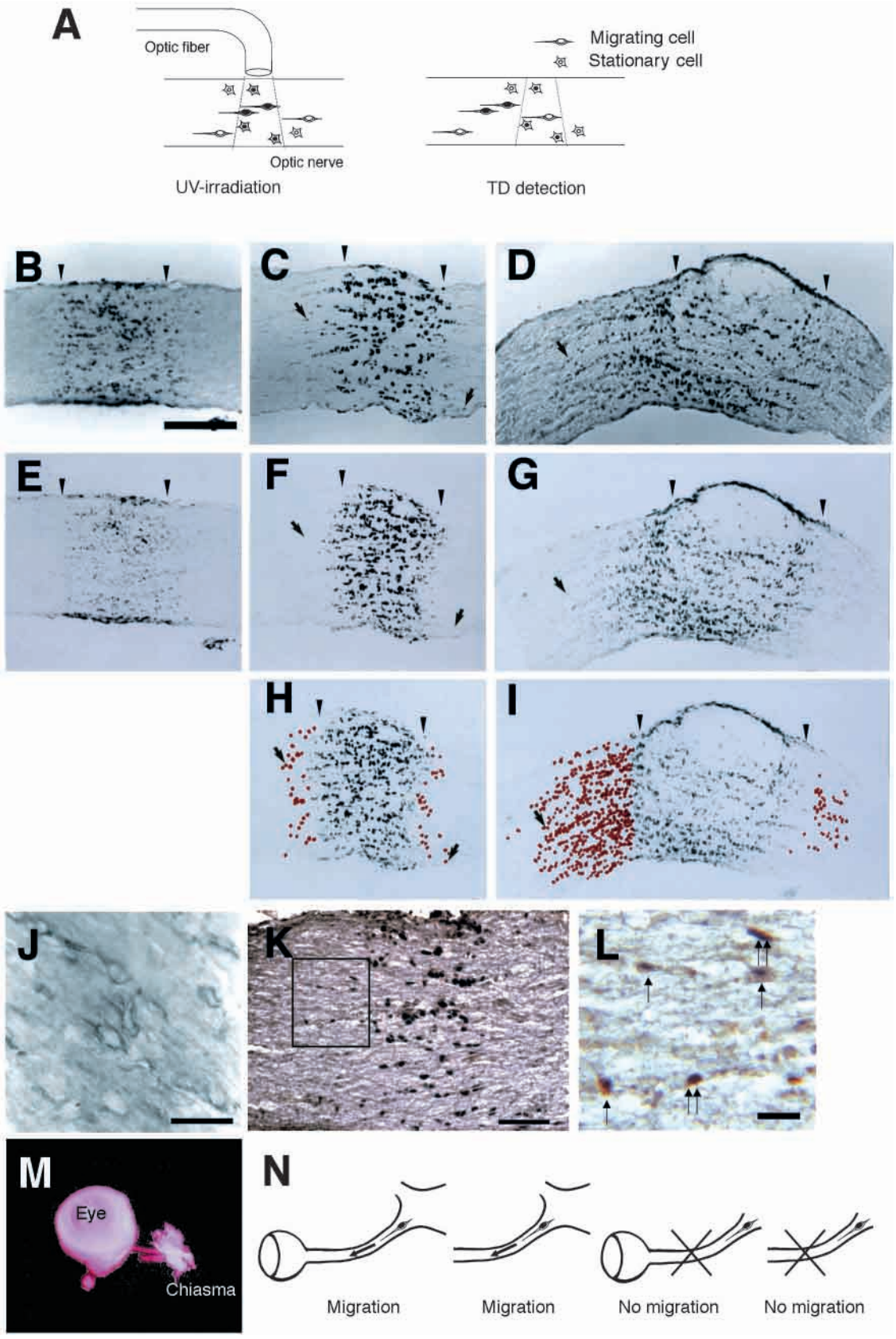
GP cell migration observed in vivo and in organ culture

The method for detecting cell migration involves UV-light irradiation of the cells through a fiber-optic cable (Tamamaki et al., 1999). The irradiated cells were subsequently identified on the basis of the TD formation and their immunohistochemical detection in the nuclei (Fig. 1A). TD-positive (TD+) migrating cells with negligible levels of damage migrated normally in vivo. This method allowed us to monitor the GP cell migration in vivo and in short-period organ cultures with the least risk of dedifferentiation in GP cells, which often occurs in GP cells in culture on plastic dishes. Newborn rat optic nerves were irradiated in vivo or in vitro for 3-5 seconds with UV light through a 200 μm diameter cable attached to the

