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# Ectopic expression of the neural cell adhesion molecule L1 in astrocytes leads to changes in the development of the corticospinal tract

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## Abstract

The cell recognition molecule L1, of the immunoglobulin superfamily, participates in the formation of the nervous system and has been shown to enhance cell migration and neurite outgrowth *in vitro*. To test whether ectopic expression of L1 would influence axonal outgrowth *in vivo*, we studied the development of the corticospinal tract in transgenic mice expressing L1 in astrocytes under the control of the GFAP-promoter. Corticospinal axons innervate their targets by extending collateral branches interstitially along the axon shaft following a precise spatio-temporal pattern. Using DiI as an anterograde tracer, we found that in the transgenic animals, corticospinal axons appear to be defasciculated, reach their targets sooner and form collateral branches innervating the basilar pons at earlier developmental stages and more diffusely than in wild type littermates. Collateral branches in the transgenic mice did not start out as distinct rostral and caudal sets, but they branched from the axon segments in a continuous rostrocaudal direction across the entire region of the corticospinal tract overlying the basilar pons. The ectopic branches are transient and no longer present at postnatal day 22. The earlier outgrowth and altered branching pattern of corticospinal axons in the transgenics is accompanied by an earlier differentiation of astrocytes. Taken together, our observations provide evidence that the ectopic expression of L1 on astrocytes causes an earlier differentiation of these cells, results in faster progression of corticospinal axons and influences the branching pattern of corticospinal axons innervating the basilar pons.

## Introduction

The formation of orderly nerve connections during development of the nervous system relies on the coordinated expression of cell recognition molecules. Cell adhesion molecules of the immunoglobulin superfamily represent one of the major classes of cell surface proteins that participate in neurite outgrowth and axonal guidance (reviewed in Brümmendorf & Rathjen, 1993). L1 is one of the most intensely studied members of this family and is predominantly expressed by neurons in the developing central and peripheral nervous system. It has been implicated in numerous important processes such as neuronal migration (Lindner *et al*., 1983), axon growth and fasciculation (Beasley & Stallcup, 1987; Rathjen *et al*., 1987) and synaptic plasticity (Luthi *et al*., 1996). The common mode of interaction is homophilic L1–L1 binding between adjacent membranes (Lemmon *et al*., 1989). However, a variety of heterophilic binding partners have been identified (reviewed in Hortsch, 1996). In addition, L1 functions not only in adhesive but also in repellent cell interactions (Castellani *et al*. 2000) and is involved in signal transduction events (reviewed in Kamiguchi & Lemmon, 1997).

We have generated a transgenic mouse that expresses L1 in its integral membrane form in astrocytes under the regulatory sequences of the glial fibrillary acidic protein (GFAP) gene (Mohajeri *et al*., 1996). Tissue sections or astrocyte cultures from these transgenic mice significantly enhance neurite outgrowth when offered as a growth substrate. In the present study, we asked whether the ectopic expression of L1 on the surface of astrocytes also interferes with axonal outgrowth and pathfinding *in vivo*. Since the GFAP-promoter is turned on late during development of the mouse central nervous system (CNS) (Pixley & de Vellis, 1984; Sancho-Tello *et al*., 1995), we choose the corticospinal tract (CST) as an experimental system. The CST is the single longest axon tract within the mammalian brain and is the last of the major developing fibre tracts to enter the spinal cord (Stanfield, 1991). Axons of the CST originate from neurons in layer V of the neocortex and establish connections with several targets in the midbrain, hindbrain and spinal cord. In the mouse, the first corticospinal axons exit the cortex via the internal capsule at embryonic day 17 (ED 17), reach the brainstem at postnatal day 0 (PD 0) and enter the spinal cord at PD 1 (Bastmeyer & O'Leary, 1996). Corticospinal axons innervate their targets by a process called

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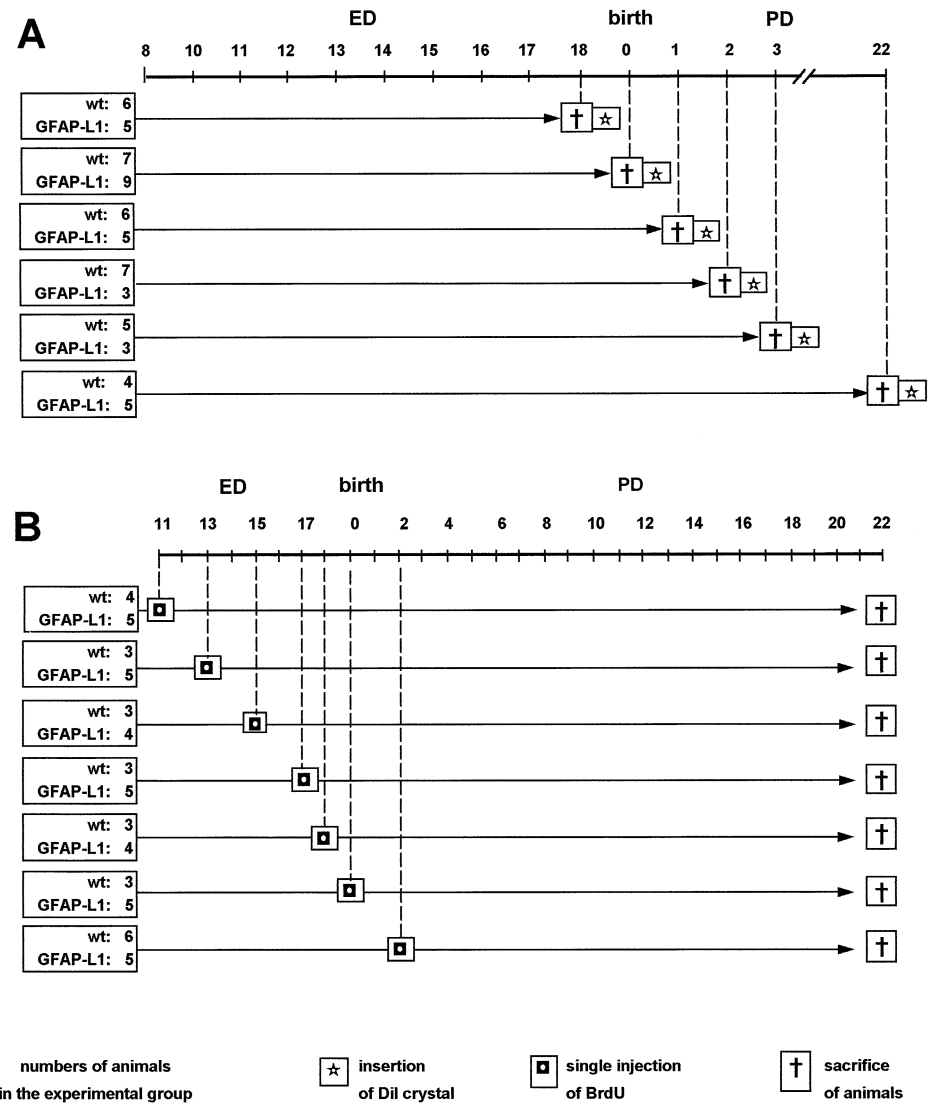


