Development 129, 965-972 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV9810

Mice that lack astrotactin have slowed neuronal migration

Niels C. Adams[1], Toshifumi Tomoda[1], Margaret Cooper[1], Gunnar Dietz[2] and Mary E. Hatten[1,*]

[1]Laboratory of Developmental Neurobiology, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, USA
[2]Medizinische Fakultät, Neurologische Universitätsklinik, Hoppe-Seyler-Straße 3, D-72076 Tübingen, Germany
*Author for correspondence (e-mail: hatten@rockefeller.edu)

Accepted 19 November 2001

SUMMARY

The cortical regions of the brain are laminated as a result of directed migration of precursor cells along glia during development. Previously, we have used an assay system to identify astrotactin as a neuronal ligand for migration on glial fibers. To examine the function of astrotactin in vivo, we generated a null mutation by targeted gene disruption. The cerebella of astrotactin null mice are approximately 10% smaller than wild type. In vitro and in vivo cerebellar granule cell assays show a decrease in neuron-glial binding, a reduction in migration rates and abnormal development of Purkinje cells. Consequences of this are poorer balance and coordination. Thus, astrotactin functions in migration along glial processes in vivo, a process required for generating laminar structures and for the development of synaptic partner systems.

Key words: Mouse, Cerebellum, Granule Cell, Purkinje Cell, Bergmann Glia, Behavior

INTRODUCTION

The creation of the laminated structure of the cerebellar and cerebral cortices is one of the most remarkable processes in the development of the brain. This lamination is achieved by the directed migration of CNS neurons along the radial glial fiber system present during the development of the brain. As rapid cell division thickens the neuroepithelium, postmitotic cells migrate from the ventricular margin out through the thickening cortical wall to form the cortical plate. Evidence from electron microscopy (Rakic, 1971) and from combined use of retroviral marking of neurons and immunocytochemical labeling of glial fibers indicates that the bulk of radial migration occurs along the glial fiber system (Misson et al., 1991). While recent studies have shown that migratory pathways in the developing brain include transverse movements of cells along axon tracts (O'Rourke et al., 1992) and massive migrations of GABAergic interneurons into the cerebral cortex (Anderson et al., 1997a; Anderson et al., 1997b; Lavdas et al., 1999), the earlier migration along the glial scaffold positions pyramidal neurons of the cortex and derives the laminar pattern of the cortical regions (Tan et al., 1998).

In the cerebellum, the two principle neurons – granule and Purkinje cells – develop in concert, with axon outgrowth of granule cells occurring concomitantly with dendritic arborization of Purkinje cells. In the early postnatal period, granule cells migrate along Bergmann glial fibers, extending parallel fibers as they migrate towards and through the Purkinje cell layer. As the parallel fibers extend, they make synaptic contact with the forming Purkinje cell dendritic arbors.

Purkinje cells also influence the number of cerebellar granule cells produced by releasing sonic hedgehog, a potent mitogen for granule cells (Wechsler-Reya and Scott, 1999). While genetic studies on mice with spontaneous neurological mutations, including purkinje cell degeneration and lurcher show the importance of interactions between granule cells and Purkinje cells to cell survival (Hatten et al., 1997; Volpe, 1995), evidence is lacking regarding the importance of the timing of migration to the differentiation of the two cell classes.

In vitro studies on purified granule neurons and Bergmann glia have established the mode of neuronal migration along glial substrates (Edmondson and Hatten, 1987; Hatten and Mason, 1990) and described a specialized junction between the neuron and glial fiber (Gregory et al., 1988), as well as a unique cytoskeletal organization of migrating neurons (Rivas and Hatten, 1995). These studies have also revealed that the morphology of cultured granule cells closely matches their morphology in vivo.

The guidance of neurons along glial fibers has been studied in both cell biological and genetic experiments. Astrotactin, neuregulin and β1-integrin (Anton et al., 1996; Edmondson et al., 1988; Fishell and Hatten, 1991; Fishman and Hatten, 1993; Pinkas-Kramarski et al., 1994) have been identified as neuronal ligands for glia-guided migrations in the cerebellar cortex; and α3-integrin functions in neuronal migration in the developing cerebral cortex (Anton et al., 1999).

Astrotactin was discovered as an activity that could be blocked by adding antibodies raised against granule cells to granule cell migration cultures (Fishell and Hatten, 1991). Molecular cloning of astrotactin indicates two functional regions, one including three EGF repeats and a fibronectin III (FNIII) domain, and the other with a single EGF repeat and an FNIII domain. Astrotactin transcripts are abundant in cortical regions of brain, in neuronal populations that have commenced migration along the glial fiber system (C. Zheng, N. C. A., N. Heinz and M. E. H., unpublished) (Zheng et al., 1996). Subsequent protein database searches have identified another functional motif, a MAC-Perforin domain, that includes the first FNIII domain and extends towards the C-

terminal of astrotactin (Ponting, 1999). Chromosomal mapping of astrotactin localizes the gene to human chromosome 1q25.2 (Fink et al., 1997), a region associated with micrencephaly, a diverse class of disorders that result in a smaller brain size (Hatten, 1999).

We have produced a targeted disruption of the gene for astrotactin and show that loss of this gene results in a decrease in the ability of granule cells to bind to glia, resulting in a drop in the rate of cell migration of granule cells in vitro and in vivo. This results in increased apoptosis of cerebellar granule cells, and altered development of Purkinje cells. Consequences of this are poorer balance and coordination. These studies show that cerebellar cortical development depends crucially on the correct and timely migration of granule cells.

MATERIALS AND METHODS

Gene targeting

cDNA fragments derived from the 5′ region of the astrotactin gene were used as probes to isolate genomic clones from a mouse 129Svj genomic lambda library. The genomic organization around the putative translation initiation site was determined. To disrupt the astrotactin locus in murine embryonic stem (ES) cells by homologous recombination, we constructed a targeting vector replacing a 168 bp exon, which includes the translation initiation site, with the neomycin phosphotransferase gene (Fig. 1A). This targeting construct was electroporated into R1 ES cells (Nagy et al., 1993; Wood et al., 1993) and targeted clones were identified by Southern blot hybridization. Chimeric mice were generated by injection of three ES cell clones into blastocysts of C57BL/6J donor mice. One of the positively targeted ES cell clones rendered four highly chimeric males. They were crossed with C57BL/6J mice and two of them transmitted the mutation through the germline. F_1 heterozygous offspring all lines were bred with C57BL/6J animals for nine to ten generations to produce homozygous astrotactin mutants.

