Netrin 1 acts as an attractive or as a repulsive cue for distinct migrating neurons during the development of the cerebellar system

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SUMMARY

Netrin 1 is a long-range diffusible factor that exerts chemoattractive or chemorepulsive effects on developing axons growing to or away from the neural midline. Here we used tissue explants to study the action of netrin 1 in the migration of several cerebellar and precerebellar cell progenitors. We show that netrin 1 exerts a strong chemoattractive effect on migrating neurons from the embryonic lower rhombic lip at E12-E14, which give rise to precerebellar nuclei. Netrin 1 promotes the exit of postmitotic migrating neurons from the embryonic lower rhombic lip and upregulates the expression of TAG-1 in these neurons. In addition, in the presence of netrin 1, the migrating neurons are not isolated but are associated with thick fascicles of neurites, typical of the neurophilic way of migration. In contrast, the embryonic upper rhombic lip, which contains tangentially migrating granule cell progenitors, did not respond to netrin 1. Finally, in the postnatal cerebellum, netrin 1 repels both the parallel fibres and migrating granule cells growing out from explants taken from the external germinal layer. The developmental patterns of expression in vivo of netrin 1 and its receptors are consistent with the notion that netrin 1 secreted in the midline acts as chemoattractive cue for precerebellar neurons migrating circumferentially along the extramural stream. Similarly, the pattern of expression in the postnatal cerebellum suggests that netrin 1 could regulate the tangential migration of postmitotic premigratory granule cells. Thus, molecular mechanisms considered as primarily involved in axonal guidance appear also to steer neuronal cell migration.

Key words: Netrin 1 receptors, Cerebellum, Pontine nucleus, Neuronal migration, Chemoattraction, Chemorepulsion, Migration

INTRODUCTION

Although the mechanisms that guide neurons during migration are not well understood, the existence of restricted migratory pathways suggests that neuronal migration is under the control of specific guiding cues (Anderson et al., 1997; Soriano et al., 1997; Wichterle et al., 1997; Pearlman et al., 1998). In addition, the heterogeneity of migratory routes followed by migratory neurons that share their origin in a given germinal matrix indicates that distinct migratory neurons may respond differentially to similar extracellular signals.

Netrins are a family of laminin-related secreted proteins that may act either as chemoattractants or as chemorepellents for distinct developing axons (Kennedy et al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995). Netrin 1 is responsible for the attraction of commissural axons towards the midline in the spinal cord (Kennedy et al., 1994; Serafini et al., 1996), although these axons also require the interaction of at least two adhesion molecules, Axonin-1/TAG-1 and Ng-CAM/L1, to reach their targets (Stoeckli and Landmesser, 1995). DCC (deleted in colorectal cancer), a neural cell adhesion molecule of the Ig superfamily (Ig-CAMs), is a component of the receptor complex that mediates the chemoattractive response to netrin 1 (Keino-Masu et al., 1996; Fazeli et al., 1997).

Loss-of-function mutations of either netrin 1 (Ntn1) or deleted in colorectal cancer (Dcc) in mice confirm their involvement in the formation of commissural connections (Serafini et al., 1996; Fazeli et al., 1997). A second DCC-related protein, neogenin, also binds netrin 1 but its function remains unknown (Keino-Masu et al., 1996). In addition, netrin 1 also acts as a chemorepulsive cue for the axons of the trochlear, trigeminal, facial and glossopharyngeal motor nuclei (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997). Such chemorepulsive action is probably not mediated by DCC, but by a second family of netrin 1 receptors, the unc5-related proteins (Leung-Hagesteijn et al., 1992; Hamelin et al., 1993; Leonardo et al., 1997).

During the development of the nervous system, considerable distances separate the final destination of migratory neurons from their proliferative matrices. Recent evidence, in both vertebrates and invertebrates, suggests that some cues governing axonal growth may also be involved in neuronal migration (Culotti and Kolodkin, 1996; Goodman, 1996; Hu and Rutishauser, 1996; Tessier-Lavigne and Goodman 1996). For instance, the lack of pontine nuclei and the severe atrophy
of the inferior olivary complex in both netrin 1 and Dcc knockout mice suggest that netrin 1 provides cues not only for axonal guidance but also for neuronal migration (Serafini et al., 1996; Keino-Masu et al., 1996; Bloch-Gallego et al., 1999). Moreover, mutations in the unc5h3 gene are responsible for the murine “rostral cerebellar malformation”, leading to aberrant migration of granule and Purkinje cells in the rostral direction and loss of the rostral cerebellar boundary (Ackerman et al., 1997; Leonardo et al., 1997; Przyborski et al., 1998).

The rhombic lip, the germinative neuroepithelium located around the alar recess of the fourth ventricle (His, 1891), gives rise to neurons that follow distinct migratory pathways and substrates (Rakic, 1990). In the mouse, the upper rhombic lip (uRL; the rostral aspect of the rhombic lip or germinal trigone; Altman and Bayer, 1997) produces progenitor cells from E13 onwards that spread in a rostromedial direction to cover the whole cerebellar surface by E17 (neurophilic tangential migration). These progenitors form the external germinal layer (EGL), which is the origin of the cerebellar granule cells (Gao and Hatten, 1994; Mathis et al., 1997). During the first two postnatal weeks in mice, postmitotic neurons from the EGL migrate inwards along Bergmann glia (gliophilic radial migration) and differentiate into granule cells under the Purkinje cell layer (Miale and Sidman, 1961; Rakic, 1971).

