Mice that lack astrotactin have slowed neuronal migration

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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

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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 selected ES cell clones rendered four highly chimeric males. They 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 littermates and astrotactin null animals. The day of birth was designated P0. Tail snips were collected for DNA extraction into blastocysts of C57BL/6J donor mice. One of the positively selected ES cell clones was added to each well. 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 and stained 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 littersmates 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 a BioRad MRC 600 and Radiance 2000 confocal laser-scanning microscopes. sections (z-series) were compiled and processed using either Confocal Assistant (Todd Brejle) or VoxselView (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 Basile) 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): BglII digests liberate 8.0 kb and 6.5 kb fragments from the wild-type and targeted alleles, respectively. PstI digests liberate 9.5 kb and 7.8 kb fragments from the wild-type and targeted alleles. SpeI 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.
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 (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.
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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, 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 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.

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).
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. 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).
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 (Gavieli 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 perform tasks requiring balance and coordinated movement 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 innervation between granule cells and their synaptic partners.

We thank all the members of the Hatten laboratory for their helpful discussion 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.).

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