Animal breeding and genotyping

Homozygous astrotactin null mice are fertile and were bred to generate litters for analysis. In some cases, we bred heterozygous animals to obtain wild-type littermates and astrotactin null animals. The day of birth was designated P0. Tail snips were collected for DNA extraction and Southern blots were performed using standard methods. For Southern analysis, genomic DNA isolated from tail snips was digested with *Pst*1 and probed with a 1450 bp genomic DNA probe isolated from a region of the 3′ of the targeting construct. In wild-type DNA, only a 9.5 kb band was detected, whereas DNA isolated from astrotactin mutant mice showed an additional 7.8 kb band.

By Northern analysis, a 7 kb astrotactin transcript was detected in the mutant (data not shown), as a result of a splicing-over event that joined two exons flanking the ATG-containing exon (168 bp). By RT-PCR and sequencing, we confirmed that the transcript in the mutant specifically lacked the 168 bp exon that contains the translation initiation methionine (Fig. 1B). To confirm that this mutant transcript was not used for translation of the protein, we made western blots with Triton X-100 extracts of wild-type and mutant brains (data not shown).

Migration assay

To measure the rate of migration in vitro, we purified granule neurons and astroglia from P6 wild-type and astrotactin null animals, generated cultures of migrating neurons and assayed migration by video microscopy as described previously (Fishell and Hatten, 1991). Cells were plated at 150,000 cells per 300 μl well (Microtest Plates, NUNC). For video recordings, the cells were maintained in L15 medium supplemented with glucose (8 mM). In brief, a field containing migrating cells was imaged every 5 minutes using Metamorph software (Image One), and the movements of cells along glial fibers were calculated using ImageTool. In all, over 200 cells were tracked. Occasionally, migration assays were fixed and stained with Tuj-1 (Babco) immunohistochemistry to reveal the morphology of granule cells.

BrdU labeling

To follow migrating cells in vivo, P6 littermates were injected with the thymidine analog 5′-bromo-2′-deoxyuridine (BrdU). Animals were killed 3, 6, 12, 24, 48, 72 or 96 hours later by an overdose of Pentobarbital (Nembutal, Abbott). The brains were fixed with Bouin's fixative (Sigma), the fixed tissue was embedded in paraffin and sectioned at 10 μm. BrdU incorporation was detected by peroxidase immunocytochemistry (Becton Dickinson) and counterstained with Hematoxylin.

Binding assay

Cerebellar granule cells were isolated from P6 wild-type and astrotactin null mice as before, and were plated on a carpet of glial cells (Hatten, 1985). Each well of a 24-well tray had 30,000 granule cells added. Granule cells were allowed to settle for 30, 60, 90 and 120 minutes before the dish was shaken at 250 rpm for 2 minutes. The supernatant was drawn off and the remaining cells were fixed with paraformaldehyde, and the wells were processed for Tuj-1 and GFAP double immunohistochemistry to reveal the presence of the remaining neurons and glia. Fluorescence photomicrographs were taken with a SPOT 2 camera (Diagnostic Instruments). The numbers of neurons remaining were counted using ImageTool and the presence of the glial carpet was confirmed. Student's *t*-test was used to compare the numbers of cells per area measured. The numbers of cells drawn off before fixation were counted to confirm that equal numbers of cells were added to each well.

Histology

The gross appearance and histology of cerebellar tissue were compared in wild-type and null littermates on postnatal days 6 (P6), 15 (P15) and adulthood. Paraffin wax embedded sections (10 μm) were stained for Nissl substance with Cresyl Violet. Sections were also de-waxed and stained for an Anti-phospho-histone H3 Mitosis Marker as recommended by the manufacturer (Upstate Biotechnology). M-phase cells were visualized with a Cy3-conjugated secondary antibody (Jackson ImmunoResearch, Pennsylvania), and sections were counterstained with DAPI (1:10 000 in PBS, Sigma). The number of stained cells was compared with the number of DAPI stained cells per unit area of the EGL. Purkinje cells were visualized by staining with antibodies against calbindin (Sigma), using a Cy3 secondary antibody (Jackson ImmunoResearch). Astroglial cells were visualized with an anti-GFAP antibody (DAKO). Images were obtained with BioRad MRC 600 and Radiance 2000 confocal laser-scanning microscopes. sections (*z*-series) were compiled and processed using either Confocal Assistant (Todd Brelje) or VoxelView (Universal Imaging).

IGL and EGL area calculations

The relative sizes of the EGL and internal granule layer (IGL) in developing cerebellar cortex of wild-type and astrotactin null mutant mice (six for each age and genotype) were determined by photographing Nissl stained sections using a SPOT-2 camera (Diagnostic Instruments) mounted on a Zeiss Axiophot microscope. Regions to be measured were revealed by selectively thresholding the images and subsequently measuring the area of these domains using ImageTool (UTHESCA). Standard parametric measures were used so confirm differences between values for wild-type and mutant animals.

Apoptosis assay

The relative number of cells undergoing programmed cell death was compared between wild-type and astrotactin null animals at P6. Cells undergoing apoptosis were identified by TUNEL labeling as described

by the manufacturer of the kit (Roche). Labeled cells were visualized using peroxidase histochemistry. The tissue was photographed (20× lens) as before and ImageTool (UTHESCA) was used to count labeled cells and to calculate the area of the EGL and IGL. Data were analyzed as before.

Behavior experiments

Ten wild-type and ten age- and size-matched astrotactin null mice were trained and tested on five consecutive days at the same time. Two steady-rate tests (2.5 rpm) on a Rota-rod treadmill (Ugo Basil) were separated by a 20 minute break. Then after 10 minutes, an accelerating test (2.5 to 20 rpm) was carried out. Data were analyzed with standard parametric measures using Excel.

RESULTS

General description

Astrotactin null animals were generated by conventional gene targeting methods (Fig. 1A,B). By western blot analysis using a polyclonal antiserum raised against the expressed peptide [containing the forth EGF repeat and the second FNIII domains (Zheng et al., 1996)], a prominent protein band (~115 kDa) was detected in extracts of wild-type tissue, but not in extracts from mutant brains (not shown). To confirm that we had generated a null mutation of the *Astrotactin* gene, we raised an additional antibody against a peptide derived from the C terminus of the astrotactin protein. The C-terminal peptide antibody recognized one prominent band (approximately 115 kDa) extracted from wild-type, but not mutant, brains (Fig. 1C). This result confirms that astrotactin is not translated in mutant animals. Astrotactin null mice survive to adulthood and breed normally; we were unable to detect any phenotype in astrotactin$^{+/-}$ mice.