FIG. 1. Schedules and animal numbers used during CST tracing with DiI and during the follow-up of neurogenesis with injections of BrdU. Progression of CST fibers and of pontine collateral formation was monitored by insertion of a crystal of the fluorescent lipophilic tracer DiI at defined developmental stages (A) and the formation of cells destined for layer V of primary motor cortex was followed by single BrdU injections (B). Both schedules included wild type (wt) and GFAP-L1 transgenic mice. ED, embryonic day; PD, postnatal day. Boxes to the left indicate numbers of animals used in the individual experimental groups. For control experiments in the barrel cortex and using the second L1-transgenic mouse line 3427 (see main text) five to seven animals, depending on litter size, were used per experiment (not shown).

'interstitial axon branching' (O'Leary & Terashima, 1988). Instead of elaborating target-orientated axons, the main stream of fibres in the tract grows past the targets which later become innervated by collateral branches arising *de novo* along the axon shafts (Bastmeyer & O'Leary, 1996).

In the present study we have used DiI as an anterograde marker in fixed brains of the GFAP-L1 transgenic mice and found that the outgrowth of corticospinal axons is significantly enhanced. In addition, these axons form collateral branches innervating the basilar pons at earlier ages than in their wild type littermates and, at least transiently, at ectopic sites.

## Materials and methods

### *Animals*

Two independent lines of GFAP-L1 transgenic mice that express L1 ectopically in astrocytes under a GFAP promoter (Mohajeri *et al*., 1996) were used. The first one (mouse line 3426), in which the expression of the transgenic L1 was stronger, was chosen for the major part of the presented study and a second one (mouse line 3427), showing a weaker expression of L1, was used as a confirming control to account for possible nonspecific positional effects of transgene insertion. Moreover, as it was impossible to detect the ectopic L1 amidst the abundant endogenous molecule expressed on host neurons, the comparison between the two lines served as an additional control for a direct causal relationship between the morphological changes and the expression of the transgene. Both lines were maintained in a heterozygous state on a C57BL/6 J genetic background. The study used 36 embryos (ED 11–18), 73 pups (PD 0–3), and eight PD 22 mice overexpressing L1 and their wild type siblings as controls (32 embryos, 70 pups, and 10 PD 22 mice).

Mice were analysed for the following developmental features: (i) elongation and collateralization of CST axons in the basilar pons region between ED 18 and PD 3 (a total of 81 animals) and at PD 22 ($n$ = 18; Fig. 1A); (ii) neuronal cell formation in layer V of neocortical motor area 6 between ED 11 and PD 2 (times of BrdU injections, all 54 animals evaluated at PD 22; Fig. 1B); (iii) neuronal cell density and width of layer V in area 6, as well as CST cross-section diameter at PD 2 (five GFAP-L1 animals and five wild types) and PD 22 [same animals as under (ii)]; (iv) differentiation of GFAP-positive cells (astrocytes) in basilar pons between ED 18 and PD 3 (GFAP-L1 animals: four ED 18, four PD 1, and five PD 3; wild types: five ED 18, five PD 1, and four PD 3). For each developmental period and each kind of evaluation, two to four litters were used depending on their size.

Animals were mated overnight, with embryonic day 0 (ED 0) defined as the day of vaginal plug detection and the first 24 h after birth defined as postnatal day 0 (PD 0). All pups used in this study were born on ED 19 ± 0.5. Embryos were obtained by Caesarian section from pregnant mice anaesthetized with pentobarbital (75 mg/kg body weight, Nembutal, Abbott Laboratories, Chicago, IL) and killed by decapitation. Newborn and perinatal mice were killed with an overdose of pentobarbital and anaesthetized, PD 22 mice were first perfused with 0.01 M phosphate buffered saline (PBS, pH 7.2) for 5 min (flow rate approx. 25 mL/min). For DiI tracing (see below), brains were immersion-fixed in 4% paraformaldehyde in PBS for 24 h at room temperature (RT). For immunostaining, they were immersed for the same time in 5% acetic acid in 70% ethanol (acetic alcohol) at 4 °C, dehydrated, and embedded in polyester wax (BDH; Poole, Dorset, United Kingdom, Ourednik *et al*., 1993). All animal experiments conformed strictly to the regulations through the Swiss Federal Law and were approved by the Veterinaeramt of the Kanton Zuerich.