Fig. 1. GP cell migration in optic nerves in in vivo or in vitro cultures together with the optic chiasma and/or the eyeball. Migrating cells exiting from the UV-irradiated area were observed when the optic nerve was cultured with the optic chiasma. (A) schematic diagram showing the principle of the UV-TD labeling method used to study cell migration. The UV-irradiated site was identified by TD-positive stationary cells (see Tamamaki et al., 1999 or the text for details). (B) TD-labeled cells observed in a paraffin section of an optic nerve fixed immediately after UV irradiation. (C) TD-labeled cells observed in an optic nerve fixed after culture for five hours. (D) TD-labeled cells in an optic nerve cultured with the optic chiasma and eyeball for five hours. (E-G) reconstructed UV-irradiated area. Photographs of serial sections were taken on transparent paper and they were overlapped to confirm the UV-irradiation area. (H-I) Reconstructed pattern of the TD-labeled nuclei distribution shown in C and D. The labeled migrating cells (red dots) were traced onto the transparent photographs, which were then overlapped to show the distribution of the labeled migrating cells. Arrows in C, F and H, and D, G and I indicate the same cells. Arrowheads indicate the UV-irradiated area, as estimated from the distribution of TD-labeled stationary cells. (J) NG2-negative immunoreactive cells in the newborn rat optic nerve. (K) Double staining study for TD and NG2 immunoreactivity. The rectangular area in the figure was enlarged in L. (L) NG2-positive migrating cells (single arrows) and NG2-negative migrating cells (double arrows). (M) An optic nerve cultured with an eyeball and the chiasma in vitro. (N) Schematic diagrams summarizing the condition under which the GP cell migration was observed. The optic chiasma was necessary to guide the GP cell migration distally. Scale bar in B, 100 μm (B-I); in K is 100 μm; in J and L are 10 μm.

nerve surface. Immediately after UV irradiation of the optic nerve, the boundary of the irradiated area was found to be contoured clearly by TD-positive nuclei in the perineurium and in the optic nerve (Fig. 1B,E). Thus, we regarded the TD-positive cells outside the irradiated area as migrating cells.

O-2A progenitors in the optic nerve were identified by



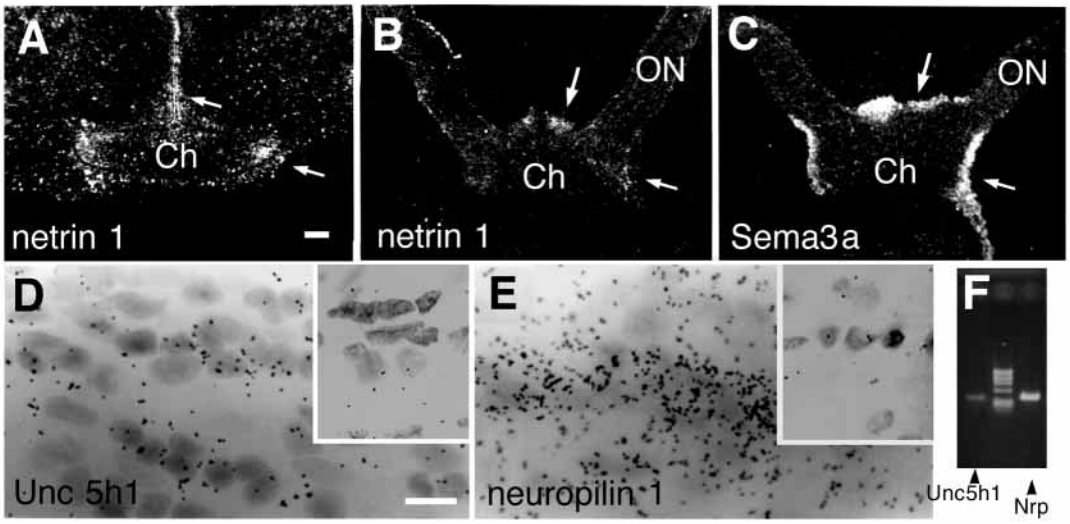


Fig. 2. In situ hybridization to detect the expression of repulsive cues in the optic chiasma and that of their receptors in the optic nerves. (A) Netrin 1 expression in a frontal section of a postnatal day 1 (P1) rat brain. The ependymal layer of the third ventricle and the lateral edges of the optic chiasma were netrin 1 mRNA-positive. (B) In a horizontal section of the optic chiasma, a netrin 1 signal was found in the anterior pole and lateral edge of the optic chiasma. (C) A Sema3a signal was also found in the anterior pole and lateral edges of the optic chiasma in a horizontal section. Arrows indicate the significant accumulation of signals. (D) Many cell nuclei in the optic nerve were positive for Unc5h1 mRNA. The inset shows the sensing probe signal. (E) Many cell nuclei were also positive for Nrp mRNA. The inset shows the sensing probe signal. (F) Results of RT-PCR using mRNA obtained from a newborn rat optic nerve. Lane 1, Unc5h1; lane 2, molecular marker 0X174 *Hac*III digested; lane 3, Nrp. CH, optic chiasma; OP, optic nerve. The calibration bar in A-C is 100 μm and that in D-E is 10 μm.

immunohistochemical staining with an anti-NG2 antibody (Stallcup and Beasley, 1987) (Fig. 1J). A double labeling study of NG2 and TD revealed that in vivo many of these migrating cells were NG2 positive (arrow in Fig. 1L), while some of them with large TD-positive nuclei were NG2 negative (two arrows in Fig. 1L). Therefore, we concluded that the migrating TD-positive cells in the optic nerves were heterogeneous and consisted of NG2-positive O-2A progenitors and NG2-negative GP cells. The double labeling study gave convincing staining only in the sample that was UV-irradiated and fixed in vivo.