The lower region of the rhombic lip (lRL) gives rise to precerebellar neurons in the inferior oliv (IO), lateral reticular nucleus (LRN), external cuneate nucleus (ECN), nucleus reticularis tegmenti pontis (NRTP) and basilar pons (BP). Although there is some spatial and temporal overlap, neurons for each nucleus have a distinct temporal pattern of birthdates and, remarkably, distinct migratory routes. Thus, neurons destined to IO are generated at E10-E11 and neurons fated to the LRN and ECN become postmitotic at E11-E12. In contrast, pontine neurons (NRTP and BP) are generated during a protracted period, extending from E12 to E16 with a late peak at E14-E15 (Taber-Pierce, 1966). Although all precerebellar neurons follow a neurophilic type of migration along circumferential routes at the periphery of the brainstem, IO neurons migrate along the submarginal stream (Bourrat and Sotelo, 1988; Altman and Bayer, 1997), while LRN and ECN neurons migrate through the marginal stream (Bourrat and Sotelo, 1991; Altman and Bayer, 1997). NRTP and BP neurons follow a distinct rostral migratory route towards their respective pontine nuclei, named the pontobulbar or pontomedullary stream (Harkmark, 1954; Rakic, 1985; Ono and Kawamura, 1990). Finally, while neurons destined to the LRN and ECN cross the midline through the most ventral region of the floor plate (Bourrat and Sotelo, 1991; Altman and Bayer, 1997), olivary and pontine neurons stop ipsilaterally on the side where they were generated (Taber-Pierce, 1966; Ellerberger et al., 1969; Bourrat and Sotelo, 1988, 1990).

The complexity of migratory routes and locations taken by rhombic lip-originated neurons suggests that multiple and specific guiding and stopping factors are involved in their migration and allocation. To understand the molecular bases of neuronal cell migration in the rhombic lip, here we study the effects of netrin 1 in vitro in explants taken from E12-E14 rhombic lip and from its postnatal derivative, the EGL. We show that secreted netrin 1 exerts an attraction on both axons and neurons arising from embryonic IRL explants, whereas it has no effect on uRL explants. In contrast, netrin 1 strongly repels parallel fibres and granule cells from postnatal EGL explants. We also show that the patterns of expression of netrin 1 and its receptors (Dcc, neogenin, unc5h2 and unc5h3) are consistent with a role for this chemotactic molecule in the migration of neurons originated from the rhombic lip in vivo.

MATERIALS AND METHODS

Animals

OF1 embryos and postnatal mice (Ifla Credo, France) were used. The mating day was considered embryonic day 0 (E0) and the day of birth postnatal day 0 (P0). After ether anaesthesia of the dams, embryos were dissected out and collected in 0.1 M phosphate-buffered saline (PBS) and 0.6% D-glucose, and used for cultures or fixed with 4% paraformaldehyde (PFA) in a 0.1 M phosphate buffer, pH 7.3. Embryos older than E14 and postnatal animals were transcardially perfused with the above fixative. After postfixation, the brains were cryoprotected in 30% glycerol, 30% ethylene glycol, 40% 0.1 M PBS, and stored at −30°C until use.

Explant cultures

The uRL and IRL from E12-E15 embryos were dissected out as a single piece and cut into 150-300 μm tissue pieces with fine tungsten needles (Fig. 1A). 250 μm parasagittal sections of postnatal cerebella were cut with a tissue chopper. After removing the meninges, 150-300 μm tissue pieces from the EGL were obtained (Fig. 1B).

Explants were cocultured at a distance (200-600 μm) with aggregates of EBNA-293 cells stably transfected with a construct encoding netrin 1-c-myc, or with the vector alone (Keino-Masu et al., 1996). Explants and cell aggregates were embedded in rat-tail collagen as described (Lumsden and Davies, 1986) and cultured in DMEM (Seromed) supplemented with L-glutamine, NaHCO3, D-glucose, and supplements B27 and N2 (all from Gibco Life Technologies), for 24-72 hours in a 5% CO2, 95% humidity incubator at 37°C. To monitor the expression of netrin 1 and the distance covered by the netrin 1 gradient, some cocultures were fixed and immunostained with the monoclonal 9E10 anti-c-myc antibody (Santa Cruz). Secreted netrin 1-c-myc was detected in a gradient of intensity over a distance of about 300-400 μm away from the EBNA-293 aggregates after 2 days in vitro (div), indicating that a long-range gradient of netrin 1 is formed in our experimental conditions. In some experiments, explants were incubated with medium conditioned for 36 hours with EBNA-293-netrin 1 or control cells diluted 1:1 with fresh medium.

28 experiments were done and up to 2000 explants analyzed.

Immunohistochemistry

Explants were fixed in 4% PFA for 1 hour and processed for the visualisation of neuronal bodies and neurites. After several rinses in PBS, cultures were incubated with monoclonal antibodies against neuron-specific class III β-tubulin (1:3000; clone Tuj-1 Babco; Moody et al., 1989), intracellular human DCC (1:500; clone GA7499, Pharmigen), C-myc (1:200, Santa Cruz) or rabbit polyclonal antibodies against intracellular human DCC (1:1000, Fabre et al., 1999), L1 (1:2000, Persohn and Schachner, 1987), C-myc (1:200, Santa Cruz) or rabbit polyclonal antibodies against intracellular human DCC (1:1000, Fabre et al., 1999), L1 (1:2000, Persohn and Schachner, 1987), TAG-1 (1:2000, Karageoge et al., 1991), calbindin D28k (1:10000, Swant) and GFAP (1:1000, Dako) (all from Gibco Life Technologies). The incubation with secondary antibodies (1:200; Vector Labs.) was followed by the streptavidin-peroxidase complex (1:400, Amersham). Enzymatic reaction was developed with diaminobenzidine and H2O2. Alternatively, FITC-conjugated secondary antibodies, or Texas Red-coupled streptavidin were used, together with fluorescent nuclear markers (bisbenzimide or ethidium bromide) to visualize both cell bodies and neurites.