### *Genotyping*

Transgenic offspring from both lines were genotyped by polymerase chain reaction (PCR) using L1 cDNA-specific primers 5′-ATG-CTGTTGGTGGGCTTGAC-3′ and 5′-GCACCCTATTCTGGCTC-CTT-3′. DNA was isolated from tail cuts by incubating the tissues overnight in tail lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, and 600 μg/mL proteinase K) at 55 °C. After precipitation with isopropanol, the DNA was resuspended in 300 μL of sterile water. A typical 50-μL PCR was run with 2 μL of DNA, 5 μL of 10× reaction buffer (Perkin Elmer, Boston, MA, USA), 200 μM dNTPs (Amersham Pharmacia Biotech, Duebendorf, Switzerland), 1.5 mM $MgCl_2$, 50 nM each of the primers, and 1 Unit of Taq polymerase (Life Technologies, Basel, Switzerland). The cycling profile was 30 cycles of 94 °C (45 s), 55 °C (30 s) and 72 °C (60 s), followed by an extension step at 72 °C for 10 min. These conditions amplified in GFAP-L1 mice a fragment of 417 base pairs which was visualized using 1% agarose gel electrophoresis and ethidium bromide.

### *Anterograde tracing of fibre tracts with DiI*

The fluorescent lipophilic carbocyanine dye, 1,1′-dioctodecyl-3′,3′,3′,3′,-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, Leiden, The Netherlands), was used as a postmortem anterograde axonal tracer (Honig & Hume, 1986; Godement *et al*., 1987) in brains fixed with 4% paraformaldehyde between ED 18 and PD 3, and at PD 22 (Fig. 1A). A small crystal of DiI (about 0.2 mm in diameter) was inserted unilaterally either into motor area 6 of the posteromedial part of the frontal cortex (Caviness, 1975) or, in mid-sagittally bisected brains, into the ventral thalamus using fine microforceps. After insertion of the DiI crystals, the tissues were kept in 4% paraformaldehyde in PBS at 37 °C for 3–8 weeks. After this incubation, 50-μm sagittal vibratome sections were prepared from the specimens and stored at 4 °C in the dark in 0.1% sodium azide in PBS. For examination, they were mounted on glass slides in distilled water and viewed with a Zeiss Axiophot microscope under UV illumination using filter conditions for detection of rhodamine.

### *Monitoring of nerve cell generation for the primary motor cortex area 6*

To follow the generation of large neurons in layer V of neocortical area 6 (Caviness, 1975; Polleux *et al*., 1997), mice received a single intraperitoneal injection of 5′-bromodeoxyuridine (BrdU; 50 mg/kg body weight, Sigma, St. Louis, MO, USA) at different stages between ED 11 and PD 2 of prenatal and early postnatal development (Fig. 1B). The solution was administered at a concentration of 10 mg/mL in physiological saline containing 7 mM NaOH. All animals were killed at PD 22. The dissected brains were fixed overnight in acetic alcohol at 4 °C, dehydrated in an ethanol series and embedded in polyester wax at 38 °C (Ourednik *et al*., 1993). Sagittal sections (20 μm) through area 6 were collected between sagittal levels 115–140 (Sidman *et al*., 1971) on slides coated with 1% gelatine containing 1% formaldehyde. Just before immunostaining, sections were dewaxed 2 × 3 min in absolute ethanol, rehydrated, and washed 2 × 5 min in PBS.

### *Immunohistochemistry*

Specific antibodies (Abs) and procedures for the various immunoreactions are described individually below. Negative controls (primary Ab replaced by diluent) were always processed in parallel with the immunoreactions for each marker. If double-immunostaining for the identification of BrdU-labelled cells was performed, the first immunoreaction was always against BrdU, followed by extensive washing in PBS (1–2 h) before the second incubation with a cell type-specific Ab was started.

#### *BrdU*

Cells incorporating BrdU were detected according to a modified version of the protocol by Ourednik *et al*. (1998). Sections were partially hydrolysed at RT in 2 N HCl for 35–40 min, rinsed for 1 min in 0.1 M PBS (pH 6), blocked for 30 min in a solution of 10% horse serum (HS) in 0.01 M PBS containing 1% $H_2O_2$, and incubated for 45 min at RT with a rat monoclonal antibody (moAb) against BrdU (clone BU1/75; Sera-Laboratory, Crawley Down, United Kingdom) diluted 1 : 75 in 0.05 M PBS (pH 7.4) containing 0.5% Tween 20. Detection of the bound primary Ab with a biotinylated rabbit anti-rat IgG Ab was conducted according to the instructions of the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The final dark blue reaction was obtained in the following nickel/cobalt-3′,3-diaminobenzidine (Ni/Co–DAB) solution: 15 mL PBS with 10 mg DAB (Sigma), 3 mg nickel-ammonium sulphate [$NiSO_4(NH_4)_2SO_4.6H_2O$], 3.5 mg cobalt chloride ($CoCl_2.6H_2O$) and 1 μL 30% hydrogen peroxide ($H_2O_2$).

#### *NeuN*

To detect NeuN-positive cells, we used our modification of a protocol by Mullen *et al*. (1992). Sections were incubated overnight at 4 °C with moAb A60 diluted 1 : 100 in 10% HS in PBS (pH 7.2). Specific binding was detected with a biotinylated horse anti-mouse IgG Ab (dilution 1 : 150, 60 min at RT) using the Vectastain ABC kit and DAB.

#### *GFAP*

For immunofluorescence staining of GFAP-positive cells, sections were reacted overnight at 4 °C with a polyclonal rabbit anti-GFAP Ab (Dako, Zug, Switzerland) diluted 1 : 100 in 10% HS, 0.05 M BSA and 0.1% Triton X-100 in PBS. After washing in PBS, a fluorescein isothiocyanate (FITC)-coupled anti-rabbit IgG Ab (Sigma) was applied in a 1 : 40 dilution for 2.5 h at RT. Sections rinsed in PBS were evaluated under UV illumination through a FITC filter on a Zeiss Axiophot microscope.

