son at the *Salmonella* Genetic Stock Centre (Calgary, Alberta, Canada). These were transduced with bacteriophage P22 into wild-type *S. typhimurium* 14028s; transductants were phenotypically confirmed to be methionine auxotrophs. Intraperitoneal inoculation of 1×10^4 *metB* mutant *S. typhimurium* was lethal in zero of four C3H/HeN mice (Charles River Laboratories) during a 14-day experiment and did not result in visible illness. In contrast, 5×10^3 *metE* mutant *S. typhimurium* killed two of four mice and made all of the infected mice ill.

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CNS Gene Encoding Astrotactin, Which Supports Neuronal Migration Along Glial Fibers

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Vertebrate central nervous system (CNS) histogenesis depends on glia-guided migration of postmitotic neurons to form neuronal laminae. Previous studies have established that the neuronal protein astrotactin functions in murine cerebellar granule cell migration in vitro. The gene encoding astrotactin predicts a protein with three epidermal growth factor repeats and two fibronectin type III repeats. Astrotactin messenger RNA is expressed in postmitotic neuronal precursors in the cerebellum, hippocampus, cerebrum, and olfactory bulb, where migration establishes laminar structures. Fab fragments of antibodies to a recombinant astrotactin peptide blocked migration of cerebellar granule neurons in vitro along astroglial fibers. Transfection of astrotactin complementary DNA into 3T3 cells indicated that astrotactin acts as a ligand for neuron-glia binding during neuronal migration.

The vertebrate cortex is patterned into several layers, each with a distinctive set of neurons (*1*, *2*). Studies on the developing brain suggest that cells destined to form specific cortical layers migrate along radial glial fibers under the direction of the protein astrotactin (*3*–*5*). A set of candidate astrotactin clones was generated by screening of a P3 to P5 granule cell complementary DNA (cDNA) expression library (*6*, *7*) with a polyclonal antiserum to astrotactin that inhibits neuronal migration (*4*). Among 39 immunopositive clones examined, one clone, GC14, encoded a brain-specific transcript of about 7 kb. The open reading frame of this transcript [2500 base pairs (bp)] predicts a polypeptide of 100 to 105 kD, with four potential N-linked glycosylation sites.

The NH_2-terminus of GC14 begins with a signal peptide of 15 amino acids (Fig. 1A). The most notable feature of the deduced GC14 protein is the presence of domains homologous to several well-characterized signaling and adhesion molecules. The sequence of GC14 contains three cysteine-rich epidermal growth factor (EGF)–like repeats (8) and two domains with homology to a motif of 90 amino acids found in fibronectin type III (FNIII) (*9*). EGF repeats are found in the extracellular domains of several proteins (*10*, *11*) and have been implicated in intercellular signaling in neuronal development. FNIII repeats have been found in axonal glycoproteins of the immunoglobulin G (IgG) superfamily (*12*). The number and arrangement of these motifs and the absence of IgG-like domains suggest that astrotactin is unlike other cell adhesion or signaling molecules.

In a Northern (RNA) blot analysis, GC14 hybridized to a single mRNA band of about 7 kb that was expressed in the brain (specifically in the cerebellum and forebrain), but not in the kidney, heart, liver, lung, thymus, or spleen (*13*). GC14 mRNA was expressed in purified cerebellar granule cells but not in glial cells.

In the developing murine cerebellar cortex, GC14 transcripts were abundant in the deeper aspect of the external germinal layer (EGL), where cells are undergoing early steps in differentiation, as well as in migratory and postmigratory cells (Fig. 2, A through C). Glial cells, including the Bergmann glia of the molecular layer and astrocytes within the white matter, were unlabeled. Proliferating precursor cells, which occupy the superficial aspect of the EGL, did not express astrotactin mRNA or protein. Rather, expression commenced in the deeper aspect of the EGL where postmitotic precursor cells bind to astroglial fibers, extend parallel fibers, migrate along the Bergmann glial fibers, detach from the glial fibers, and move past Purkinje neurons. Astrotactin expression continued as differen-

B EGF repeats:

EGF1: CSKD-NGGCSKNFRCI--------SDRKLDSTGCVCPSGLSPMKDSSGC
(328 to 367)
EGF2: CSDGFNGGC--EQLCLQQMAPFPEDPLYNILMFCGCIEDYKLGVDGR-C
(376 to 423)
EGF3: TQRLLQEATMSSLWC--------SGTGDVIEDWCRCDSTAFGADGLPTC
(701 to 742)
EGF-mouse: C GC C C C G C

C FNIII repeats:

ASTFNI: PDFLTGMVNFSEVSGYPVLQHWKVRSVMYHIKLNQAAISQAFSNALHSLDGATSRADFVALLDQFGNHYIQEAVYGFEESCS
(511 to 593)
ASTFNII: PVLRLSTVHEP--SSNLVVLEWEHSEPPIG----VQIVDYLYRQEKVTDRMDHSKVETETV-LSFVDDIISGAKAPCAMP-S
(747 to 821)
FNIII: P--L---------T-----V-W--P..4-8aa..I--Y-V-Y----------..5-14aa..L-P---Y---V-A------S

Fig. 1. Deduced amino acid structure of astrotactin and homology with known proteins. (**A**) Schematic representation of the domain structure of astrotactin. Black, signal peptide; gray, EGF-like repeat; hatched, FNIII-like repeat. (**B**) Alignment of the three EGF repeats and (**C**) alignment of the two FNIII repeats in astrotactin over the consensus of the respective motifs (*8*, *9*). Identical amino acids are shown in bold; underlined amino acids are substitutes found in FNIII repeats of other proteins at the same positions. Dashes denote gaps to optimize the alignment. ASTFN, astrotactin FNIII repeat; aa, amino acid. Numbers indicate the positions of each repeat within astrotactin cDNA.

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tiated granule cells transited the Purkinje cell layer and extended short dendrites, which contact the terminals of the ingrowing, afferent mossy fibers, forming glomeruli that are enwrapped by vellate astrocytes.

A similar pattern of expression was seen in the developing hippocampal formation (Fig. 2, D and E), where heavily labeled cells were observed in the subgranular layer of the immature dentate gyrus. In the neonatal period, within the overlying cortex, cells in the superficial layers were labeled intensely. Scattered labeled cells were seen within the deeper layers (Fig. 2F). Labeled cells were not detected in the collapsing ventricular zone, the major germinal zone of the cortex. Expression was not detected in the adult cortex. Transcripts were abundant in the developing olfactory bulb, where postmitotic immature neurons within the granule cell layer, mitral cell layer, and glomeruli were labeled. GC14 mRNA was not detected in the deep nuclei of the cerebellar cortex, subcortical brain regions, thalamus, midbrain, or brainstem.

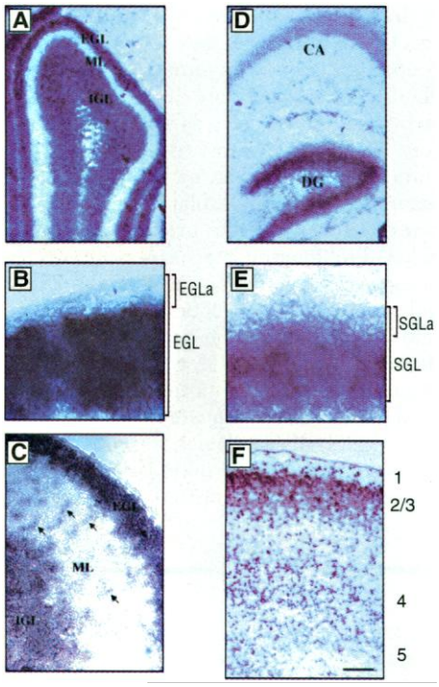

Fig. 2. Expression of astrotactin mRNA in situ. Astrotactin mRNA is localized in parasagittal sections of postnatal day 10 mouse brain. (**A**) Labeled cells are detected in the inner layer of the EGL, molecular layer (ML), and internal granule cell layer (IGL) of the cerebellum. (**B**) Proliferating precursor cells in the superficial zone of the EGL (EGLa) are not labeled; however, EGL cells migrating across the ML are labeled [arrows in (**C**)]. (**D**) Astrotactin mRNA is expressed in the granule cells of the dentate gyrus (DG) in the developing hippocampal formation. CA, cornis ammonis. (**E**) Proliferating cells in the subgranular layer (SGLa) are unlabeled. (**F**) In the developing cortex, newly generated cells of layer 2/3 are most intensely labeled, with fewer labeled cells in deeper layers. Scale bar in (F) represents 50 μm in (A), (D), and (F) and 12.5 μm in (B), (C), and (E).

We used antibodies to GC14 to examine the role of GC14 protein in neuronal migration along glial substrates (*4*, *14*). Whereas neurons treated with Fab fragments of preimmune serum and depleted antiserum to GC14 underwent directed migration along the glial fibers at speeds between 20 and 50 μm/hour (Fig. 3, A and C), Fab fragments of antiserum to GC14 arrested migration (Fig. 3B). To compare the inhibition of neuronal migration by the Fab fragments of antibody to GC14 with that by the original polyclonal astrotactin antibodies, we applied the original astrotactin antiserum to an affinity column of glutathione-S-transferase–GC14 fusion protein. Fab fragments prepared from both the original polyclonal antibodies and the activity in the serum that bound to the GC14 affinity column arrested migration (Fig. 3, D and E). These results establish that GC14 cDNA encodes astrotactin, a ligand for neuronal migration in the CNS along astroglial fibers. Moreover, the in vitro studies show that membrane-associated astrotactin supports cell migration along cerebellar astroglial fibers.