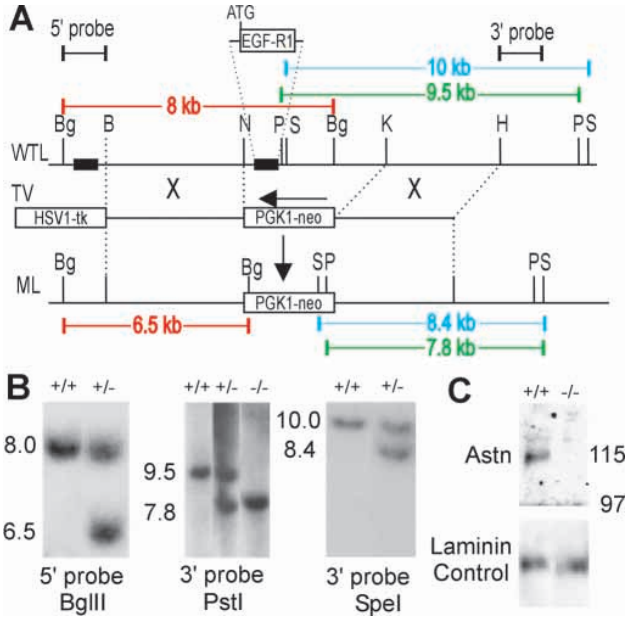

Fig. 1. Targeting strategy of astrotactin locus. (A) Wild-type astrotactin locus (WTL): black rectangles represent exons. The black lines on either side represent the 5′ and 3′ external probes used to detect homologous recombination by Southern blot. Astrotactin targeting vector (TV): a 168 bp exon containing the translation initiation site (ATG) was designed to be replaced with a pgk1-*neo* cassette. pgk1-*neo* and HSV1-*tk* were used as positive and negative selection markers, respectively. (B) Targeted locus after homologous recombination (ML): *Bgl*II digests liberate 8.0 kb and 6.5 kb fragments from the wild-type and targeted alleles, respectively. *Pst*I digests liberate 9.5 kb and 7.8 kb fragments from the wild-type and targeted alleles. *Spe*I digests give 10.0 kb for wild-type allele and 8.4 kb for the mutant allele. (C) Western blot analysis indicates that this mutant produces no astrotactin protein in astrotactin null animals.

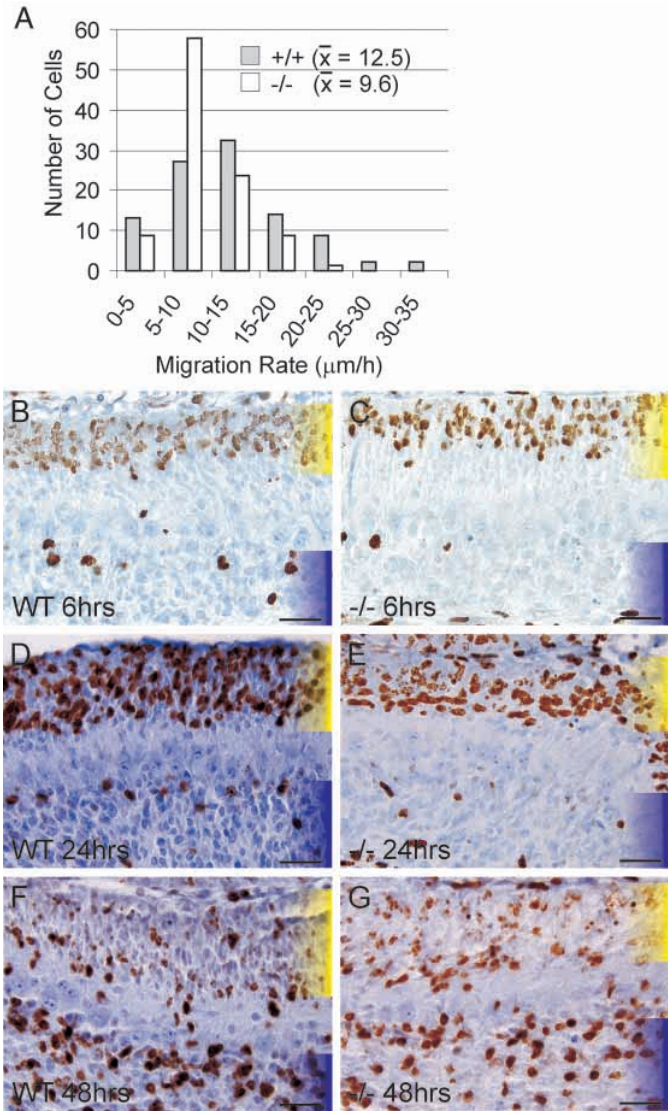

Fig. 2. In vitro and in vivo migration assays show that granule cells migrate more slowly in mice lacking astrotactin. (A) In vitro migration assay. The majority of wild-type granule cells migrate faster than 10 μm/h (gray) whereas the majority of granule cells from astrotactin null mice migrate more slowly than 10 μm/h. (B-G) Midline sagittal sections of cerebella from mice after varying survival times after intraperitoneal injection of BrdU. Brown peroxidase product shows cells that have taken up BrdU, sections were counterstained with Hematoxylin. In all figures, the EGL is at the top and is marked yellow, the IGL is marked by a blue bar. Six hours after BrdU injection there are similar numbers of heavily labeled cells in wild type (B) and astrotactin null (C) EGL. After 24 hours survival there are more labeled cells in the molecular layer and IGL of wild-type (D) cerebella than in astrotactin null mice (E). Forty-eight hours after injection, there are many labeled cells in the IGL and heavily labeled cells in the EGL of wild-type mice (F), whereas there are still a substantial number in the EGL and fewer in the IGL of the mutant mice (G). Scale bars: 30 μm.