### *Microscopy and photomicrography*

Immunostained sections using DAB and haematoxylin-stained sections were mounted in Entellan (Merck, Darmstadt, Germany), sections used for immunofluorescence were mounted in Fluoromount

(BDH Laboratory Supplies, Poole, England) and DiI-stained sections were viewed in PBS. All material was analysed with Zeiss Axiophot or Axioscope microscopes. Photomicrographs were taken on Kodak Technical Pan at 50 ASA, Kodak EPJ 64, and Kodak EPJ 400.

### Quantitative evaluation

For each animal group and each histological measurement three to five sections from sagittal (DiI tracing of CST fibres, layer V thickness, and number of neurons) or coronal serial sections (DiI tracing of thalamocortical fibres and CST diameter measurement) were used and counts (performed with experimentor blind to experimental conditions) obtained in a Zeiss Axioscope microscope (magnification 40 × 10) equipped with a reticle eye-piece. For cell numbers, 1000–2000 cells per individual category of quantification were counted per one animal. For each quantitative evaluation, the arithmetic mean ± standard deviation (SD) were calculated using ANOVA and the statistical significance of value differences between wild type and L1-transgenic mice was assessed in a Student's *t*-test ($P < 0.05$ was taken as the significance level). CST diameters were determined with the same setup using the Neurolucida program.

#### *DiI tracing in the CST*

DiI-labelled axons overlying the basilar pons and penetrating the presumptive barrelcortex were observed and photographed at levels corresponding to sagittal sections 132–143 and 45–63 (Sidman *et al*., 1971).

#### *Measurement of cross-section diameters in the CST at PD 2 and PD 22*

From PD 22 brains, 20-μm coronal sections through the lower brain stem at and caudal to the level of the inferior olive (levels 495–510, Sidman *et al*., 1971) were prepared. In PD 2 brains, analogous sections were cut from a 200–300 μm strip caudal to the posterior pontine flexure. All sections were stained with Mayer's haematoxylin prior to measurement.

#### *Measuring of the radial thickness of layer V in primary motor area 6*

Sections (20 μm) were cut between sagittal levels 115–140 (Sidman *et al*., 1971) through the primary motor area 6 of PD 22 mice and stained with Mayer's haematoxylin. Laminar borders of layer V were determined on the basis of general interlaminar differences in cytoarchitecture; lower cell density and presence of relatively high numbers (30–40% of all cells including glial cells) of large (diameters of 18–21 μm) pyramidal cells clearly and sharply distinguishes this layer from the more granular surrounding levels VI and IV. (also, see Rice & Van der Loos, 1977). Approximately 3 mm of neocortex, beginning 1 mm anterior to the border of the lateral ventricle, were divided into three equal radial strips and three measurements of the total thickness of layer V were performed by sweeping the visual field (magnification 40 × 10) radially through the middle of these strips.

At PD 2, the recognition of layer V was more difficult but nevertheless possible; at this stage, about 75% of the radial extent of the developing cortex is composed of layers VI and V, separated clearly from the cortical plate by a transition zone formed by small cells (4–6 μm diameter), the incipient layer IV. In contrast to layer IV, layer V consists mainly of cells varying in size (5–15 μm diameter) with evidently lower density than the surrounding layers IV and VI. The cells in layer VI also vary in size (6–14 μm diameter), nevertheless, about 70% of those are small cells (6–8 μm diameter) that are packed much more densely than those in layer V. The measured distance between the cell-dense layers IV and VI was defined as radial thickness of layer V. Measurements were performed at the level of the anterior border of the hippocampus.

#### *Quantification of BrdU/NeuN-double-positive cells in layer V of motor area 6 in PD 22 mice*

The ratio of double-labelled (BrdU/NeuN) to all NeuN-positive cells (labelling index L.I.%, Fig. 3A) for each experimental interval and animal group was evaluated in immunohistochemically stained material taken from sagittal section levels 115–140 (Sidman *et al*., 1971).

#### *Quantification of the densities of large neuronal (NeuN$^+$) cells in layer V of motor area 6 in PD 2 and PD 22 mice*

Relative cell densities (cells per mm$^2$) of large neurons in PD 22 (diameter >18 μm) and in PD 2 (diameter >10 μm) were obtained across layer V using a reticle eyepiece (magnification 40 × 10) in strips of 0.125 mm width going through the middle of the three radial neocortical areas in PD 22 animals and through the middle of the area frontal to the hippocampus in PD 2 animals as already defined for measurements of the thickness of layer V (see above). Neuronal counts per unit area were corrected for split cell error (Abercrombie, 1946; Clarke, 1992).

## Results

In initial experiments, we looked for gross anatomical differences between the GFAP-L1 transgenic mice and their wild type littermates. As we could not see any obvious changes, we measured the rate of the overall brain growth in both experimental groups. Between ED 18 and PD 3, brains of mice from both experimental groups grew about 0.40 mm per day in length and 0.45 mm in width. No significant difference in brain length (measured from olfactory bulbs to the medullary flexure) or width (maximal distance across both hemispheres) could be detected in any experimental interval between L1-transgenic and wild type mice. This indicated that the ectopic expression of L1 on astrocytes does not interfere with the gross anatomy and overall growth of the brain.

To compare the development of the CST between the two mouse strains, a crystal of DiI was inserted into the posteromedial part of the frontal neocortex, containing motor area 6 (Caviness, 1975) of fixed mouse brains at ED 18, PD 1–3 and PD 22. This procedure labelled a large number of corticospinal axons and allowed us to follow their path through the internal capsule into the cerebral peduncle overlying the basilar pons and into the spinal cord.