Sections from different developmental stages, some of them
corresponding to the rat netrin 1 using T3 polymerase (Ambion), a cDNA fragment of 0.72 kb encoding the rat netrin 1 unstranslated region of mouse netrin 1 (Serafini et al., 1996) using T3 polymerase (Ambion), a cDNA fragment of the rat Dcc cytoplasmic region of 0.85 kb, and a fragment of 0.72 kb encoding the rat neogenin cytoplasmic region (Keino-Masu et al., 1996) using SP6 polymerase (Promega). cDNA fragments of the rat unc5h2 (1.5 kb) and rat unc5h3 (1.6 kb) (Leonardo et al., 1997) were transcribed using T7 polymerase (Ambion). Labelled antisense cRNA was added to the prehybridisation solution (250-500 ng/ml) and hybridisation was carried out at 60 ºC overnight. Sections were then washed in 2× SSC (30 minutes, room temperature), digested with 20 mg/ml RNase A (37°C, 1 hour), washed in 0.5× SSC/50% formamide (4 hours, 55°C) and in 0.1× SSC/0.1% sarkosyl (1 hour, 60°C). After rinsing in Tris-buffered saline (TBS)/0.1% Tween 20 (1 hour), sections were blocked in 10% normal goat serum (2 hours) and incubated overnight with alkaline phosphatase-conjugated antibody to digoxigenin (Boehringer-Mannheim, 1.2000). After washing, sections were developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Life Technologies), mounted on gelatinised slides and coverslipped with MowiolTM.

Controls
Control hybridisations, including hybridisation with sense digoxigenin-labelled riboprobes or RNAs A digestion prior to the hybridisation, prevented alkaline phosphatase staining above background levels. For immunocytochemical controls, omission of the primary antibodies prevented immunostaining.

**RESULTS**

Netrin 1 effects on neurons and neurites derived from the lower rhombic lip

The temporal sequence of proliferation of neuronal precursors in the IRL allows us to pinpoint the classes of precerebellar neurons affected by netrin 1 in their migration. In our experiments, we selected rhombic lip explants taken from E12-E14 embryos. At E12, all IO neurons have left the IRL and most of the cells in these explants corresponded to LRN and ECN neurons. In contrast, the neurons generated at E14 are

Table 1. Quantitative analysis of the neuritic outgrowth (A) and cellular migration (B) in explants from the lower rhombic lip at different ages

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Mean area ± s.d. (mm²)</th>
<th>Statistical significance</th>
<th>Number of explants</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal quadrant</td>
<td>Distal quadrant</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>(A) Neurite outgrowth (Tuj-1 immunostaining)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRL E12 net 1 2 div</td>
<td>0.302±0.103</td>
<td>0.110±0.072</td>
<td>**</td>
<td>23</td>
</tr>
<tr>
<td>IRL E12 cont 2 div</td>
<td>0.129±0.058</td>
<td>0.211±0.128</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>IRL E14 net 1 1 div</td>
<td>0.072±0.052</td>
<td>0.024±0.018</td>
<td>**</td>
<td>17</td>
</tr>
<tr>
<td>IRL E14 cont 1 div</td>
<td>0.031±0.001</td>
<td>0.025±0.004</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>IRL E14 net 2 div</td>
<td>0.296±0.178</td>
<td>0.097±0.039</td>
<td>**</td>
<td>23</td>
</tr>
<tr>
<td>IRL E14 cont 2 div</td>
<td>0.115±0.073</td>
<td>0.131±0.083</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>(B) Neuronal cell migration (bisbenzimid stained)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRL E14 net 2 div</td>
<td>0.380±0.177</td>
<td>0.056±0.025</td>
<td>**</td>
<td>6</td>
</tr>
<tr>
<td>IRL E14 cont 2 div</td>
<td>0.056±0.018</td>
<td>0.045±0.014</td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

div, days in vitro.
The area occupied by the bulk of the neuritic processes and/or by the cell bodies in the proximal and distal quadrants in explants cocultured with aggregates of EBNA-293 control cells or with cells transfected with the netrin 1-c-myc construct was measured and expressed as means ± s.d. Significant differences between proximal and distal quadrants were calculated from crude data using the LSD test. **P<0.01.

The preferential orientation of the outgrowth was determined for each explant dividing the area occupied by the neuritic outgrowth or cellular migration in the proximal quadrant into that of the distal quadrant, which gives a ratio of 1 for radial outgrowth. The limits between categories were calculated from the s.d. obtained for the ratios in explants cocultured with control cells. div, days in vitro.

=, radial: ratio = 1±s.d.; +, attraction: ratio >1±s.d.; -, repulsion: ratio <1–s.d.; %+, percentage of the explants attracted by netrin 1.

Netrin 1 guides neuronal migration 1361
exclusively destined to pontine nuclei (Taber-Pierce 1966, see also Discussion).

We first tested whether netrin 1 acts as an attractive or repulsive cue for LRN and ECN neurons, by coculturing E12 lRL explants with aggregates of netrin 1-secreting cells. Explants cultured with control EBNA-293 cell aggregates for 2 div showed no preferential orientation (radial) of outgrowing neurites immunostained with the Tuj-1 mAb (Table 1; Fig. 2). Most importantly, the outgrowing neurites were almost devoid of migrating cell bodies. In contrast, when E12 lRL explants were cultured with netrin 1-secreting cells, 91% of the E12 lRL explants (Table 1) showed a marked asymmetrical pattern of neurite outgrowth with far fewer processes in the distal than in the proximal quadrant (data not shown). This was so even when tissue explants were located at long distances (around 600 μm) from the cell aggregates. Moreover, the neurites in the proximal quadrant formed tight, prominent fascicles, which were associated with strands of migrating neurons.