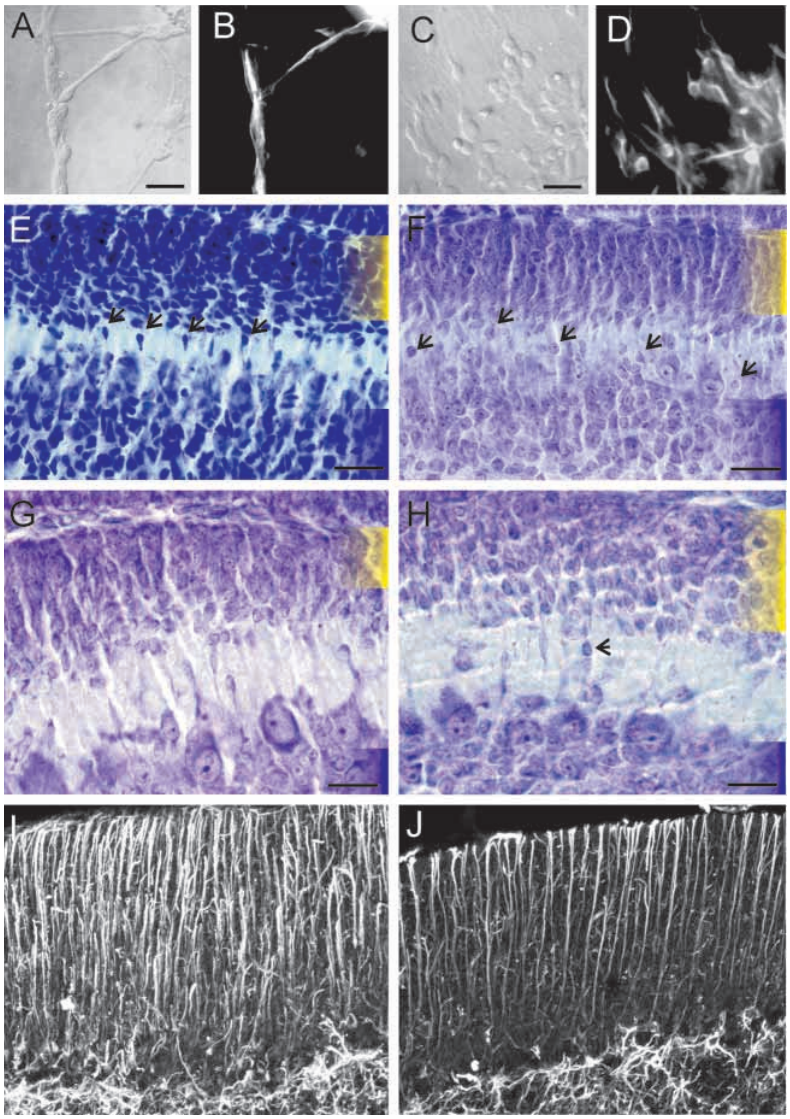

Fig. 3. Morphology of granule cells in vitro (A-D), in vivo (E-H) and glia (I,J). (A) Brightfield micrograph of wild type granule cells in a migration culture. Note the elongated cell profiles as granule cells migrate along glial fibers. The same culture following Tuj-1 immunohistochemistry is seen in B. (C) An equivalent migration culture with astrotactin null granule cells. Cell profiles are more rounded and are not as closely associated with the glial process. Tuj-1 stain of the same culture reveals that the rounded profiles are differentiated neurons. (E-J) Sagittal sections through cerebellum with EGL at top of figure. (E) Wild-type P6 Nissl stained section showing normal morphology of migrating granule cells in the molecular layer. Note that cells are 'tear drop' in shape (arrows). (F) Section showing the more rounded granule cells (arrows) in the molecular layer of P6 astrotactin null mice. (G) Nissl section of a P8 wild-type mouse with elongated migrating profiles in the molecular layer above the much larger Purkinje cells. (H) Section from an astrotactin null P8 mouse showing rounded granule cell profiles in the EGL (arrow). (I,J) Bergmann glia labeled with GFAP in normal and mutant mice. Scale bars: 30 μm.

In vitro migration assay

Astrotactin was discovered as an activity that could be blocked when antibodies to granule cells were added to granule cell migration cultures. To ensure that our observations of astrotactin null mice were consistent with the initial observations, we tested the ability of granule cells from these mice to migrate along glial processes in vitro. To measure the rate of migration of granule cells, we purified EGL and astroglia cells from P6 wild-type and mutant mice recombined them in vitro, and assayed for cell migration (Fishell and Hatten, 1991). Wild-type granule cells migrated between 0 and 35 μm/h, whereas cells isolated from astrotactin null mice all migrated at less than 25 μm/h, with 66% migrating at less than 10 μm/h, compared with 33% of granule cells from wild-type mice. Over a 2 hour period, the average migration rate for wild-type granule cells was 12.5 μm/h. Granule cells isolated from the astrotactin null mice migrated 30% more slowly (9.6 μm/h) (see Fig. 2A).

In vivo migration assay

To ensure that granule cells from astrotactin null mice also migrate more slowly in vivo as well as in vitro, we injected BrdU into P6 mice. The dividing granule cells in the EGL take up BrdU before they migrate towards the IGL. Both short-term and long-term in vivo migration assays were undertaken (3, 6, 12, 24, 48, 72 and 96 hours post injection). In all cases, tissue was processed to visualize heavily labeled cells only. Granule cells were the only cell type to take up BrdU in the EGL.

Short-term survival assays (3-6 hours after BrdU injection at P6) result in similar numbers of heavily labeled granule cells in the EGL of both normal and astrotactin null mice (Fig. 2B,C). This observation confirms our finding that there are similar rates of cell division in the EGL of wild-type and mutant mice when visualized with an anti-phospho-histone H3 Mitosis Marker (data not shown). Other (non granule cell, presumably astrocyte) profiles are also present in the inner layers of the cerebellum.

After 24 hours, there are heavily labeled granule cell profiles in the developing IGL of wild-type (Fig. 2D) and mutant mice (Fig. 2E). However in sections from astrotactin null mice there were frequently fewer heavily labeled profiles in the IGL; this corresponded with more heavily labeled profiles in the EGL (Fig. 2E) when compared with wild-type material. Similar differences in the numbers of heavily labeled profiles in the IGL of wild-type and mutant mice can be seen after 48 hours of survival (Fig. 2F,G). After 48 hours post injection, there are still more heavily labeled profiles in the EGL of astrotactin null mice than in wild type (Fig. 2F,G).

Morphology of granule cells

Fig. 3A,B shows the morphology of cerebellar granule cells after 24 hours in a low-density culture. They display the characteristic elongated profiles seen when granule cells are migrating. Fig. 3C,D shows a similar culture of granule cells from astrotactin null mice. The granule cell profiles from the mutant mice are more rounded and are not as closely associated with the glial cells.

Fig. 3E shows a parasagittal section of a wild-type P6 cerebellum. In the molecular layer elongated profiles of migrating granule cells are clearly visible. However, when a corresponding section from an astrotactin null cerebellum is examined, rounded profiles similar to those seen in in vitro preparations are present

(indicated by arrows in Fig. 3F). Sections from older wild-type and mutant animals (P8) show similar profiles (Fig. 3G,H).

Glial cell morphology

Although the slowed rate of migration was consistent with changes in cell morphology seen in mutant granule cells, it was also possible that a loss of astrotactin had affected the Bergmann glial fiber system. To visualize the radial glial fibers, we stained sections with antibodies against the glial fibrillary acidic protein. No differences were seen in the number, individual morphology or overall disposition of glial fibers in wild-type and mutant animals (Fig. 3I,J). Thus, the slowed rate of migration appeared to be intrinsic to the granule neuron rather than the result of abnormalities in glial fibers.