We first investigated the development of the CST in wild type C57BL/6 J mice (Fig. 2, left column). At ED 18, the first corticospinal axons had just entered the cerebral peduncle overlying the rostral part of the basilar pons. At birth (PD 0), most axons had passed the pons and reached the pyramidal decussation. At that time, the first collateral branches became visible on axonal segments overlying the caudal part of the basilar pons. At PD 1, corticospinal axons had grown through the medulla and entered the spinal cord about 1.2 mm past the pons (not shown). At this stage, the collateral branches growing into caudal parts of the pons were well elaborated while new collaterals innervating its rostral parts just appeared. At PD 2–3, the mature pattern, characterized by long collaterals into the rostral and caudal parts of the basilar pons, begun to emerge. Both sets of collateral branches were clearly distinct from each other and separated by about 150–200 μm.

We then studied the development of the pontine CST in GFAP-L1 mice (Fig. 2, right column). In these animals, the trajectory of the

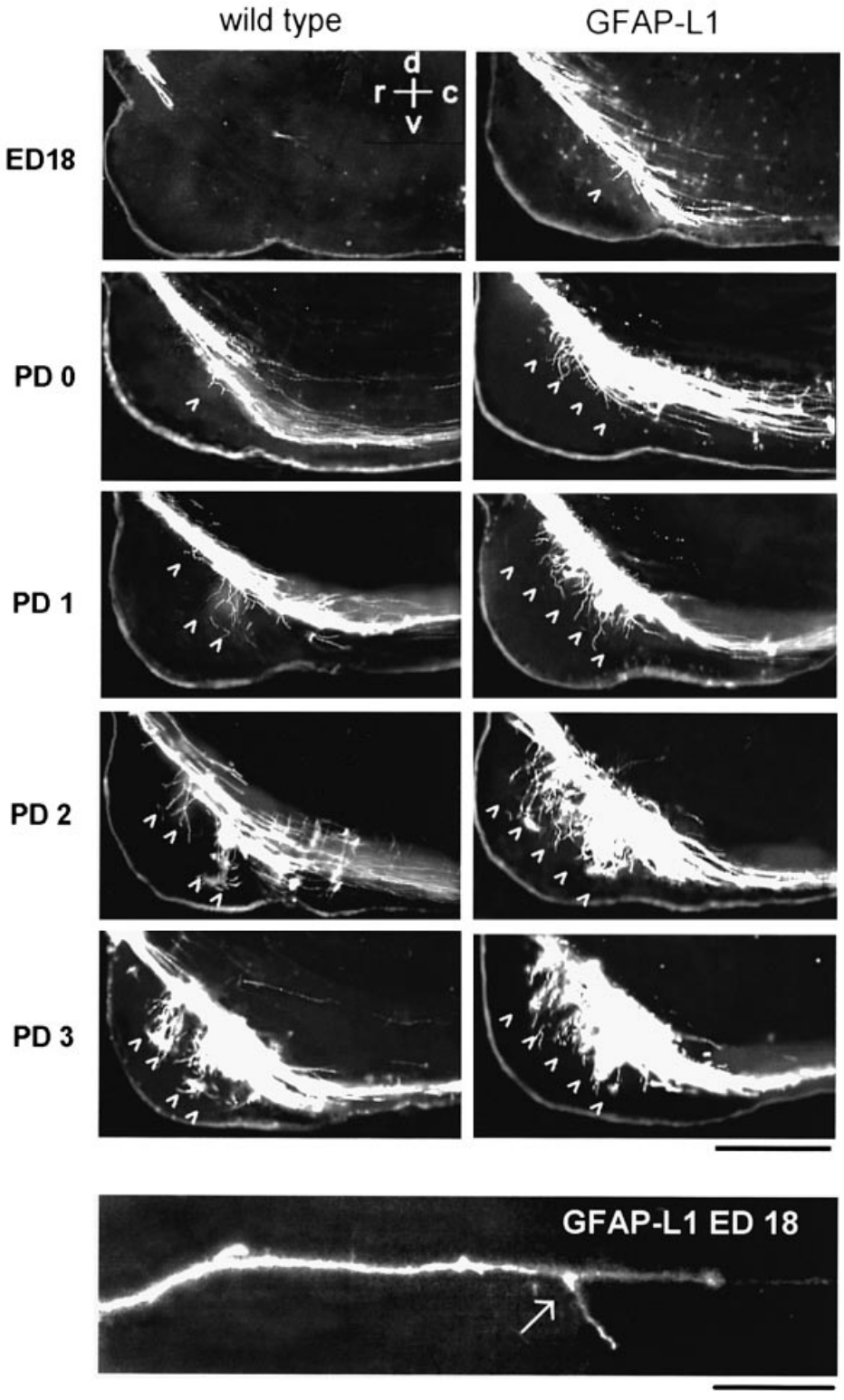


FIG. 2. Development of the corticospinal tract in wild type and GFAP-L1 mice. Sagittal sections through the CST overlying the basilar pons. Corticospinal axons and their collaterals were labelled anterogradely by DiI placed in the sensorimotor cortex of wild type (left column) and GFAP-L1 mice (right column). (Top) At ED 18 first corticospinal axons reached the rostral part of the basilar pons in wild type animals, while they had already passed the pons and formed the first collaterals in the GFAP-L1 transgenics. During further development, long collaterals into the rostral and caudal parts of the basilar pons began to emerge in the wild type mice (arrowheads). In contrast, collateral branches in the trangenics did not start as two distinct sets but in a continuous fashion across the entire region of the tract overlying the basilar pons (arrowheads). r, rostral; c, caudal; d, dorsal; v, ventral; arrowheads, regions of collateral formation. (Bottom) Example of a collateral branch (arrow) arising from an axonal segment overlying the basilar pons in a GFAP-L1 mouse at high magnification. Bars, 200 μm (top) and 20 μm (bottom).