To test the effect of netrin 1 on the migration of pontine precerebellar neurons, similar in vitro experiments were carried out using E14 lRL. In cocultures with control cell aggregates, IRL neurites grew symmetrically in 75% of the explants after 1 div and in 100% of the explants after 2 div (Table 1; Fig. 3A). After both culture times, radial emerging neurites were poorly fasciculated (Fig. 3A), and the few Tuj-1-positive neurons leaving the explants showed long neuritic processes, probably corresponding to the leading process of migrating pontine cells (Ono and Kawamura, 1990) and roughly bipolar morphology (Fig. 3C,E). These neurons did not migrate for long distances and formed a narrow ring of less than 100 μm thick around the explants (Table 1; Figs 3A, 4A). In contrast, 59% (1 div) and 100% (2 div) of the explants cultured with netrin 1-secreting aggregates (Table 1; Fig. 3B) showed prominent neurite outgrowth directed towards the cell aggregates. Most of these attracted processes had fasciculated and attained the netrin 1 producing cell masses (Fig. 3B,F). Isolated migrating neurons were practically absent in cocultures in which cell nuclei were visualized with DNA-staining dyes. On the contrary, the migrating neurons were tightly apposed, following each other to form thick neuronal

**Fig. 2.** Histograms illustrating the area of neuritic processes and cell bodies in the proximal and distal quadrants of E12 and E14 lower rhombic lip explants cocultured with EBNA-293 control cells or with cells secreting netrin 1 for 48 hours. Data are means ± s.e.m.; **P=0.01, LSD test.
Netrin 1 guides neuronal migration

Chains, which emerged from the explants, mainly in their proximal quadrants (Table 1B; Fig. 3B,D), but also occasionally in their lateral quadrants (Fig. 4B). In double-stained cocultures, most of the chains of migrating cells were intermingled with the fasciculated processes (Fig. 3B,D,F) as occurs in the pontine stream in vivo (Ono and Kawamura, 1990), and spanned the distance between the IRL explants and the EBNA-293 transfected cell masses (Fig. 3B). Thus, precerebellar neurons use a neurophilic type of migration (Rakic, 1990). Moreover, a few neurites also grew in the distal quadrants, but they were not associated with massive neuronal migration (Figs 3B, 4B), which supports the suggestion that, in the cocultures, neuritic outgrowth and neuronal migration are not a unique process. These results show that netrin 1 functions as a chemotactic cue for migrating precerebellar neurons, because it imposes directionality to their migration.

Table 2. Quantitative analysis of the outgrowth in explants from the uRL and EGL

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Mean area ± s.d. (mm²)</th>
<th>Statistical significance</th>
<th>Number of explants</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal quadrant</td>
<td>Distal quadrant</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>uRL E14 net 1 1 div</td>
<td>0.051±0.017</td>
<td>0.099±0.023</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>uRL E14 cont 1 div</td>
<td>0.073±0.036</td>
<td>0.062±0.027</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>uRL E14 net 1 2 div</td>
<td>0.083±0.052</td>
<td>0.115±0.043</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>uRL E14 cont 2 div</td>
<td>0.133±0.053</td>
<td>0.149±0.062</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>EGL P0 net 1 3 div</td>
<td>0.069±0.025</td>
<td>0.099±0.030</td>
<td>*</td>
<td>18</td>
</tr>
<tr>
<td>EGL P0 cont 3 div</td>
<td>0.112±0.040</td>
<td>0.122±0.045</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>EGL P2 net 1 2 div</td>
<td>0.065±0.023</td>
<td>0.103±0.024</td>
<td>**</td>
<td>22</td>
</tr>
<tr>
<td>EGL P2 cont 2 div</td>
<td>0.087±0.033</td>
<td>0.077±0.030</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>EGL P4 net 1 2 div</td>
<td>0.050±0.018</td>
<td>0.095±0.042</td>
<td>**</td>
<td>32</td>
</tr>
<tr>
<td>EGL P4 cont 2 div</td>
<td>0.173±0.047</td>
<td>0.172±0.042</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>EGL P5 net 1 2 div</td>
<td>0.062±0.026</td>
<td>0.131±0.051</td>
<td>**</td>
<td>31</td>
</tr>
<tr>
<td>EGL P5 cont 2 div</td>
<td>0.119±0.026</td>
<td>0.130±0.037</td>
<td></td>
<td>11</td>
</tr>
</tbody>
</table>

The area occupied for the bulk of the neuritic processes and cell bodies in the proximal and distal quadrants of explants cocultured with aggregates of EBNA-293 control cells or with cells transfected with the netrin 1-c-myc construct were measured and expressed as means ± s.d. Significant differences between proximal and distal quadrants were calculated from crude data using the LSD test.

*P=0.05; **P=0.01. The preferential orientation of the outgrowth was determined for each explant by dividing the area of the outgrowth in the proximal quadrant into that of the distal quadrant, which gives a ratio of 1 for radial outgrowth. The limits between categories were calculated from the s.d. obtained for the ratios in explants cocultured with control cells. div, days in vitro.

=, radial: ratio = 1±s.d.; +, attraction: ratio >1+s.d.; –, repulsion: ratio <1±s.d.; %, percentage of the cultures repelled by netrin 1.

Fig. 3. Netrin 1 is chemotactic for neurites and cells originating from E14 IRL explants. E14 IRL explants cocultured 48 hours with aggregates of EBNA-293 control cells (A,C,E) or cells transfected with the netrin 1-c-myc expression vector (B,D,F). Confocal images at a medium plane of the culture showing the neuronal processes immunostained with anti-β-tubulin III antibodies, in green, and the cellular nuclei stained with ethidium bromide, in red. (C,E) and (D,F) are respectively higher magnifications of the boxes shown in A and B, which illustrate the short distance and the random orientation of migrating neurons confronted with control cells (E) and the long chains of migrating cells oriented to the source of netrin 1 (F). c, aggregate of control cells; n, aggregate of netrin 1-secreting cells. Scale bars, 200 µm (A,B) and 40 µm (C-F).
To substantiate the notion that migrating neurons in E14 IRL explants are pontine neurons, cultures were immunostained with anti-TAG-1 antibodies (Wolfer et al., 1994). As shown in Fig. 4C,D, TAG-1 antibodies stained thick strands of migrating neurons with long leading edges, which were directed towards the netrin 1 aggregates. Finally, the strong migration of postmitotic pontine precerebellar neurons produced by netrin 1 was reproduced when single E14 IRL explants were cultured with medium conditioned for 36 hours with EBNA-293 cells secreting netrin 1 (Fig. 4E-H). Some of the IRL explants, cultured either with aggregates of netrin 1-secreting cells or in conditioned medium, were immunostained with anti-GFAP antibodies. GFAP-positive cells were missing, suggesting that the migration was not gliophilic.