When the optic nerve was cultured alone for 5 hours (Fig. 1C,F,H), a small number of TD-positive cells migrated both distally and proximally from the irradiated area. In each 7 μm thick section of five optic nerves, 11±6.6 cells migrated 43.3±15.3 μm distally and 7.7±3.2 cells migrated 33.3±5.8 μm proximally. Differences in the cell number and in migration distance were not significant. Even without any guidance, TD-positive cells were assumed to migrate a short distance because O-2A progenitors in the optic nerves are highly motile (Small et al., 1987). Thus, we regarded any cell migration less than 60 μm as migration under no guidance. However, when the optic nerve was irradiated in vivo (Fig. 1K,L) or in culture together with the optic chiasma and the eyeball (Fig. 1D,G,I,M), 100±51.1 TD-positive cells migrated 162±26.8 μm distally and 15±3.7 cells migrated 56.0±16.7 μm proximally. A significant number of TD-positive cells were guided distally (*t*-test: $P<0.05$).

When the optic nerve was cultured with the optic chiasma (Fig. 1L), 68.8±20.4 (n=5) GP cells migrated 109±22.3 μm (n=5) toward the distal cut end. The migration toward the proximal cut end was less than 60 μm (n=5). However, the eyeball alone did not have the ability to attract the GP cells toward the eyeball. Both the migration toward the distal and proximal cut ends was less than 60 μm. All the data consistently indicated that some repulsive cues were secreted from the optic chiasma or surrounding tissues to guide the GP cells in the optic nerve toward the eyeball (Fig. 1N).

Guidance cues released from the chiasma

Therefore, we investigated the expression of several guidance cues in the optic chiasma of newborn rats by in situ hybridization and detected the expression of netrin 1 (Serafini et al., 1994) and Sema3a (Kolodkin et al., 1993) (Fig. 2A-C). Netrin 1 was expressed not only in the ependymal layer of the third ventricle, but also in the lateral edges of the optic chiasma (Fig. 2A). In horizontal section, the anterior pole and lateral edges of the optic chiasma also appeared as netrin 1 positive (Fig. 2B). The netrin 1 expression at the junction of the eyeball and optic nerve (Serafini et al., 1994; Serafini et al., 1996; Deiner et al., 1997; Deiner et al., 1999) was at a background level on the day of birth. Sema3a was also expressed in the anterior pole and lateral edges of the optic chiasma, and the sheath of the optic nerve in its proximal part (Fig. 2C).

Currently, DCC, Unc5h1, Unc5h2, and Unc5h3 are known to be netrin 1 receptors (Leonardo et al., 1997), and Nrp (Kawakami et al., 1995; He et al., 1997; Kolodkin et al., 1997) is known to be a Sema3a receptor. We investigated the expression of these receptors and found expression of Unc5h1 (Fig. 2D) and Nrp (Fig. 2E) in the optic nerve. The mRNA signal of these receptors covered the nuclei of many cells in the optic nerve. RT-PCR also revealed expression of these receptors in the optic nerves (Fig. 2F). In situ hybridization using sensing probes for these secreted factors and receptors did not show any significant accumulation of silver grains in the tissue sections (insets).

Assay of the guidance cue effects in the organ culture

In order to determine whether netrin 1 and Sema3a guided the GP cells expressing Unc5h1 or Nrp, netrin 1 and/or Sema3a, expression vectors were transfected into COS1 cells (Fig. 3N). In co-cultures of UV-irradiated optic nerve with a netrin 1-secreting COS1 cell cluster placed at the proximal cut end of the optic nerve (Fig. 3A), most migrating cells were found on the distal side to the irradiated area (39.2±18.6 cells, n=5).

They migrated a maximum distance of 88.0±11.0 μm (n=5) in 5 hours. In co-cultures with a Sema3a cluster (Fig. 3B), most migrating cells were directed distally from the irradiated area (35.4±14.2 cells; 94.0±11.4 μm, n=5). In co-cultures with clusters secreting netrin 1 and Sema3a (Fig. 3C), most migrating cells were also directed distally (23.4±11.3 cells, n=5; 74.0±18.2 μm, n=5). However, we could not detect any significant enhancement either in the number of migrating cells, or in the migration distance, over a 5-hour period. To confirm that the cues were repulsive, COS1 cell clusters secreting netrin 1 or (and) Sema3a were placed at the distal cut end of the optic nerve and cultured. As expected, the TD-positive cells migrated toward the proximal cut end of the optic nerves (Fig. 3D-F), which was opposite to the direction of normal cell migration in vivo (Fig. 1K).

To further identify the cell populations guided by netrin 1 and Sema3a, COS1 cell clusters secreting netrin 1 and those secreting Sema3a were placed at opposite cut ends of the optic nerve. As shown in Fig. 3G, the TD-positive cells migrated and appeared both distal and proximal to the UV-irradiated area. The TD-positive cells responsive to netrin 1 (64.6±17.9 cells; 90±25.5 μm, n=5) were not repelled by Sema3a. The TD-positive cells responsive to Sema3a (61.2±13.4 cells; 93±12.0 μm, n=5) were not repelled by netrin 1, either. Thus, the cells responsive to netrin 1 and those responsive to Sema3a were considered to be different cell types. Each migrating GP cell had a leading process directed away from the netrin 1 or Sema3a source (Fig. 3J,K). The TD-positive nuclei of the cells responsive to netrin 1 were 4.8±1.1 μm (n=30) long. The TD-positive nuclei of the cells responsive to Sema3a were 5.7±1.2 μm (n=30) long. The difference in the nuclear size was significant (t-test: P<0.01).