CST in the midbrain and hindbrain was not altered and no pathfinding errors of corticospinal axons were observed, althought the CST in transgenic animals appeared thicker. At PD 2, its cross-section diameter at the level of the inferior olive (Fig. 3A) was slightly but significantly ($P < 0.05$) larger in GFAP-L1 mice ($66720 \pm 4070$ $\mu m^2$) than in wild type animals ($57199 \pm 3495$ $\mu m^2$). The outgrowth of corticospinal axons was shifted to earlier developmental ages in the trangenics (Fig. 2). While at ED 18 the corticospinal axons in wild type animals were just entering the rostral portion of the basilar pons, axons in their GFAP-L1 littermates had, at that time, passed this region and collateral branches were already forming over the caudal part of the basilar pons. The rostral set of corticopontine collaterals appeared as early as PD 0 in the transgenics. That these collaterals are true interstitial branches and not corticospinal axons

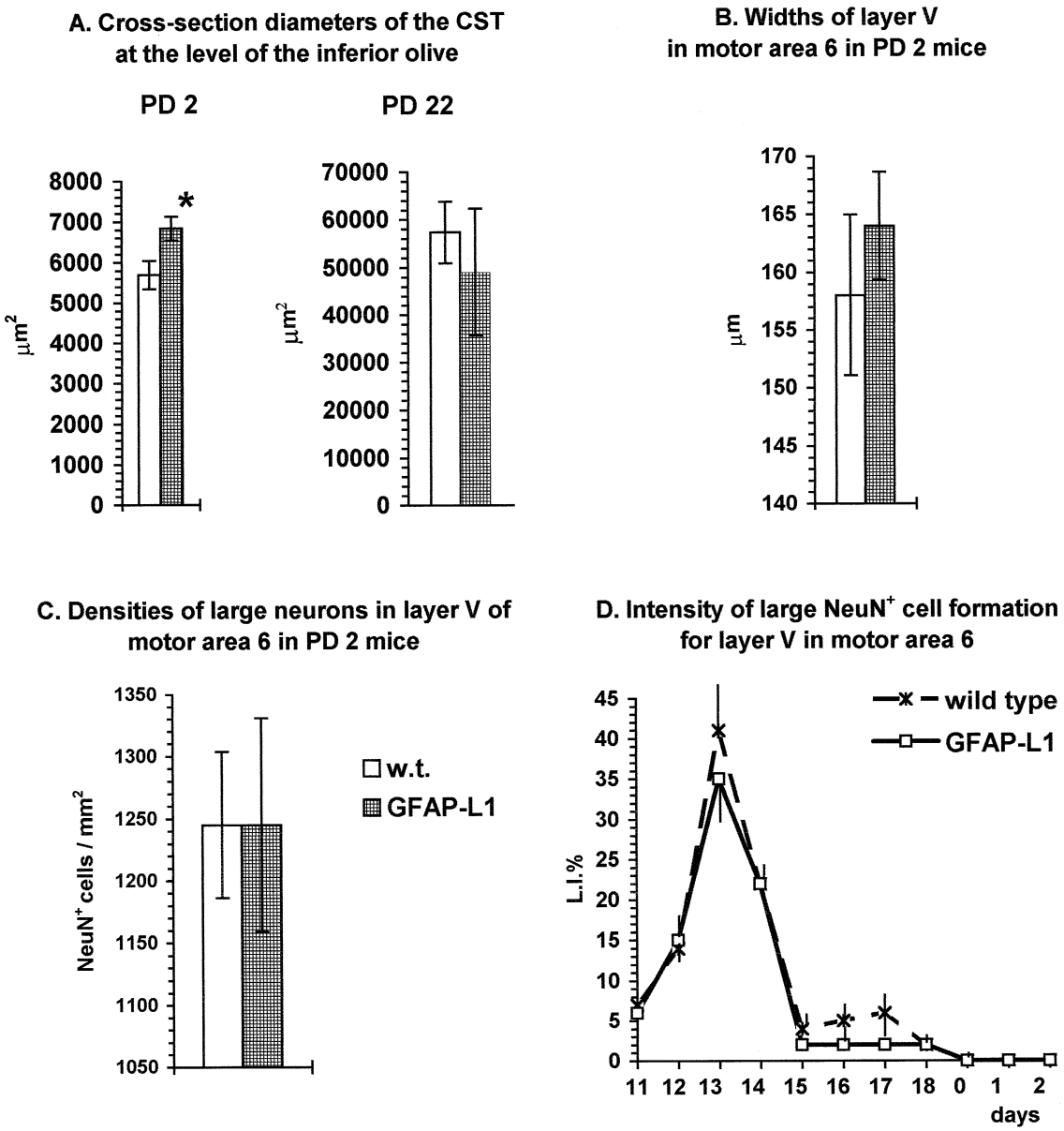


FIG. 3. Quantitative comparisons between wild type and GFAP-L1 mice performed in this study. Measured were the following parameters: cross-section diameter of the CST at PD 2 and PD 22 (A); width of and neuronal density in layer V of primary motor cortex (area 6) at PD 2 (B and C) and the labelling index (L.I.) for large neurons destined for layer V (D). The cross-section diameter of the CST at the level of the inferior olive was slightly but significantly larger in the transgenic mice (*$p < 0.05$). The other parameters were similar in both experimental groups.

that have redirected their growth towards the basilar pons was obvious from the high magnification photographs (Fig. 2, bottom). Collaterals branch off the parental axons at a right angle and are often associated with a varicosity, as described previously (Bastmeyer & O'Leary, 1996). In sharp contrast to the morphology in wild type animals, collateral branches in the transgenic mice did not start out as two distinct rostral and caudal sets. Instead, they branched from the axon segments in a continuous caudorostral direction across the entire region of the CST overlying the basilar pons. At PD 1, the density of these collaterals was higher in transgenic mice and their branches were intensely intertwined. The observed anatomical and developmental differences of the pontine collaterals between the transgenic and wild type mice were even more pronounced at PD 2. Although the formation of the corticopontine projections in the transgenics was altered, we never observed ectopic branches in parts of the tract rostral or caudal to the basilar pons.