These experiments demonstrate that netrin 1 is not only a chemoattractant for neurites emerging from LRN, ECN, NRTP and BP neurons generated in the IRL, but also that it chemoattracts and promotes the massive cell migration of these neurons.

**Netrin 1 effect on neurons and neurites from the upper rhombic lip and external germinal layer**

To test the role of netrin 1 in the two modes of migration of the cerebellar granule cell precursors and neurons, in vitro experiments were carried out with either E14 uRL explants or P0-P6 EGL explants. E14 uRL explants cocultured with control cells emitted neurites without preferential orientation after either 1 or 2 div (Table 2). When cultured with netrin 1-producing cells, the neuritic outgrowth from uRL explants was less homogeneous (Fig. 5A,B), although no significant changes were noticed in the area occupied by the emitted processes (Fig. 6; Table 2).
To dissect EGL explants from the parasagittal slices of postnatal cerebella, we used the easiest cleavage plane, which passes just below the multicellular (P0–P2) or monocellular (P4–P6) Purkinje cell layer. Thus, these EGL explants initially contain the subpial basal and glial laminae, the entire EGL, the nascent molecular layer and most of the Purkinje cells (Fig. 1B). To favour the radial outgrowth of granule cell processes and neurons, explants were placed either on their upper subpial or their deeper Purkinje cell surface. The outgrowth from such EGL explants cultured in a tridimensional collagen gel matrix was symmetrical and radial, which allowed us to monitor the effects of netrin 1.

To identify the classes of cerebellar neurons and processes that grew from EGL explants, cultures were immunostained with Tuj-1 mAb or with antibodies to different markers: TAG-1 or L1 to visualise granule cell bodies and processes, calbindin for Purkinje cells and GFAP for Bergmann glia (Wassef et al., 1985; Buttiglione et al., 1996; Soriano et al., 1997). When P0 EGL explants were cocultured for 1 to 3 days with netrin 1-expressing cells, 67% of the explants showed Tuj-1-positive emerging neurites that grew preferentially towards the distal quadrant (Table 2; Fig. 5C,D). Older EGL explants (P2, P4 or P5) showed a similar, although more dramatic, repulsive effect (Figs 6, 7A-D). For instance, P4 and P5 EGL explants showed a preferential outgrowth in the distal quadrant in about 90% of cases (Table 2). The repulsive effect of netrin 1 on neurite extension was manifested in preferential neuritic outgrowth in the distal quadrant rather than in veering away from the source of netrin 1, and was easily mimicked when EGL explants were cultured with conditioned medium from netrin 1-secreting cells, which reduced neurite outgrowth (Fig. 5E,F).

The use of distinct cellular markers showed that calbindin-positive Purkinje cells did not survive in these conditions and degenerated during the second day in culture (not shown). GFAP-immunostaining revealed positive cell bodies within the explants, most probably belonging to Bergmann glial cells, but no GFAP-positive processes grew out of the explants (data not shown). In contrast, TAG-1 (Fig. 7E) and L1 (not shown) antibodies, which in the developing cerebellum mark the most immature and mature parallel fibres, respectively (Buttiglione et al., 1996), labelled the majority of processes arising from EGL explants. This indicates that most, if not all, the neurites repelled by netrin 1 in our assay correspond to the parallel fibres. Finally, together with the parallel fibers, TAG-1- or L1-positive cell bodies were observed leaving the tissue explants (Fig. 7E). In the presence of control EBNA-293 cells, few labelled cell bodies were present near to the explants, in the radial-emerging bundles of parallel fibres. In contrast, in the

Fig. 6. Histograms illustrating the area of neuritic outgrowth in the proximal and distal quadrants of E14 upper rhombic lip and postnatal EGL explants cocultured for 48–72 hours with EBNA-293 control cells or with cells transfected with the netrin 1-c-myc construct. Data are presented as means ± s.e.m.; *P=0.05; **P=0.01, LSD test.

Fig. 7. Netrin 1 repels neuritic outgrowth from postnatal mice EGL explants. Explants from P2 (A,B), P4 (C,D) and P6 (E) cerebella were cocultured for 48 hours with EBNA-293 control cells (A,C) or with cells transfected with netrin 1-c-myc expression vector (B,D,E) and immunostained with antibodies against β-tubulin III (A-D) or TAG-1 (E). Note the strong chemopulsion exerted by netrin 1 in B,D. (E) Photomicrograph showing TAG-1-positive granule cells (arrows) migrating along fibres in the quadrant distal to the source of netrin 1. Perimeters of aggregates of control cells (c) and netrin 1-secreting cells (n), outlined with white circles. Scale bars, 250 μm (A–D); 25 μm (E).
presence of netrin 1-expressing cells, the immunopositive cell bodies were more abundant and exclusively found in the quadrant distal to the netrin 1 cell aggregate (Fig. 7E). TAG-1- or L1-immunoreactive cell bodies were very small (6-8 μm) and therefore correspond to migrating granule cells. Taken together, the above results show that netrin 1 has no effect on E14 uRL explants, indicating that this factor does not play a role in the initiation of the tangential migration of granule cell progenitors originating from the germinative trigone. In contrast, netrin 1 exerts a strong repulsive effect on the outgrowth of parallel fibres (the axons of granule cells) and on the intraneuritic translocation of granule cell nuclei in these axons. The netrin 1-generated repulsion is already evident at birth (P0) but is more dramatic at later postnatal stages.