The guidance of GP cells with large nuclei by Sema3a-Nrp interaction was confirmed by blocking Nrp with an anti-Nrp antibody (Chen et al., 1998). Following rinsing of the optic nerves in the antibody solution for 30 minutes and application of the antibody in the bathing medium of the collagen gel cultures, the number of migrating cells guided by Sema3a was significantly reduced (8.9±3.2, n=5; t-test: P<0.05) (Fig. 3H). The migrating cells, even after Nrp blocking serum treatment, had significantly smaller nuclei (3.8±1.1 μm, n=30; t-test: P<0.01) than those of the cells responsive to Sema3a in the normal condition. Nonetheless, following the application of this antibody, the GP cell migration guided by netrin 1 was normal (36.7±11.2; 116.7±20.8 μm, n=5) (Fig. 3I).

Control COS1 cell clusters did not show any guidance effect on cell migration in the optic nerve (migration less than 60 μm).

Analysis using mutant mice and blocking serum

We also investigated mutant mice deficient in guidance cues or corresponding receptors. *Unc5h1* mutant mice have not yet been bred. A *netrin 1* gene knockout was lethal after birth and caused hypoplasia of the optic nerves (Serafini et al., 1996; Deiner et al., 1997; Deiner et al., 1999). Until now, because of the technical difficulties involved, there has been no success in experiments to detect cell migration in the optic nerve of *netrin 1* mutant mice in vitro. A *Nrp* gene knockout was also lethal in the early embryonic stage, and optic nerves with GP cells were not available from *Nrp* mutant mice (Kitsukawa et al., 1997). A *Sema3a* gene knockout was not lethal (Behar et al., 1996; Taniguchi et al., 1997). Thus far, the number of cells responsive to Sema3a in the optic nerves of *Sema3a* mutant mice has been normal (Fig. 3L). However, the size of the nuclei of cells responsive to Sema3a in mutant mouse optic nerves was small (3.6±1.1 μm; n=30) and comparable to those found in rat optic nerves under treatment with Nrp blocking serum (Fig. 3M).

Identification of GP cells responsive to guidance cues

At this point, we clarified that migrating GP cells were heterogeneous. In vivo, some of them appeared NG2 positive with small nuclei, and others as NG2 negative with large nuclei. Netrin 1-guided GP cells with small nuclei in organ culture. Sema3a-guided GP cells had large nuclei. However, a double labeling study using TD and NG2 immunohistochemistry, in cultured optic nerves, did not give convincing staining, and did not show that netrin 1 guided the NG2-positive GP cells. A double labeling study using NG2 immunohistochemistry and an Unc5h1 in situ hybridization study did not give convincing staining, either. To prove that the NG2-positive GP cells were guided by netrin 1 directly, we investigated the migration of dispersed GP cells in a collagen gel culture system.

GP cell migration was observed by inducing them out of the optic nerve into a collagen gel using a guidance cue. A short piece of optic nerve was placed in contact with a BHK cell cluster expressing netrin 1, Sema3a, or GFP (Fig. 4A). To obtain BHK cells expressing netrin 1, Sema3a, or GFP, we produced recombinant Sindbis virus expressing netrin 1, Sema3a or GFP. The efficiency of Sindbis virus infection on BHK cells was close to 100% in the titer we used. After the infection, BHK cells stopped migration and proliferation, and finally caused cell death after 3 days. Therefore, they did not migrate out from cell-clusters and did not disturb our observation of GP cells during the three days of the culture period. During the 3 days of culture, the short piece of optic nerve became spherical in most cases.

During the culture period, even in cases in which a piece of optic nerve was cultured alone, some GP cells (42±18; n=6) migrated from the optic nerve (Fig. 4A), because the GP cells were highly motile (Fig. 1; Small et al., 1987). Control BHK cells expressing GFP did not enhance the GP cell migration from a piece of optic nerve (data not shown). In the experiments with BHK cells expressing netrin 1 or Sema3a, another source of netrin 1 or Sema3a was placed in a collagen gel and cultured for 3 days as shown in Fig. 4B,C. Numerous GP cells migrated out of the optic nerve into the collagen gel under the effect of the guidance cues. In the culture with netrin 1, 110±36 (n=13) GP cells migrated out of the optic nerve. In the culture with Sema3a, 110±51 (n=12) GP cells migrated out of the optic nerve. The effects of netrin 1 and Sema3a were quantified by counting cells contained in the sectors shown in the figure (Fig. 4D). Sectors containing the source of guidance cues were set 100 μm wider on both sides. Cells in the sectors containing the source of guidance cues and those in the sector with less effect of guidance cue were counted, and the numbers of cells contained in 10º of the sectors were compared. However, the difference in the number of cells contained in the 10º of the sectors was not significant with either netrin 1 or Sema3a as the guidance source (Fig. 4B,C). Cells migrating without guidance could have disturbed our observation.