Binding assay

Our in vitro migration assays of astrotactin null mice contained cells that were more rounded than the cells seen in wild-type assays. To test whether this was due to an alteration in granule cell to glial cell binding, we carried out a glial binding assay to examine an aspect of the granule cell-glial cell interaction, the binding rates of granule cells to glial carpets (Stitt and Hatten, 1990). We found that there was a marked decrease in the ability of granule cells extracted from P6 astrotactin null mice to bind to wild-type glia compared with wild-type granule cells (see Fig. 4A). After shaking the dish after allowing cells to settle for 30 minutes, there was little difference in the numbers of wild-type and mutant granule cells adhering to the dishes. However allowing cells to settle for 60, 90 and 120 minutes and then agitating them revealed significant decrease in the abilities of mutant cells to adhere to glial cell carpets.

Morphometric analysis

When we compared mid sagittal sections from wild-type and astrotactin null mice stained for Nissl substance, it was clear that there was a difference in the thickness of the IGL. This was consistent with slowed migration of granule cells into the IGL of astrotactin null mice. However, to quantify this observation, we made a Morphometric analysis of the area filled by granule cells at P6, P15 and adult in mid sagittal Cresyl Violet stained sections. Fig. 4B shows that at P6 there is a significant (P<0.01) difference in the area of IGL when wild-type and mutant cerebella are compared. This difference is not seen in the EGL at the same stage. By P15, this difference in IGL area is more marked, however it should be noted that the EGL is still present in mutant mice. Sections from the midline of adult wild-type and mutant mice show that there are approximately 12% fewer granule cells in sections from astrotactin null mice when compared with corresponding sections from wild-type mice. This results in an overall decrease in the size of the cerebellum from astrotactin null mice by approximately 10% compared with wild type.

Cell division and TUNEL assays

We used a TUNEL labeling to assay whether the pyknotic profiles seen amongst the migrating granule cells were cells undergoing programmed cell death. At P6 there was a dramatic increase (approx. 50%, P<0.05) in the rate of cell death in the EGL of astrotactin null mice (Fig. 4C). This suggests that the slowed migration seen in granule cell precursors impairs their survival. We did not detect any significant difference in cell death rates in the Purkinje cell populations. As the loss of cells from apoptosis suggested a larger difference in the size of the cortex than we observed, we examined whether a loss of astrotactin null was stimulating cell proliferation. To measure the number of granule cell precursors undergoing cell division, we labeled the tissue with an M phase marker, an anti-phospho-histone H3 antibody (Chadee et al., 1995). In sections of P6 cerebellar tissue, there was no significant difference in the number of labeled, mitotic figures per unit area of EGL (data not shown).

Histology

Further examination of the sections stained for Nissl substance used for the morphometric data revealed more changes to the morphology in astrotactin null mice. As can be seen in Fig. 5A, there is no EGL present at P15 in the wild-type cerebellum, this contrasts with what can be seen in the astrotactin null cerebellum, where a significant number of granule like cells are still present along the margins of the cerebellum (Fig. 5B), which is consistent with slower rates of migration. Fig. 5B also reveals the presence of pyknotic profiles in the molecular layer of astrotactin null mice, consistent with increased rates of cell death seen by TUNEL staining. Another indication that there are problems with granule cell migration is the occasional presence of ectopic accumulations of granule cells in the molecular layer (see Fig. 5C).

In addition to abnormal granule cell profiles, Purkinje cells, the synaptic partner of granule cells, are also affected by the loss of astrotactin function. In wild-type cerebella stained for Nissl substance, Purkinje cells appear as a well ordered, single layer of cells that are oriented in the sagittal plane. In mice that lack astrotactin, Purkinje cells are frequently found displaced out of plane (Fig. 5D,H) alongside cells that appear to have a more normal morphology. This result is clearer in material stained with Calbindin antibodies. Wild-type adult Purkinje cells are well ordered in the sagittal plane and have extensive arbors that extend

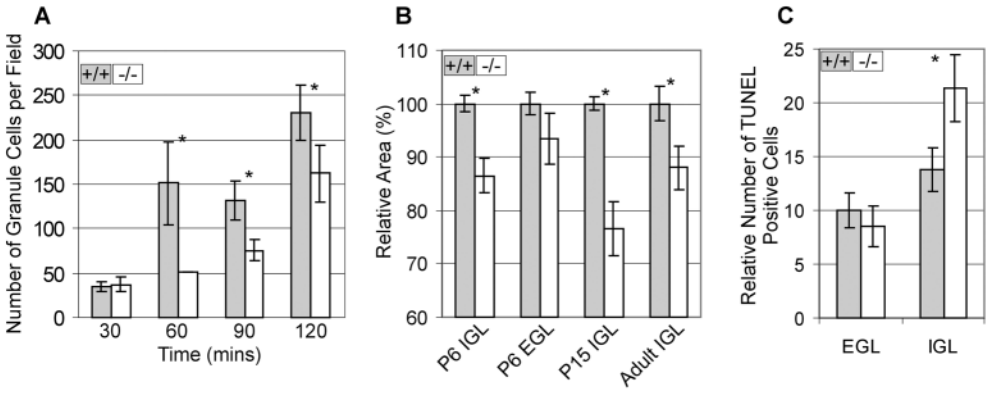

Fig. 4. (A) Assay to assess binding of granule cells to glial substrates. Asterisks indicate significant differences (P<0.01) in the ability of wild-type and mutant cells to adhere to glia. (B) Comparisons of the relative area occupied by granule cells in the cerebella of P6, P15 and adult normal and mutant mice (*P<0.01). (C) Comparison of the relative number (corrected for areas observed) of TUNEL-positive cells in the EGL and IGL of normal and mutant P6 mice (*P<0.05).