**Developmental expression of netrin 1, Dcc, neogenin, unc5h2 and unc5h3 genes**

To examine whether in vivo netrin 1 mediates the attractive and repulsive effects observed in vitro, we studied by in situ hybridisation and immunohistochemistry the patterns of expression of netrin 1 and its receptors in the lower brainstem and cerebellum of embryonic and postnatal mice. At E12, netrin 1 was highly expressed in the epithelial cells at the origin of the floor plate. The expression spread mediolaterally (with a decreasing gradient) through the ventricular basal plate, up to the sulcus limitans. Dcc mRNA and protein were not expressed in the ventricular zone, but in the subventricular zone of both the basal and alar plates. DCC protein labelled fibres in the marginal and submarginal migratory streams. At this stage of development, the hybridisation signals for unc5h2 and unc5h3 were almost absent (not shown; Bloch-Gallego et al., 1999).

At E14, the expression of netrin 1 was similar to E12 (Fig. 8A). Dcc expression remained high in the subventricular zone and along the pontomedullary migratory stream, up to thepons. Less intense expression was detected in the nucleus of the solitary tract and the vestibular region (Fig. 8C,D). neogenin mRNA was highly expressed in cells of the ventricular zone in both the basal and alar plates and in two dorsalmost lines, underlying the lower rhombic lips (Fig. 8B). Thus, Dcc and neogenin genes show non-overlapping patterns of expression. Unc5h2 signals were high and occupied most of the ventricular alar plates, including the lRL, whereas unc5h3 signals were practically absent (Fig. 8E,F). The expression of unc5h2 was complementary to that of netrin 1 and partially overlapping with neogenin mRNA distribution (Fig. 8A,B,E). At pontine levels, netrin 1 was expressed in the midline (Fig. 8G) and Dcc signals were intense in the pontine migratory stream (Fig. 8H). Unc5h2 and unc5h3 were faintly expressed in the pons at E14, and increased with age (Fig. 8I).

At E14, the expression of netrin 1 and its receptors was weak in the developing cerebellar plate. netrin 1 was virtually absent, with the exception of a faint staining in deep nuclear regions.
Netrin 1 guides neuronal migration

**Fig. 9.** Expression of netrin 1 and its receptors in the cerebellar anlage at E14. (A) netrin 1 mRNA (NET-1) is almost absent in the cerebellar anlage. (B) Neogenin mRNA (NEOG) is strongly expressed in the ventricular zone with a mediolateral gradient of expression and also in the EGL and deep nuclei of the cerebellum. (C) Dcc mRNA and (D) protein (DCC-P), as neogenin, are localized in the ventricular zone and EGL. Note that several fibres tracts, probably cerebellar afferents and the trochlear nerve (arrow), are strongly immunoreactive for DCC. DN, deep nuclei; EGL, external granule layer; IRL, lower rhombic lip; VZ, ventricular zone; URL, upper rhombic lip; IV, IVth ventricle. Scale bars, 250 μm.

(Fig. 9A). Dcc mRNA and protein were found in the cerebellar primary neuroepithelium, the germinal trigone and the EGL. DCC protein present in fibre bundles might correspond to the vestibular fibres, which are the first extracerebellar projections to arrive (Fig. 9C,D). Neogenin colocalized its expression with Dcc, mainly at the middle region of the ventricular zone. In addition, neogenin was also expressed in deep nuclear neurons (Fig. 9B). As development proceeded, the cerebellar expression of netrin 1 and its receptors changed. Thus, at P5 netrin 1 expression was prominent in the EGL and in both the stellate and basket cells (the inhibitory interneurons of the molecular layer), but almost absent in the IGL (Fig. 10A). Neogenin mRNA was expressed in granule cells in both the EGL and IGL (Fig. 10B). Dcc mRNA was found throughout the EGL, whereas DCC protein was only found in the parallel fibres at the interface between the deep EGL and the nascent molecular layer. DCC-positive fibres were also observed in the granule cell layer (IGL) and in the white matter (Fig. 10C, D). Unc5h2 and Unc5h3 mRNAs were confined to granule cell precursors (EGL) and postmigratory granule cells (IGL) (Fig. 10E, F).

**DISCUSSION**

**Netrin 1 is a guiding cue for the migration of neurons fated to the pontine nuclei**

In the present study, we have addressed the role of netrin 1 in the migration of precerebellar neurons by culturing...
explants of the inferior lateral recess of the IVth ventricle at E12-E14 (IRL explants). Although we cannot rule out the possibility that some olivary neurons may be present in our explants, several arguments indicate that we have monitored the migration of neurons fated to the pontine precerebellar nuclei. First, although a few olivary neurons are late-generated at E12 in the IRL, this stage contributes mainly to the generation of precerebellar neurons fated to the external cuneateus (EC), the lateral reticular (LR) and the raphe nuclei (Taber-Pierce, 1966; Bourrat and Sotelo, 1988; Altman and Bayer, 1997). This is especially so at E14, when the IRL only generates neurons destined to the pontine nuclei (PN) (Taber-Pierce, 1966; Altman and Bayer, 1997). Second, during the formation of the precerebellar system, the surface glycoprotein TAG-1/Axonin 1 is strongly expressed along the extramural migratory streams (which contains neurons destined to the EC, LR and PN nuclei), but not along the intramural migratory stream, the pathway followed by migrating olivary neurons (Wolfer et al., 1994), which agrees with the present data showing that the neurons that respond to netrin 1 are strongly immuno reactive for TAG-1. We thus conclude that the IRL explants selected in our experiments correspond to the precerebellar neuroepithelium that gives rise to the LR and EC nuclei at E12, and to the basilar pons at E14 (Taber-Pierce, 1966).