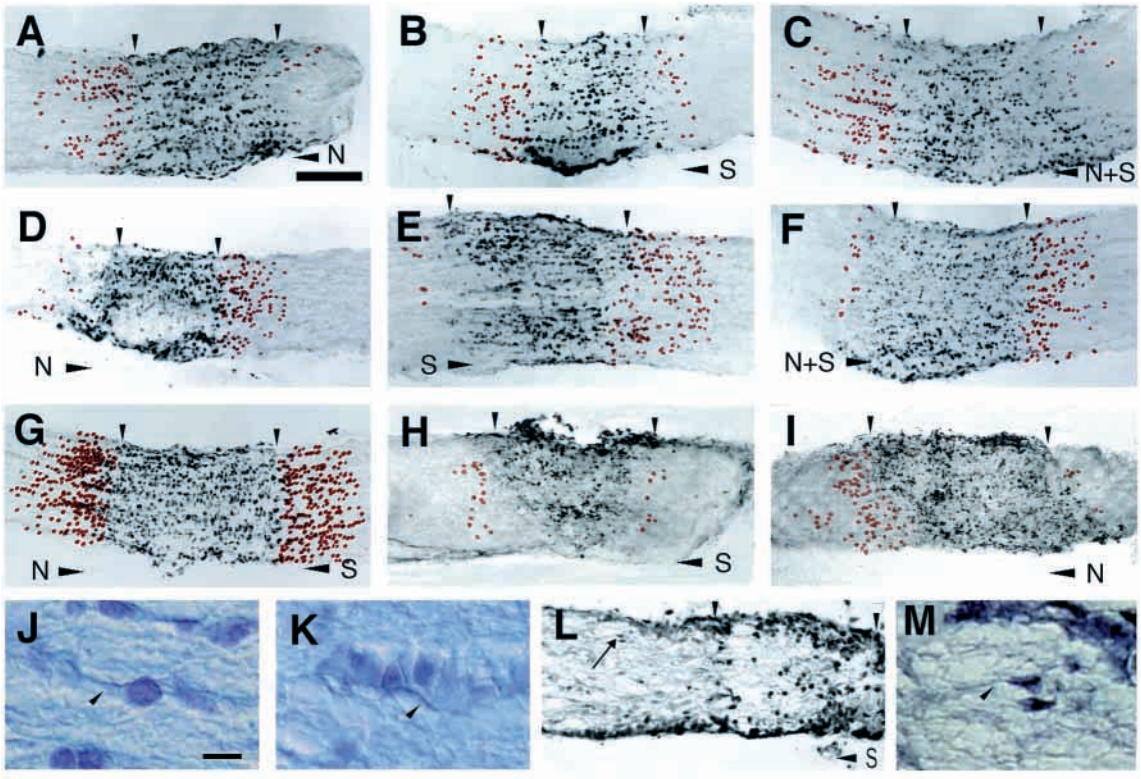


Fig. 3. GP cell migration in an optic nerve co-cultured with COS1 cell clusters secreting netrin 1 and /or Sema3a. The cell migration was shown by reconstructed distribution of TD-labeled migrating cells 5 hours after UV irradiation. The left sides of all the figures represent the distal direction of the optic nerve. (A-I) Small arrowheads indicate the UV-irradiation area. Large arrowheads indicate the direction of diffusion of guidance cue. (A) Cell migration in an optic nerve co-cultured with COS1 cell clusters secreting netrin 1. The COS1 cell clusters were placed at the proximal cut end of an optic nerve. (B) Cell migration in an optic nerve co-cultured with COS1-cell-clusters secreting Sema3a. The COS1 cell clusters were placed at the proximal cut end of an optic nerve. (C) Cell migration in an optic nerve co-cultured with COS1 cell clusters secreting both netrin 1 and Sema3a. (D-F) The same conditions as in A-C, except that the COS1 cell clusters were placed at the distal cut end of the optic nerves. (G) Cell migration in an optic nerve co-cultured with two COS1 cell clusters, one of which was secreting netrin 1 and the other was secreting Sema3a, placed at opposite cut ends of an optic nerve. (H) Cell migration in an optic nerve treated with anti-neuropilin blocking serum and co-cultured with COS1 cell clusters secreting Sema3a. The number of migrating cells significantly decreased. (I) Cell migration in an optic nerve treated with the anti-Nrp serum and co-cultured with COS1 cell clusters secreting netrin 1. The number of migrating cells was similar to that in A. (J) Migrating cells seen with Nomarski optics. Large TD-positive migrating cells responsive to Sema3a had a leading process (arrowhead) directed away from the Sema3a source. (K) Small TD-positive migrating cells responsive to netrin 1 had a leading process (arrowhead) directed away from the netrin 1 source. (L) Cell migration in an optic nerve obtained from a *Sema3a* mutant mouse and co-cultured with COS1 cell clusters secreting Sema3a. (M) The smallest TD-positive migrating cells responsive to Sema3a (indicated by an arrow in L) had a leading process (arrowhead) directed away from the Sema3a source. (N) Schematic diagram of the experimental arrangement. Scale bar in A is 100 μm (A-I,L); in J is 5 μm (J,K,M).