One possible reason for the increased thickness of the CST in GFAP-L1 mice during early development could be a larger number of cortical pyramidal neurons in the primary motor cortex. To test this possibility, the density of large neurons in layer V and the width of layer V in motor area 6 were determined in anti-NeuN- and cresyl violet-stained sections from wild type and transgenic animals at PD 2, when these strains displayed a clear difference in CST architecture (Fig. 2). The width of layer V was 164 ± 4.6 μm in trangenics and 158 ± 7.0 μm in wild type animals (Fig. 3B), which is approximately one third of the total width of the sensorimotor cortex at this age. Relative numbers of large NeuN-positive cells (diameter ⩾10 μm) in this layer were also similar in both animal

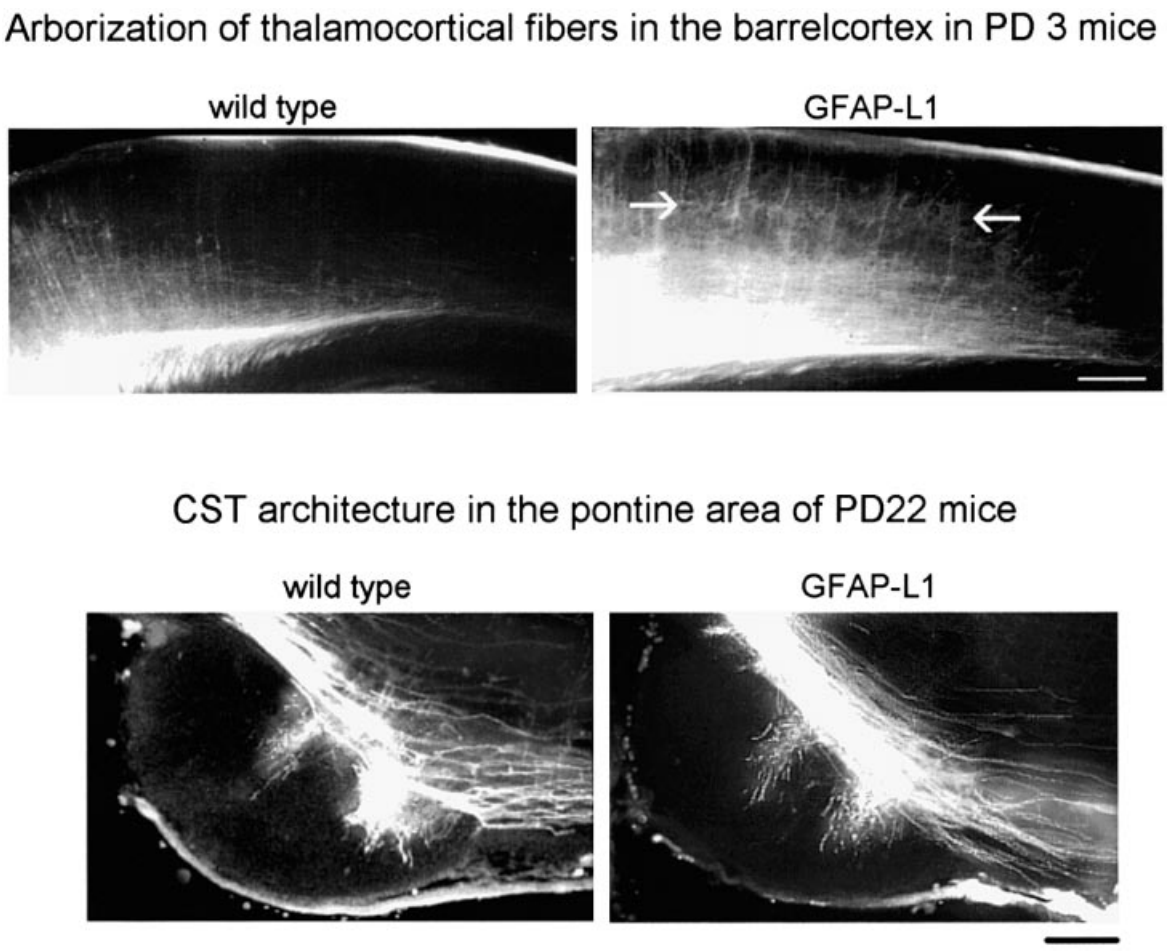


FIG. 4. Arborization of thalamocortical fibres in the barrel cortex and CST architecture in the pontine area. (Top) In additional control experiments, sagittal sections from wild type (left) and L1-transgenic mice (right) were compared with respect to the pattern of arborizing terminals from DiI-labelled thalomocortical axons penetrating the barrel cortex at PD 3. Note the more advanced state in the transgenics with the upper tier of periodic clusters of terminal arbors being already formed in layer IV (lamina between the white arrows), while in the wild type they are just beginning to appear. This observation points towards a more general character of the disturbance of growing fibre tracts by ectopic L1. (Bottom) This comparison shows sagittal sections throught the CST overlying the basilar pons from the above mouse strains at PD 22. Both examples of DiI-labelled corticospinal axons and their collaterals show the typical adult organization characterized by two sets of collaterals innervating rostral and caudal parts of the basilar pons. All initial morphological differences had disappeared which was also true for the barrel cortex, and both were also confirmed in the second transgenic mouse line (data not shown). Bars, 150 μm (top); 100 μm (bottom).

groups with 1245 ± 85.8 cells per $mm^2$ in trangenics and 1245 ± 58.8 cells per $mm^2$ in wildtype animals (Fig. 3C). These data suggest that in both wild type and GFAP-L1 mice the CST originates from the same number of pyramidal neurons and that increased axonal bifurcation and/or an altered degree of axonal fasciculation are likely to be the reasons for its broader appearance in the transgenics.