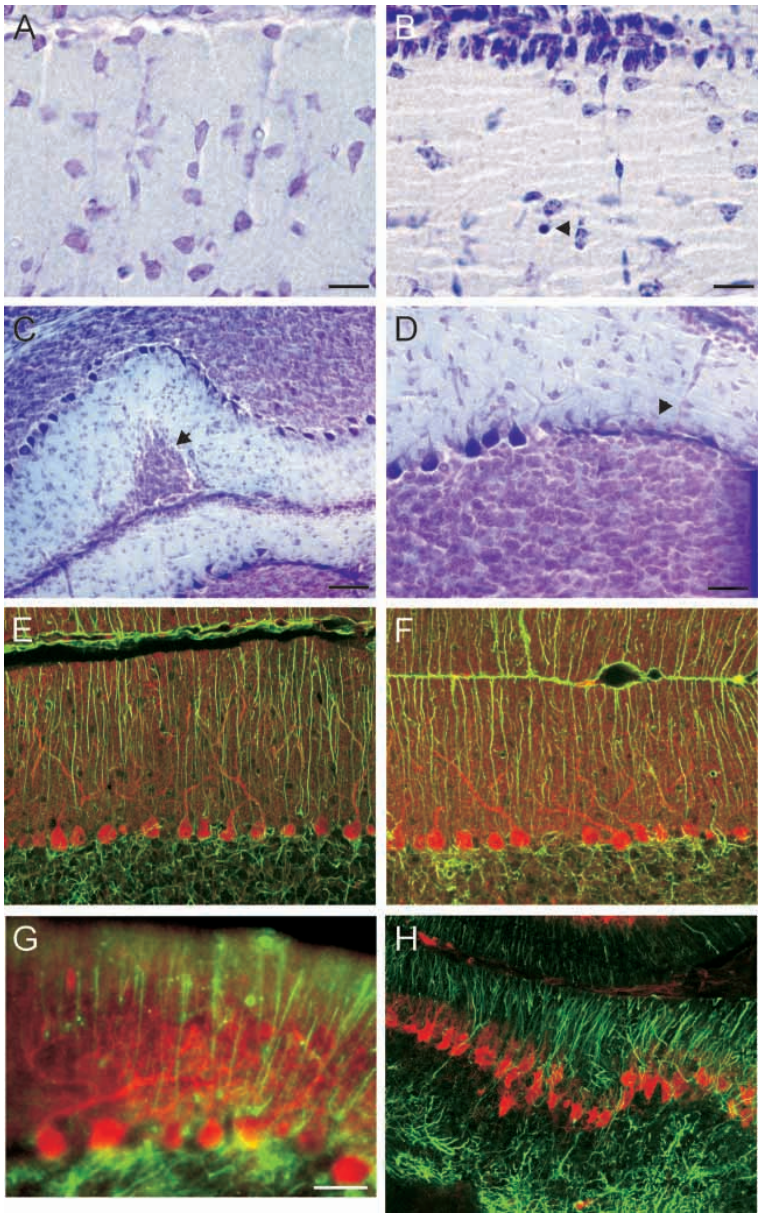

Fig. 5. Morphological changes to the cerebellum of astrotactin null mice. Nissl stained sections of normal (A) and mutant P15 mice (B) reveal that the dispersion of the EGL is delayed in astrotactin null mice. Note also the presence of pyknotic nuclei in B (arrowhead). (C) Nissl stained parasagittal section from a P15 astrotactin null mouse showing an ectopic clump of granule cells in the molecular layer (arrow). (D) Nissl stained parasagittal section from a P15 astrotactin null mouse showing Purkinje cells with abnormal morphology (arrowhead) adjacent to Purkinje cells that appear normal. Note how the abnormal cells are oriented out of the plane of the section. (E-H) Confocal images of double immunohistochemistry showing glia (GFAP staining in green) and Purkinje cells (calbindin staining in red) on parasagittal sections. (E) Wild-type adult, showing normal Purkinje cell morphology. In F,G, the dendrites of Purkinje cells from astrotactin null mice are seen to spread across into territories normally occupied by neighboring cells. Note that the columnar organization (revealed by the GFAP staining) of the cerebellum is otherwise normal. (H) Ectopic Purkinje cells in a section cut from a P19 astrotactin null mouse. Scale bars: 30 μm (A,B,D,E-H); 60 μm (C).

directly above the cell body (see Fig. 5E). In astrotactin null mice, there are frequently Purkinje cells that are displaced from the sagittal plane and their dendritic fields invade areas normally filled by adjacent Purkinje cells (Fig. 5F,G). To ensure that breakdown of order is not due to a gross disruption of the cerebellar organization, we double stained with Calbindin and GFAP antibodies. This confirmed that the columnar organization of the cerebellum is maintained, despite the presence of abnormal Purkinje cells (see Fig. 5H). The dismorphic arborization patterns seen in astrotactin null mice only becomes apparent after the third postnatal week. Calbindin staining of astrotactin null cerebella before this age (P6 to P19) reveal developing arborization patterns similar to those seen in normal mice (data not shown).

Behavioral assay

As morphological defects in the Purkinje cells are associated with locomotion and learning deficits, we tested the astrotactin null mice on a Rota-rod treadmill (Crawley, 1999), an assay for coordinated movement. On the standard fixed speed tests, the astrotactin null mice were significantly less able to stay on the rod (Fig. 6) throughout the duration of the assay. On the accelerated test, astrotactin null mice were significantly less able to stay on the bar during the first 4 days and on day 5 there was no significant difference in their ability to remain in the rod.

DISCUSSION

In this study we demonstrate that astrotactin is required for appropriate and timely migration of cerebellar granule cells. Absence of astrotactin results in abnormal granule and Purkinje cell morphology, resulting in mice that are less able to complete tasks requiring coordinated movement and balance. Granule cells are less able to attach to their migratory substrate, Bergmann glia, and are more likely to undergo an early death.

Astrotactin is required for granule cell migration

As polyclonal antibodies to astrotactin recombinant proteins block migration of granule cells in vitro (Fishell and Hatten, 1991), we used a migration assay to examine the effect of granule cells lacking astrotactin on migration.

The observed migration rates for wild-type granule cells in migration assays in this study are similar to those reported for untreated granule cells in previous studies (Fishell and Hatten, 1991). This allows us to compare the effects of adding astrotactin function-blocking antibodies to genetically induced loss of astrotactin function. Granule cells from astrotactin null mice migrate on average at 9.6 μm/h, compared with 4-5 μm/h seen when astrotactin-function-blocking antibodies are added to migration cultures (Fishell and Hatten, 1991). There are two possible explanations for this difference in the decrease in migration rates. The first is that the astrotactin function blocking antibodies bind to other astrotactin family members (we are aware of at least one gene related to astrotactin; T. T. and M. E. H., unpublished). The second is that by binding to astrotactin, the antibodies interfere with more than just astrotactin and its (as

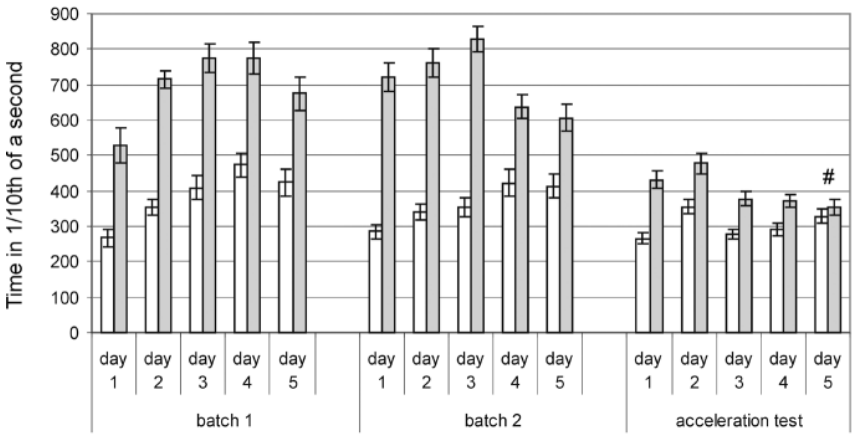

Fig. 6. Histogram showing the performance of wild type and astrotactin null mice in a Rota-rod behavioral assay. Note the consistent significantly (P<0.01) lower performances of astrotactin null mice in all exercises, except on the final day of the acceleration test (#) when compared with age- and size-matched wild-type mice. Batch 1 refers to the first set tests for each mouse and Batch 2 refers to the second test for each mouse.

yet unidentified) binding partner. We favor the first explanation, as using these antibodies for immunohistochemistry results in labeling that is more extensive than that obtained with in situ hybridization (data not shown).