We further added an immunohistochemical procedure to the analysis. Immunoreactivity of NG2, O4 (Sommer et al., 1981) and PLP (Fuss et al., 2000), which are possible markers of oligodendrocyte precursors, were found in some of the cells migrating out of a piece of the optic nerve; 29±5.6%, 46±2.4%, and 39±2.9%, respectively (Fig. 4E-U; Table 1). GFAP-positive cells also appeared in the gel and made up 13±2.3% of the migrating cells (Fig. 4R-U; Raff, 1989). Most of the NG2-positive, O4-positive, PLP-positive and GFAP-positive cells were bipolar (Fig. 4G,K,O,S). According to the sector analysis of the distribution of the labeled cells, NG2-positive and PLP-positive cells significantly avoided the sectors containing the netrin 1 source. That is they were responsive to netrin 1 (Table 1). O4-positive cells also seemed to avoid the netrin 1 source. GFAP-positive cells appeared in this experiment to migrate at random.

We conducted the same set of experiments with the Sema3a sources. Immunoreactivity of NG2, O4, PLP and GFAP were found to be less than with netrin 1 as the source. 5.1±7.6%, 14±5.3%, 5.3±1.1%, and 6.7±2.6% of the GP cells migrated out of the optic nerve, respectively (Fig. 4V-Y; Table 1). According to the sector analysis in the distribution of the labeled cells, NG2+, O4+, PLP+, and GFAP-positive cells migrated at random and ignored the Sema3a sources (Table 1). The GP cells responsive to Sema3a seemed to be only those around the netrin 1 source, where the GP cells responded to netrin 1 repulsively (Fig. 4E,F). We found larger GP cells negative for the four markers around the netrin 1 source. They