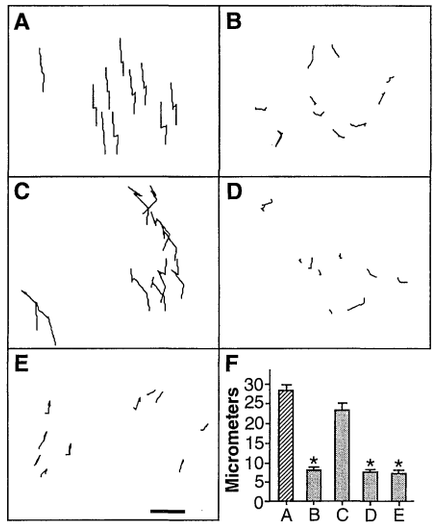

Fig. 3. Astrotactin functions in migration along astroglial fibers. The migration of purified granule cells along glial fibers in microcultures was measured with a video microscopy assay. After 36 hours in vitro, the Fab fragments of (**A**) preimmune serum, (**B**) affinity-purified antibody to GC14, (**C**) depleted antibody to GC14, (**D**) antibody to astrotactin affinity-purified with a GC14 fusion protein column, and (**E**) the original antibody to astrotactin were added. Cells were tracked with a computer-assisted system. (**F**) The rate of migration was calculated by tracking the position (*x* and *y* pixels) of a set of 10 to 15 cells in each of five random fields (for a total of >50 cells) per experimental treatment over a 4-hour period. Scale bar in (E) represents 50 μm in (A) through (E). Asterisk indicates blocking activity.

To examine the mode of action of astrotactin, we transfected full-length transcripts of astrotactin cDNA into 3T3 cells and examined whether the protein provided a heterophilic adhesion receptor system for neuron-glia binding (*5*, *15*) or a component of a program of gene expression for neuronal differentiation (*6*, *15*). When granule cells purified from early postnatal cerebellar cortex were plated on transfected 3T3 cells, the neurons adhered poorly and failed to extend neurites or to express the axonal glycoprotein TAG1, a marker of later steps of differentiation (*16*) (Table 1). The absence of a role for astrotactin in inducing further steps in granule cell differentiation was supported by the finding that addition of antibodies to astrotactin peptide did not affect neurite extension and TAG1 expression in a reaggregation culture system. Together, these experiments suggest that astrotactin does not induce a program of neuronal differentiation through homophilic neuron-neuron binding.

To test whether astrotactin provides a ligand for glial binding, we plated astroglial cells purified from the same cell preparations used to generate neurons onto transfected 3T3 cells. In contrast to granule cells, astroglial cells adhered to transfected 3T3 cells, and more than 70% of the cells extended long processes, which is typical of glial cells in coculture with neurons. Immu-

Table 1. Astrotactin functions in neuron-glia adhesion. Transfection of astrotactin cDNA into 3T3 cells confers glial binding and supports astroglial differentiation. Granule neurons and astroglial cells were purified from early postnatal cerebellum, labeled with the fluorescent dye PKH26, and plated on a monolayer of 3T3 cells transfected with astrotactin cDNA or with the control vector. After one division, the binding of neurons or glial cells and the formation of processes by these cells were assessed by means of fluorescence microscopy. Labeled granule neurons failed to extend neurites, but labeled glial cells bound to the transfected 3T3 cells and more than 65% of the cells extended long processes, which is typical of differentiated glia in coculture with neurons. Differentiation of neurons or glial cells was assayed by immunostaining of the cultures with antibodies to the axonal glycoprotein TAG1, a marker for granule cell differentiation; GFAP, a marker for glial differentiation; and neural cell adhesion molecule (N-CAM), a general neuronal marker. A plus value indicates that >75% of the cells were labeled; a minus value indicates that <25% of the cells were labeled.

Functional assays	Astroglial cells	EGL cells
Adhesion	+	−
Process formation	+	−
Cellular antigen expression		
GFAP	+	−
N-CAM	−	+
TAG1	−	−

nostaining with antibodies to glial fibrillary acidic protein (GFAP), a marker of glial differentiation, confirmed that astrotactin both conferred glial binding and induced glial differentiation (Table 1). In control experiments, purified glial cells did not bind to untransfected 3T3 cells. These experiments demonstrate that the mode of action of astrotactin is to provide a heterophilic ligand for glial binding. As a secondary effect, astrotactin appears to maintain the differentiation of glial cells.