Analysis of the precerebellar nuclei in mice deficient in expression of netrin 1 has revealed that basilar pontine neurons are missing (Serafini et al., 1996) and that less than 14% of inferior olivary neurons reach their ventromedial position (Bloch-Gallego et al., 1999), although it is not known how such a phenotype arises. The present data show that netrin 1 acts as a long-range chemotactic factor for pontine nuclei migrating neurons, which represents the first direct evidence for netrin 1 influencing the direction of migrating neurons. Our data support the notion that the absence of pontine nuclei in netrin 1– hypomorphic mice directly reflects abnormal neuronal migration from the IRL caused by the lack of netrin 1. Since Dcc knockout mice share similar defects in pontine structures (Fazeli et al., 1997), it is likely that this receptor is involved in such chemotaxis. In favour of this conclusion are the observations on inferior olivary neurons in netrin 1– deficient mice. The visualisation of these neurons by their Brn-3b expression (Bloch-Gallego et al., 1999) suggested that the atrophy of the inferior olive is mainly the result of impaired migration, because Brn-3b-expressing cells were distributed along the submarginal migratory stream. Thus, the present results, together with those in inferior olivary neurons (Bloch-Gallego et al., 1999), allow us to conclude that netrin 1 is an essential cue for the circumferential migration of precerebellar neurons.

However, other mechanisms involving netrin 1 may contribute to the mutant phenotype. For instance, DCC may induce apoptosis in the absence of ligand-binding, but blocks cell death when engaged to netrin 1 (Mehlen et al., 1998). As the early postmitotic cells in the IRL express DCC, the netrin 1/DCC interaction may regulate the survival of early pontine nuclei cells (Cho and Fearon, 1995). In addition to exerting a strong chemotactic effect, our explants incubated with netrin 1-conditioned media show that this factor dramatically promotes the exit of migrating neurons from the neuroepithelium, even in the absence of well-defined gradients. Thus, although our findings show that netrin 1 acts as a chemoattractive cue for the migration of pontine nuclei along the anterior extramural stream, as occurs for the navigation of ventrally growing axons (Kennedy et al., 1994; Shirasaki et al., 1995, 1996), we cannot rule out additional mechanisms of action of netrin 1 that may contribute to the generation of the phenotype in netrin 1 mutant mice.

The present observations also show that netrin 1 induces the formation of thick strands and fascicles of migrating neurons and neurites, in which these cells attach closely to each other, which is reminiscent of the neurophilic migration that takes place in the extramural migratory streams in vivo (Rakic, 1985; Ono and Kawamura, 1989, 1990) and in other migratory pathways such as the rostral migratory stream to the olfactory bulb (Jankovski and Sotelo, 1996; Lois et al., 1996; Wichterle et al., 1997). Previous studies have reported the presence of TAG-1 in developing axons that respond to netrin 1 (Placzek et al., 1990; Serafini et al., 1994; Wolfer et al., 1994; Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995; Tamada et al., 1995; Shirasaki et al., 1996) and the participation of this molecule in the transduction of floor-plate-derived signals has been hypothesised (Shirasaki et al., 1996). The upregulation of TAG-1 protein in migrating cells by netrin 1 reported here represents the first direct link between TAG-1 expression and a guidance protein secreted by the floor plate. TAG-1 forms homophilic trans-interactions and heterophilic interactions with other IgCAMs, such as F3 or L1, which results in changes in adhesivity and fasciculation (Lemmon et al., 1989; Felsenfeld et al., 1994; Buttiglione et al., 1998). TAG-1 may contribute to the formation of neuritic fascicles and, particularly, of the strands of migrating pontine neurons, similar to the participation of PSA-NCAM to the formation of neuronal chains in the rostral migratory stream of the olfactory bulb (Hu et al., 1996).

**Correlation of netrin 1 effects with the pattern of expression in vivo**

The pattern of expression of netrin 1 and their receptors in the early development of the pontine nuclei is summarised in Fig. 11. Some precerebellar neurons originating in the IRL migrate along the marginal stream to cross the midline and allocate in the LRN and ECN contralateral to their site of origin. In contrast, neurons migrating within the submarginal (inferior olivary neurons) and pontomedullary streams (basilar pontine neurons) do not cross the midline, and populate nuclei ipsilateral to their side of origin (Taber-Pierce, 1966; Bourrat and Sotelo, 1990). The present study shows that pontine nuclei cell progenitors express unc5h2 and neogenin receptor genes but not Dcc, whereas, postmitotic pontine neurons along the anterior extramural migratory pathway express the opposite combination of receptors, i.e., high Dcc and no neogenin, unc5h2 or unc5h3. Finally, as these neurons end their circumferential migration near the midline, they re-express low levels of unc5h2 and unc5h3 genes. Conversely, netrin 1 is expressed in the midline area near the target region (pontine nuclei), which suggests that a gradient of netrin 1 produced in this area may be responsible for the unidirectional migration of neurons towards the precerebellar nuclei. Also, the pattern of receptor expression in vivo is consistent with current views that DCC is a component of the receptor complex mediating chemoattractive responses by netrin 1 along the migratory
pathway. Finally, the re-expression of the \(unc5h2\) and \(unc5h3\) receptor genes when migrating pontine neurons approach the target region suggests that these receptors may prevent these neurons from crossing the midline, thereby allowing netrin 1 to act as a stop signal, as proposed for olivary neurons (Bloch-Gallego et al., 1999).

However, netrin 1 is also expressed in the floor plate region close to the precerebellar neuroepithelium, so additional mechanisms may prevent young postmitotic neurons from migrating towards this source of netrin 1. Possible mechanisms include the sequestration of netrin 1 by association with the extracellular matrix or by neogenin receptors (Kennedy et al., 1994; Serafini et al., 1994; Keino-Masu et al., 1996), which are highly expressed near the site of netrin 1 expression, or the presence of a non-permissive substrate for neuronal migration in this brainstem area.