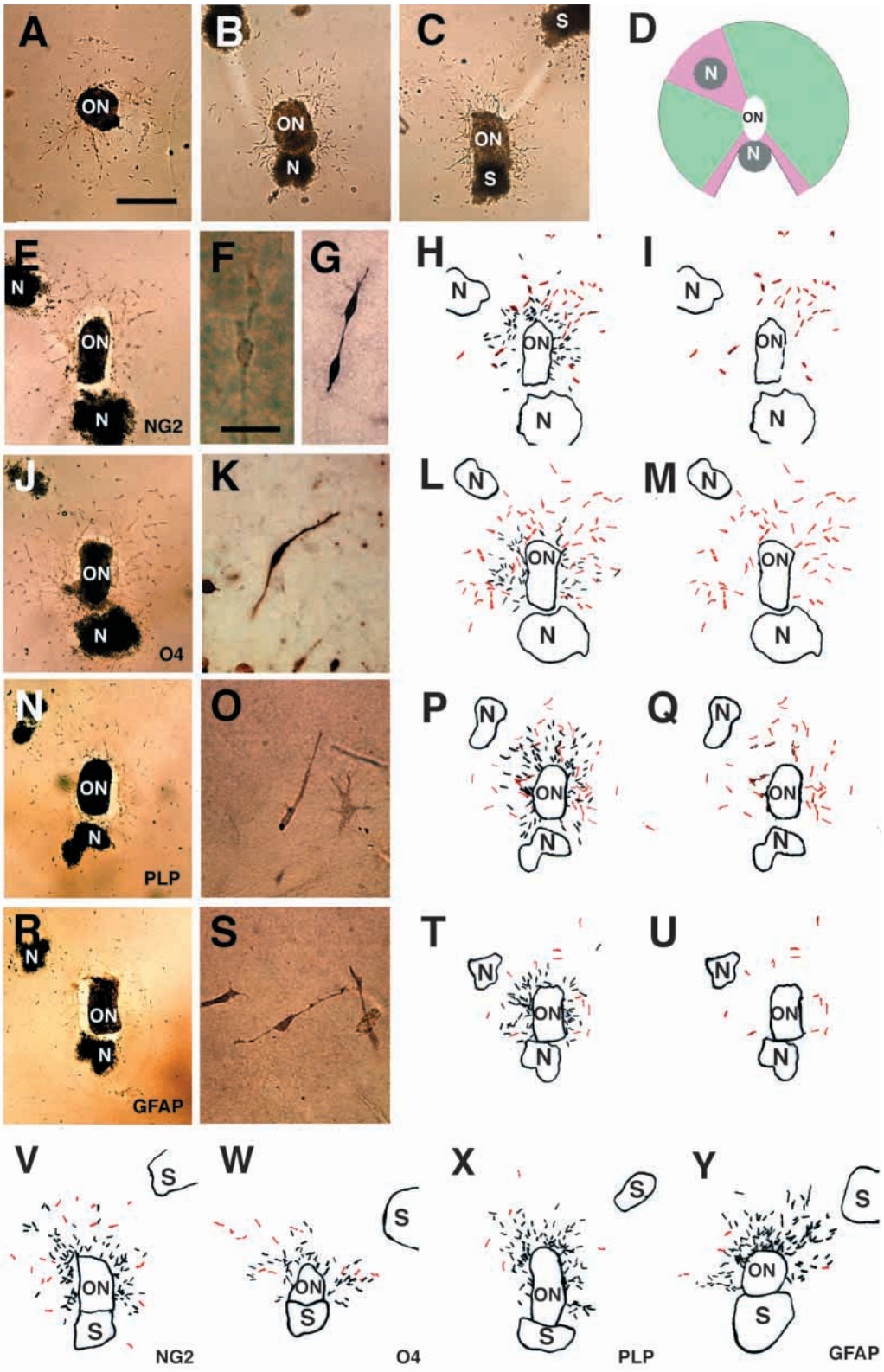


Fig. 4. GP cell migration in collagen gel co-cultured with BHK-cell-clusters secreting netrin 1 or Sema3a. (A) GP cell migration observed without the effect of a guidance cue. (B) GP cell migration observed under the effect of netrin 1 expressed by BHK cell clusters at a proximal cut end and at an additional point. (C) GP cell migration observed under the effect of Sema3a expressed by BHK cell clusters. (D) A schematic diagram showing the arrangement of BHK cell clusters secreting a guidance cue and a piece of optic nerve (ON) in a collagen gel culture. Gray indicates the guidance cue source. Pink indicates the sectors containing the guidance cue source and green indicates the sector with a smaller guidance cue effect. The sectors containing the guidance cue source were drawn 100 μm wider on both sides. (E-I) The distribution of NG2-positive cells in the co-culture with netrin 1 sources. (E) Low magnification photograph. (F) NG2-negative cells with large nuclei found near the netrin 1 source. (G) Bipolar NG2-positive cells. (H) Diagram of the culture in E. Red in H and I indicates the NG2-positive migrating cells. (I) Diagram of the culture in E showing the distribution of NG2-positive cells. (J-M) The distribution of O4-positive cells in the co-culture with netrin 1 sources. (J) Low magnification photograph. (K) Bipolar O4-positive cells. (L) Diagram of the culture in J. Red in L and M indicates the O4-positive migrating cells. (M) Diagram of the culture in J showing the distribution of O4-positive cells. (N-Q) The distribution of PLP-positive cells in the co-culture with netrin 1 sources. (N) Low magnification photograph. (O) Bipolar PLP-positive cells. (P) Diagram of the culture in N. Red in P and Q indicates the PLP-positive migrating cells. (Q) Diagram of the culture in N showing the distribution of PLP-positive cells. (R-U) The distribution of GFAP-positive cells in the co-culture with netrin 1 sources. (R) Low magnification photograph. (S) Bipolar GFAP-positive cells. (T) Diagram of the culture in R. Red in T and U indicates the GFAP-positive migrating cells. (U) Diagram of the culture in R showing the distribution of GFAP-positive cells. (V) Diagram of a co-culture of an optic nerve and Sema3a sources showing the distribution of NG2-positive cells (red). (W) Diagram of a co-culture of an optic nerve and Sema3a sources showing the distribution of O4-positive cells (red). (X) Diagram of a co-culture of an optic nerve and Sema3a sources showing the distribution of PLP-positive cells (red). (Y) Diagram of a co-culture of an optic nerve and Sema3a sources showing the distribution of GFAP-positive cells (red). N, netrin 1; ON, optic nerve; S, Sema3a. Scale bar in A is 500 μm, (A-C,E,J,N,R); in F is 50 μm (F,G,K,O,S).

Table 1. Guidance cue effects on GP cells labeled with oligodendrocyte markers

	IR cells	Responsive	Nonresponsive	*t*-test
Response to netrin 1				
NG2	25±5.6 (%)	1.50±0.11 (/10º)	0.13±0.11 (/10º)	$P<0.05$
O4	47±2.4	2.5±0.68	1.3±0.41	$P=0.1$
PLP	39±2.9	1.5±0.14	0.52±0.17	$P<0.05$
GFAP	13±2.3	0.24±0.13	0.16±0.07	$P=0.50$
Response to Sema3a				
NG2	5.1±7.6 (%)	0.21±0.30 (/10°)	0.32±0.24 (/10°)	
O4	14±5.3	0.34±0.16	0.36±0.29	
PLP	5.3±1.1	0.31±0.11	0.22±0.08	
GFAP	6.7±2.6	0.28±0.04	0.11±0.10	

Cells in the sectors containing the source of guidance cues and that in sectors with smaller guidance cue effects were counted, and the numbers of cells contained in 10° of the sectors were compared.

had significantly larger somata measuring 23.4±5.5 μm (n=30; t-test: $P<0.01$) than NG2-positive, PLP-positive or O4-positive cells (15.9±1.9 μm; n=30).

DISCUSSION

Technical considerations

To investigate the mechanisms of GP cell migration in the rat optic nerve, we used two methods, a UV-TD labeling method and a collagen gel co-culture method. Both methods were essential to reach the conclusion reported in this study.

The UV-TD labeling method has several advantages and disadvantages. First, it can be used to visualize all migrating cells at any point and at any time, regardless of their origin without any retrograde transport of the labeling by neurons. Of the techniques we tried, the UV-TD labeling method was the only method that could monitor the GP cell migration in in vivo and in vitro organ cultures for 5 hours. One disadvantage of the method was that UV irradiation caused some damage to some of the cells. However, by reducing the irradiation period, the method allowed monitoring of only TD-positive migrating cells that had escaped UV damage. We showed previously that in both in vitro and in vivo experiments UV damage did not disturb the direction of cell migration significantly (Tamamaki et al., 1999). Migrating TD-positive cells in the neocortex took a similar trajectory to reach the cortical plate.