Observations of labeling granule cells with BrdU confirm that the decrease seen in the migration of granule cells from astrotactin null mice in vivo correlates with decreased migration rates in vitro. A criticism of this conclusion might be that there were fewer granule cells labeled in astrotactin null mice and therefore fewer heavily labeled cells were found in the IGL. This is not born out by the observation that fewer granule cells were seen in the IGL and there were more heavily labeled cells in the EGL.

The decreased rate of migration is very likely to be a result of an inability of the granule cells to bind to Bergmann glia. Binding assays showed that granule cells from astrotactin null mice were less able to bind and stick to cultured glial carpets (Fig. 4A). This inability to bind with glia is also evident when granule cells are observed in migration cultures (Fig. 3C,D). Wild-type granule cells elongate on contact with glia both in vivo and in vitro (see Fig. 3A,B,E), whereas mutant granule cells frequently remain rounded and thus migrate at a slower rate (Fig. 3C,D,F,H). The inability of granule cells to bind to glia was not caused by an alteration made to the glia, as glial morphology in astrotactin null mice appeared to be normal, and the glia in the migration cultures were from wild-type animals. However, there is clearly not a complete failure of granule cells to attach to glia and to initiate migration. This is born out by the presence of an IGL and the occasional elongated migratory profile in sections stained for Nissl substance.

The results presented in Fig. 2 clearly show that granule cells lacking astrotactin can (and do) migrate away from the EGL, but they do so after the wild-type cohort. This might argue that the primary role of Astrotactin is to facilitate binding to Bergmann glia this in turn allows granule cells to migrate. That removing astrotactin function does not result in a complete abolishment of migration is not surprising, as previous studies have indicated that multiple sets of molecules are involved in the migration of granule cells from the EGL to IGL, including neuregulin (Rio et al., 1997), thrombospondin (O'Shea et al., 1990) and α-integrin (DeFreitas et al., 1995). It is also highly likely that there are other proteins similar to astrotactin that partially rescue our astrotactin null mice. There is at least one more astrotactin-like protein expressed in the cerebellum at the time when granule cells are making their way across the molecular layer (unpublished observations). However, removing astrotactin does result in the delay of granule cells leaving the EGL, the creation of ectopic accumulations of granule cells in the EGL and the protracted presence of the EGL.

A secondary role for astrotactin in migration is likely to be in maintaining the contact between granule cells and Bergmann glia. Pyknotic nuclei are more frequently found in the molecular layer of mice lacking astrotactin (see Fig. 5B). This is an indication that the granule cells have detached from the Bergmann glia prematurely and then undergo cell death. This opens the possibility that Astrotactin has a role in supporting the survival of granule cells, as well as supporting their migration along Bergmann glia. That the highly compact and densely labeled cells we see in the molecular layer are dying is confirmed when the tissue is stained for the presence of TUNEL-positive cells. However, this staining does not always overlap, as the fragmentation of DNA (what the TUNEL method stains) occurs before pyknosis (Gavrieli et al., 1992). We found no difference in TUNEL staining of Purkinje cells between normal and mutant animals (data not shown), indicating that there is no apparent need for astrotactin in the maintenance of these cells.

Morphological changes to the cerebellum in astrotactin null mice

A consequence of a decrease in the ability to migrate and the death of granule cells as they traverse the molecular layer is that the IGL of astrotactin null mice is smaller (see Fig. 4B). There is no apparent difference in the generation of granule cells in astrotactin null mice, as revealed by BrdU incorporation (Fig. 2B) and staining for the presence of M phase granule cells (data not shown). In addition to this, there is no significant difference in the size of EGL at P6 in astrotactin null mice. Another consequence of the failure to initiate and maintain migration is that there are ectopic accumulations of granule cells in the molecular layer of the majority of astrotactin null adult mice.

The most striking consequence of the delay in migration seen in cerebellar granule cells is that their synaptic partners, the Purkinje cells, are profoundly affected. This is manifested in Purkinje cells tilting out of the sagittal plane (see Fig. 5D, arrow) and their dendritic arbors stretching perpendicular to their normal orientation. Surprisingly, this disruption is not accompanied by the concomitant disruption of Bergmann glia among which they lie. The disrupted dendritic morphology of Purkinje cells is not present between P6 (data not shown) and P19 (note that the dendrites of the appropriately positioned Purkinje cells in Fig. 5H have normal arbors), when Purkinje cells in astrotactin null mice appear normal in morphology and number, but develops in parallel with the gradual innervation from granule cells. The cause of this spreading of dendritic arbors is likely to be due to the delay and decrease in the number

of connections made with the parallel fibers that originate from the granule cells. The displaced Purkinje cells seen in Fig. 5H are due to an ectopic accumulation of granule cells in the molecular layer (similar to that in Fig. 5C) of this P19 cerebellum. Despite this, the Bergmann glia soma and processes remained in their normal position and orientation.

Behavioral changes in mice that lack astrotactin

The Rota-rod test measures the ability of an animal to maintain balance by coordinating the movement of all four feet and making the necessary postural adjustments. It also measures the ability of the animal to improve on these skills with practice. Mutant and wild-type mice were examined for Rota-rod performance using two test protocols: rod rotation at a constant rate and rod acceleration. Astrotactin null mice were much less able to remain on the rotating rod at the beginning of the series of tests (>50% less time) when compared with age and size matched wild-type mice. This deficit, compared with wild-type mice, in the ability to maintain coordinated movement remained throughout the duration of the tests. When the same mice were confronted with a steadily accelerating rod, the astrotactin null mice were initially much less able to stay on. However, towards the end of the period, there was no statistical difference in the ability of astrotactin null and wild-type mice to stay on an accelerating rod. This result indicates that while astrotactin null mice are inherently less able to perform tasks requiring balance and coordinated movement, on shorter tasks they are able to learn to make up for this deficit.

This study has shown that Astrotactin is required for granule cell migration. It has also shown that the timing and amount of innervation between granule cells and their synaptic partners (Purkinje cells) is crucial. Untimely innervation results in altered morphology of Purkinje cells and a related decrease in the ability to perform coordinated balance and movement.