The mechanism of action of astrotactin seen in assays in vitro is consistent with the structure of the protein. EGF repeats have been implicated in cell-cell signaling (*11*), suggesting that astrotactin functions in a signaling pathway that is crucial for neuron-glia interactions before and during migration. Because glial cells bind to neurons and undergo differentiation in response to astrotactin, we anticipate an astrotactin receptor on the glial surface. With the cloning of the gene encoding astrotactin, at least three genes can be proposed to function in the migration and assembly of neurons during murine cortical histogenesis: (i) *Weaver* acts at the earliest stage, blocking the expression of a ligand needed for entry of postmitotic neurons into a program of gene expression that includes expression of astrotactin and TAG1 (*17*). (ii) The gene encoding astrotactin is expressed in initial steps of differentiation required for utilization of the glial scaffold for migration and laminar formation. (iii) *Reelin* acts at later steps, during the arrest of locomotion of the migrating neuron along glial fibers and formation of neuronal layers (*10*, *18*).

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14. To produce recombinant GC14 protein, a cDNA fragment containing the region of one EGF and one FNIII repeat was subcloned into pGEM-3X vector (Pharmacia). Affinity purification of antibodies to GC14 was according to the protocol in *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, Eds. (Wiley, New York, 1994), vol. 2, suppl. 27. Cell culture experiments were carried out with primary cells from cerebellums harvested on postnatal days 5 to 6. The in vitro migration culture assay was performed as described previously. Fab fragments prepared from these antibodies were added at a concentration of 0.4 μg/ml. Cell images were recorded with a Panasonic optical memory disk recorder, and measurements of neuronal migration were made with Metamorph software (Universal Imaging).
15. Full-length GC14 cDNA was subcloned into pRC/CMV vector (Invitrogen) and transfected into 3T3 cells by calcium phosphate–mediated gene transfer. Colonies resistant to G418 were screened for astrotactin expression by protein immunoblot. As a control, cell lines containing the vector alone were also tested. For the cell-binding assay, a monolayer of 3T3 cells was preplated. Granule and glial cells were labeled with PKH26 dye and added to the 3T3 cells. After 36 hours in vitro, the number of the labeled cells that bound and the percentage of bound cells that were differentiated were quantified by random counting of cells in 10 fields at a magnification of ×20.
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Circadian Rhythms in Cultured Mammalian Retina

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Many retinal functions are circadian, but in most instances the location of the clock that drives the rhythm is not known. Cultured neural retinas of the golden hamster (*Mesocricetus auratus*) exhibited circadian rhythms of melatonin synthesis for at least 5 days at 27°C. The rhythms were entrained by light cycles applied in vitro and were free-running in constant darkness. Retinas from hamsters homozygous for the circadian mutation *tau*, which shortens the free-running period of the circadian activity rhythm by 4 hours, showed a shortened free-running period of melatonin synthesis. The mammalian retina contains a genetically programmed circadian oscillator that regulates its synthesis of melatonin.

Virtually all organisms have circadian (about 24-hour) rhythms that are driven by endogenous oscillators. In nonmammalian vertebrates, circadian oscillators have been identified in the pineal organ (*1*), the retina (*2*), and tentatively the hypothalamus (*3*). In mammals, only the suprachiasmatic nucleus of the hypothalamus (SCN) has been proven to contain circadian oscillators (*4*), although there is indirect evidence that they may exist in the retina as well (*5*).

Researchers at several laboratories including our own have failed to demonstrate circadian rhythmicity of melatonin synthesis in mammalian retinas cultured at 37°C. However, because golden hamsters (*M. auratus*) are hibernators, their tissues tolerate lowered culture temperatures, and we therefore attempted to culture their neural retinas at a constant temperature of 27°C. In chambers identical to those used for *Xenopus* retina (*6*), the hamster retinas were continuously perifused with medium at 0.5 ml/hour. Medium was collected with a fraction collector at 3-hour intervals and radioimmunoassayed for melatonin (*7*).

In a light:dark cycle (LD 14:10; light intensity of 1800 $\mu W/cm^2$) cultured hamster retinas were robustly rhythmic for the duration of the experiment (4 days), with period lengths of 24 hours and peaks of melatonin synthesis during the dark phase of the cycle (Fig. 1, A and B); the hamster retinas exhibited the same general pattern as that found in the cultured retina of *Xenopus* (*2*) and in cultured pineal glands of many nonmammalian vertebrates (*1*). In most of these other systems, light directly suppresses melatonin synthesis. In our experiments such suppression, if it occurs, is not complete (Fig. 2, A and B).

The rhythm of melatonin synthesis can be entrained in vitro (Fig. 2). Three retinas were cultured in a light cycle that was advanced 7 hours from the cycle of the intact animals, and three other retinas were cultured in a light cycle that was 7 hours

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