The response of dispersed GP cells to guidance cues could also be monitored in a collagen gel culture without any harmful effects (Fig. 4). However, when dispersed from an optic nerve and placed in culture, it is well known that O-2A progenitor cells prematurely stop dividing and differentiate within 2 days (Raff et al., 1983; Raff et al., 1984). If these cells are cultured in 10% FCS they become type-2 astrocytes, whereas if they are cultured in the absence of FCS they become oligodendrocytes. Therefore, it is possible that the response of GP cells to guidance cues in vitro may be different from that in vivo. TD-labeled GP cells in the optic nerve migrated 50 μm/hour at most (unpublished data). However, they migrated only several hundred μm in the collagen gel co-culture in 3 days. It is easy to speculate that the substrates for cell migration and gradients of guidance cues were different in the collagen gel culture and in the optic nerves. Thus far, the results of the UV-TD labeling studies in vivo and in vitro were consistent with those from in the dispersed culture. The two methods were reliable in different aspects, and contributed to the conclusion described below in a complementary manner.

GP cells guided by netrin 1 and Sema3a

The netrins and the semaphorins belong to families of phylogenetically conserved guidance cues that can function as both attractants and repellents in guiding different classes of axons toward their targets (Serafini et al., 1994; Kolodkin et al., 1993). They also guide neuronal cell migration (Yee et al., 1999; Tamamaki, 1999). Here, in addition, we reported that two of these guidance cues (netrin 1 and Sema3a) guided GP cell migration in the infant rat optic nerve toward the eye.

The source of netrin 1 was found in the ventricular zone of the third ventricle, lateral and anterior edges of the optic chiasma. Sema3a was also found around the optic chiasma. The junction of the eyeball and optic nerve (Serafini et al., 1994; Serafini et al., 1996; Deiner et al., 1997; Deiner et al., 1999) ceased expressing netrin 1 by the day of birth. Netrin-1 and Sema3a released from these expression sites act as guidance cue gradients in the optic nerve. However, we are still uncertain how netrin 1 or Sema3a guides the GP cells from the ventricular zone of the third ventricle into the optic nerve. A few days after birth, the ventricular zone starts to supply GP cells to the optic tract (unpublished data). The mechanisms that guide the GP cells first to the optic nerve, and then to the optic tract at the optic chiasma, cannot be explained simply by netrin 1 and Sema3a. We speculate that netrin 1, Sema3a and other guidance cues participate in guiding the GP cells in the proper directions in the optic chiasma.

Our data showed that the migrating GP cells in the optic nerve at P0 were heterogeneous and divided at least into two types. One was a GP cell type that was responsive to netrin 1 and had a smaller nucleus and soma. The GP cells that were repelled by netrin 1 appeared as NG2 positive in a dispersed culture (Fig. 1). In the collagen gel, they further differentiated and most of them became oligodendrocyte precursors (O4+, PLP+). In our study, the culture medium with 10% FCS did not prevent O-2A progenitors from differentiating into oligodendrocyte precursors, and did not induce as many GFAP-positive cells as reported by Raff (Raff, 1989). This suggested that collagen gel in dispersed culture or netrin 1 (substrates or guidance cues) interact with the molecular mechanisms of O-2A progenitor differentiation.

The other type of GP cells responsive to Sema3a have large nuclei and somata (Figs 3J and 4K). The large NG2-negative, TD-positive migrating cells found in vivo (Fig. 1L) may belong to the GP cell type. O-2A progenitors in the optic nerve had been thought to be only motile GP cells, and were identified by the expression of NG2 in culture (Small et al., 1987). We found, however, that NG2-negative GP cells were also migrating in the optic nerves under the guidance of Sema3a. The markers we used in this study did not label the GP cells. Therefore, the GP cells responsive to Sema3a may not belong to any cell types previously found in the optic nerve.

The cells responsive to Sema3a, even after blocking the NP1 with antiserum and in *Sema3a* mutant mice, had smaller nuclei than those of the two GP cell types described above. This observation suggests the presence of some unknown receptors that interact with Sema3a on the smallest cells. Although the

smallest cells may be cells of the third type responsive to guidance cues in the optic nerve, we do not have a proper marker and need to make progress in the research of these cells before we can clarify this point.

The O-2A progenitors were repelled by netrin 1 from the base of the third ventricle (Ono et al., 1997) toward the eye and stopped at the barrier found at the junction of the eyeball and optic nerve (Ffrench-Constant et al., 1988; Huang et al., 1991; Laeng et al., 1996). This is a distance of a few millimeters in newborn rats. If the same molecular mechanism were adopted in human optic nerves, the distance which GP cells must migrate would be a few centimeters. Such a powerful molecular mechanism for guiding O-2A progenitor migration may become an important tool for establishing treatments for brain damage associated with a marked loss of oligodendrocytes (Rizzo et al., 1989; Lessmann et al., 1997; Bulte et al., 1999; Franklin et al., 1999).

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