We thank all the members of the Hatten laboratory for their helpful discussions and especially Perrin Wilson for her help with the glial binding assays. This work was supported by NIH Grant NS 15429 (to M. E. H.).

REFERENCES

Anderson, S., Eisenstat, D., Shi, L. and Rubenstein, J. (1997a). Interneuron migration from basal forebrain to neocortex: dependence on DLx genes. *Science* **278**, 474-476.

Anderson, S. A., Qiu, M. S., Bulfone, A., Eisenstat, D. D., Meneses, J., Pedersen, R. and Rubenstein, J. L. R. (1997b). Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late born striatal neurons. *Neuron* **19**, 27-37.

Anton, E. S., Cameron, R. S. and Rakic, P. (1996). Role of neuron-glial junctional domain proteins in the maintenance and termination of neuronal migration across the embryonic cerebral wall. *J. Neurosci.* **16**, 2283-2293.

Anton, E. S., Kreidberg, J. A. and Rakic, P. (1999). Distinct functions of alpha3 and alpha(v) integrin receptors in neuronal migration and laminar organization of the cerebral cortex. *Neuron* **22**, 277-289.

Chadee, D. N., Taylor, W. R., Hurta, R. A., Allis, C. D., Wright, J. A. and Davie, J. R. (1995). Increased phosphorylation of histone H1 in mouse fibroblasts transformed with oncogenes or constitutively active mitogen-activated protein kinase kinase. *J. Biol. Chem.* **270**, 20098-20105.

Crawley, J. N. (1999). Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. *Brain Res.* **835**, 18-26.

DeFreitas, M. F., Yoshida, C. K., Frazier, W. A., Mendrick, D. L., Kypta, R. M. and Reichardt, L. F. (1995). Identification of integrin alpha 3 beta 1 as a neuronal thrombospondin receptor mediating neurite outgrowth. *Neuron* **15**, 333-343.

Edmondson, J. C. and Hatten, M. E. (1987). Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. *J. Neurosci.* **7**, 1928-1934.

Edmondson, J. C., Liem, R. K., Kuster, J. E. and Hatten, M. E. (1988). Astrotactin: a novel neuronal cell surface antigen that mediates neuron-astroglial interactions in cerebellar microcultures. *J. Cell Biol.* **106**, 505-517.

Fink, J. M., Hirsch, B. A., Zheng, C., Dietz, G., Hatten, M. E. and Ross, M. E. (1997). Astrotactin (ASTN), a gene for glial-guided neuronal migration, maps to human chromosome 1q25.2. *Genomics* **40**, 202-205.

Fishell, G. and Hatten, M. E. (1991). Astrotactin provides a receptor system for CNS neuronal migration. *Development* **113**, 755-765.

Fishman, R. and Hatten, M. (1993). Multiple receptor systems promote CNS neural migration. *J. Neurosci.* **13**, 3485-3495.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493-501.

Gregory, W. A., Edmondson, J. C., Hatten, M. E. and Mason, C. A. (1988). Cytology and neuron-glial apposition of migrating cerebellar granule cells in vitro. *J. Neurosci.* **8**, 1728-1738.

Hatten, M. E. (1985). Neuronal regulation of astroglial morphology and proliferation in vitro. *J. Cell Biol.* **100**, 384-396.

Hatten, M. E. (1999). Central nervous system neuronal migration. *Annu. Rev. Neurosci.* **22**, 511-539.

Hatten, M. E., Alder, J., Zimmerman, K. and Heintz, N. (1997). Genes involved in cerebellar cell specification and differentiation. *Curr. Opin. Neurobiol.* **7**, 40-47.

Hatten, M. E. and Mason, C. A. (1990). Mechanisms of glial-guided neuronal migration in vitro and in vivo. *Experientia* **46**, 907-916.

Lavdas, A. A., Grigoriou, M., Pachnis, V. and Parnavelas, J. G. (1999). The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J. Neurosci.* **19**, 7881-7888.

Misson, J. P., Austin, C. P., Takahashi, T., Cepko, C. L. and Caviness, V. S., Jr (1991). The alignment of migrating neural cells in relation to the murine neopallial radial glial fiber system. *Cereb. Cortex* **1**, 221-229.

Nagy, A., Rossant, J., Nagy, R., Abramow, N. W. and Roder, J. C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **90**, 8424-8428.

O'Rourke, N. A., Dailey, M. E., Smith, S. J. and McConnell, S. K. (1992). Diverse migratory pathways in the developing cerebral cortex. *Science* **258**, 299-302.

O'Shea, K. S., Rheinheimer, J. S. and Dixit, V. M. (1990). Deposition and role of thrombospondin in the histogenesis of the cerebellar cortex. *J. Cell Biol.* **110**, 1275-1283.

Pinkas-Kramarski, R., Eilam, R., Spiegler, O., Lavi, S., Liu, N., Chang, D., Wen, D., Schwartz, M. and Yarden, Y. (1994). Brain neurons and glial cells express Neu differentiation factor/heregulin: a survival factor for astrocytes. *Proc. Natl. Acad. Sci. USA* **91**, 9387-9391.

Ponting, C. P. (1999). Chlamydial homologues of the MACPF (MAC/perforin) domain. *Curr. Biol.* **9**, R911-R913.

Rakic, P. (1971). Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in Macacus Rhesus. *J. Comp. Neurol.* **141**, 283-312.

Rio, C., Rieff, H., Pelmin, Q. and Corfas, G. (1997). Neuregulin and erbB receptors play a critical role in neuronal migration. *Neuron* **19**, 39-50.

Rivas, R. J. and Hatten, M. E. (1995). Motility and cytoskeletal organization of migrating cerebellar granule neurons. *J. Neurosci.* **15**, 981-989.

Stitt, T. N. and Hatten, M. E. (1990). Antibodies that recognize astrotactin block granule neuron binding to astroglia. *Neuron* **5**, 639-649.

Tan, S. S., Kalloniatis, M., Sturm, K., Tam, P. P. L., Reese, B. E. and Faulkner-Jone, B. (1998). Separate progenitors for radial and tangential cell dispersion during development of the cerebral neocortex. *Neuron* **21**, 295-304.

Volpe, J. J. (1995). Neuronal proliferation, migration, organization, and myelination. In *Neurology of the Newborn* (ed. J. J. Volpe), pp. 43-92. Philadelphia: W.B. Saunders.

Wechsler-Reya, R. and Scott, M. (1999). Control of neuronal precursor proliferation in the cerebellum by sonic hedgehog. *Neuron* **22**, 103-114.

Wood, S. A., Allen, N. D., Rossant, J., Auerbach, A. and Nagy, A. (1993). Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* **365**, 87-89.

Zheng, C., Heintz, N. and Hatten, M. E. (1996). CNS gene encoding astrotactin, which supports neuronal migration along glial fibers. *Science* **272**, 417-419.