Age-dependent chemorepulsion of granule cell progenitors and axons mediated by netrin 1

During embryonic development, granule cell progenitors are generated in the uRL and migrate tangentially over the cerebellar anlage to form the EGL, where they re-enter the mitotic cycle. The present data with E14-uRL explants show that embryonic granule cell progenitors do not respond to netrin 1, although they express the \(Dcc\) and \(neogenin\) receptor genes. This is consistent with observations in the cerebellum of newborn netrin 1 defective mice showing an almost intact EGL layer (Bloch-Gallego et al., 1999). Since netrin 1 is not expressed in the cerebellar anlage at early embryonic stages, \(Dcc\) expression in granule cell progenitors derived from the uRL may reflect a netrin 1-independent action for this receptor. It has been suggested that granule cell progenitors migrate along preexisting axonal tracts (Hynes et al., 1986). At embryonic stages, the dorsal surface of the cerebellar anlage is crossed by DCC-containing axons that overlap with and run in parallel with migrating granule cell progenitors (Fig. 10D). As occurs with other IgCAMs such as L1 or NCAM (reviewed in Walsh and Doherty, 1997), homophilic trans DCC interactions between axons and neurons may contribute to the tangential migration of embryonic granule cell precursors. In the \(unc5h3\) null mutant embryos, granule cell progenitors extend more rostrally than in wild-type mice, indicating that \(unc5h3\) receptors transduce a chemorepulsive signal for tangentially migrating granule cell progenitors (Przyborski et al., 1998). The results in the \(unc5h3\) mutant in vivo and our negative finding with netrin 1 in vitro, suggest that \(unc5h3\) receptors may bind additional ligands, as recently postulated in Caenorhabditis elegans (Colavita et al., 1998).

Recent publications (Wu et al., 1999; Hu, 1999) have shown that tangentially migrating neuronal progenitors in the rostral migratory stream and some neurons in the cerebral cortex might be guided by long-range repulsive factors, the Slit proteins. These results, together with those reported here for precerebellar neurons, strongly suggest that chemotactic mechanisms exert a guidance effect on tangentially migrating neurons. Although we have not observed chemotropic effects of netrin 1 on embryonic granule cell progenitors, it will be interesting to determine whether other diffusible factors, such as Slit proteins, are guiding these progenitors.

Starting at birth, postmitotic granule cells descend to the deep EGL and extend their axons, the parallel fibres (Ramón y Cajal, 1911; Miale and Sidman, 1961). Granule cell somata then become oriented perpendicular to the axons and migrate radially through the molecular layer, attached to the Bergmann glia, to form the IGL (Rakic, 1971, 1981, 1985; Hatten and Liem, 1981). The present data show that netrin 1 exerts a strong
chemorepulsive effect on axons growing from postnatal EGL explants, which were characterised as parallel fibres on the basis of their immunoreactivity for L1 and TAG-1 proteins (Persohn and Schachner, 1987; Yamamoto et al., 1990). Such a chemorepulsive effect is age-dependent, and thus more dramatic at P5 than at P0, which again correlates with the period of parallel fibre formation in vivo (Altman and Bayer, 1997). In the postnatal cerebellum, netrin 1 is expressed in the EGL and interneurons of the molecular layer. We suggest that netrin 1 contributes to the initiation of the correct parallel fibre extension, which could be a prerequisite for the ordered exit of postmitotic premigratory granule cells from the EGL (Engelkamp et al., 1999). The coexpression of Dcc, unc5h2 and unc5h3 receptor genes in granule cell precursors as well as in postmitotic granule cells is consistent with the view that co-participation of DCC and unc5 receptors is required for netrin 1 to exert a chemorepulsive response (Hong et al., 1999).

In addition to axons, our data show migrating neurons leaving the explants in the quadrants distal to the source of netrin 1, which are identified as granule cells because of L1 and TAG-1 immunostaining. We believe that this observation reflects an action of netrin 1 on migrating neurons for the following reasons. (1) Granule cell perikarya outside the explants are exclusively observed in the distal quadrants, but not in the proximal quadrants where granule cell axons also grow, which rules out the possibility that migrating cells move passively through the parallel fibres. (2) The rostral cerebellar malformation, which results from the disruption of the unc5h3 gene, leads to abnormal migration and positioning of granule cells, which implies a possible role for netrin 1 signalling through this receptor in cerebellar migration. (3) unc5h2 and unc5h3 receptors are highly expressed in premigratory granule cell bodies. However, Bergmann fibres did not leave our EGL explants and the granule cells migrate along fascicles of TAG-1-positive fibres belonging to parallel fibres. We thus interpret our results as showing that the non-glialphobic migration observed in vitro mimics the tangential migration of granule cells within the deeper EGL, just before their inwards radial migration (Ryder and Cepko, 1994), and that this tangential migration is under the control of netrin 1 and its receptors.

Netrin 1, a factor involved in axonal growth and neuronal migration

Netrin 1 secreted by the floor-plate acts as a chemoaattractant factor for ventrally directed axonal navigation (Kennedy et al., 1994; Keino-Masu et al., 1996; Shirasaki et al., 1995, 1996) and as a chemorepellent cue for dorsally oriented axons (Colamarino and Tessier-Lavigne, 1995). The present data show that netrin 1 is involved in the growth and targeting of intrinsic cerebellar circuits, which are independent of the midline, by providing chemorepulsive signals to developing parallel fibres. Thus, together with reports on corticofugal fibres (Métin et al., 1997; Richards et al., 1997) and ongoing studies on the hippocampus (J. Barallobre, personal communication), the present data indicate that netrin 1 is also involved in the guidance of some ipsilateral projections and in target-layer selection.

More importantly, the present results show that netrin 1 exerts a direct chemoaattractant influence on precerbellar migrating neurons along the circumferential extramural stream, and a chemorepulsive effect on granule cell migration. These data, together with our previous study in the olivary nucleus (Bloch-Gallego et al., 1999) and the analysis of unc5h3 mutants (Przyborski et al., 1998), show a novel role for netrin 1 in neuronal migration, which, as for axonal growth, may be either chemoaattractant or chemorepellent. Thus, together with other molecules with a role in neuronal migration and axonal growth, such as reelin or semaphorin III (D’Arcangelo et al., 1995; Del Rio et al., 1997; Eickholt et al., 1999), netrin 1 is likely to have a dual role in the construction of neural networks and in the guidance of migrating neurons towards their target nuclei and layers.

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Netrin 1 guides neuronal migration 1371

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