The earlier appearance of the CST and collaterals innervating the basilar pons in GFAP-L1 mice could reflect either a premature generation of pyramidal neurons or an accelerated neurite outgrowth. To investigate the first possibility, we studied the generation of neurons for layer V in area 6 by BrdU-pulse labelling at different developmental stages (Figs 1B and 3D). Double immunostaining of sections from PD 22 mice showed, that the BrdU/NeuN-double-positive neurons destined for layer V were born between E 11 and E 15 in a similar spatiotemporal program in both animal groups in agreement with other published data (e.g, Polleux *et al*., 1997).

To test, whether other late developing projection systems would also show a similar acceleration of their outgrowth pattern in L1 transgenic mice, we looked at the thalamocortical projection into the barrel cortex of the primary somatosensory neocortical area (Woolsey & Van der Loos, 1970). We inserted a crystal of DiI into the ventral thalamus of fixed brains between PD 0–4 to label thalamocortical axons of the ventrobasal nucleus. During this time, thalamocortical axons arrive in the barrel cortex, branch into layer IV and organize into the characteristic pattern of periodic dense clusters of terminal arborizations (Agmon *et al*., 1993). In wild type animals, thalamocortical axons reached layer IV at PD 0 and started to form a lower tier of terminations at PD 2. By the end of PD 4, the upper tier with the barrel-like fibre condensations became clearly visible. In GFAP-L1 mice, this developmental sequence was accelerated by ≈ 1 day, i.e. the lower tier of terminations in layer IV was already evident at PD 1 and the upper condensations were almost complete at PD 3 (Fig. 4, top). Interestingly, no obvious change in the pattern of these terminal condensations was observed. At PD 22, this fibre system also regained wild type characteristics. These findings suggest that the expression of ectopic L1 *in vivo* has transitory perturbing effects on axonal growth rate of all late developing fibre tracts.

Earlier *in vitro* experiments and histological analysis using immunohistochemistry combined with electron microscopy demon-

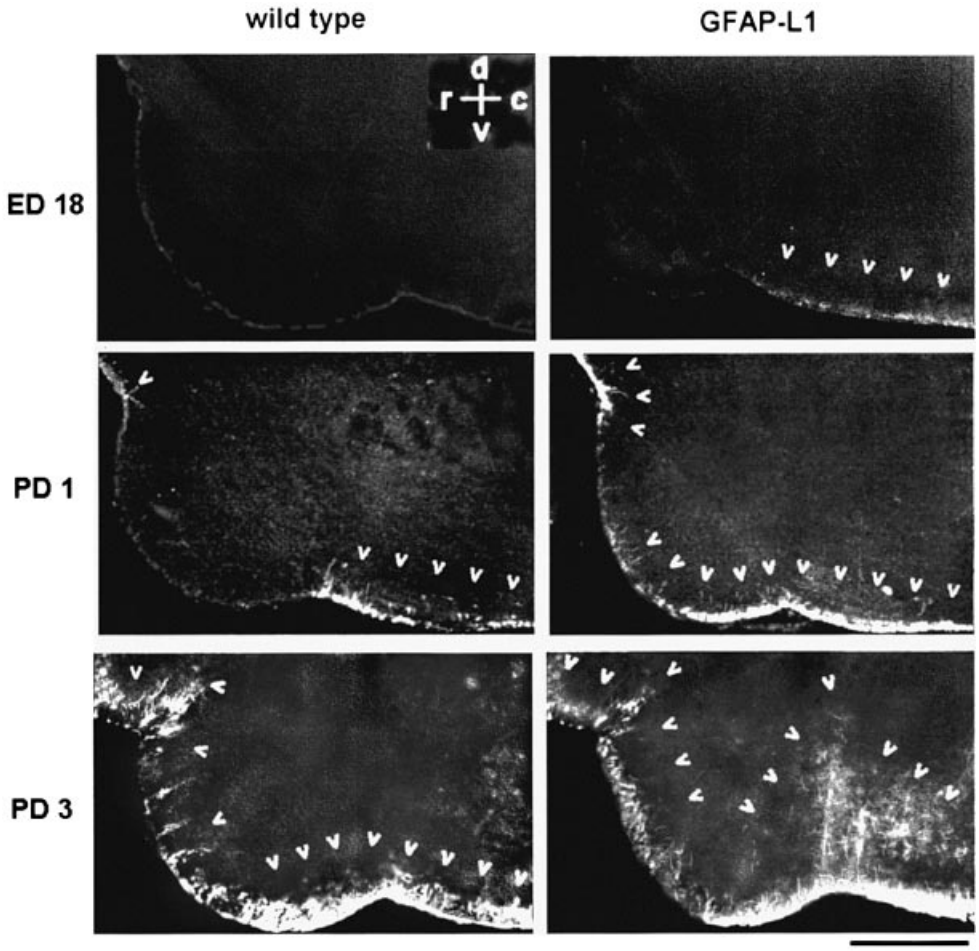


FIG. 5. GFAP expression in wild type and GFAP-L1 mice. Sagittal sections through the area of the basilar pons in wild type (left column) and GFAP-L1 mice (right column) labelled with GFAP-antibodies. First GFAP-immunoreactivity is confined to the pial surface caudal to the basilar pons (arrowheads) and appears at ED 18 in the transgenics and at PD 1 in the wild type mice. During further development, GFAP-immunoreactivity spreads further rostrally and is found at PD 3 along the pial surface and in the pontine grey in both animal groups (arrowheads). r, rostral; c, caudal; d, dorsal; v, ventral. Bar, 200 μm.

strated that astrocytes from GFAP-L1 mice express GFAP and the L1 transgene concomitantly (Mohajeri *et al*., 1996). The abundant expression of endogenous L1 on neurons in the CNS obscures, however, the presence of ectopic L1 on astroglia, making standard anti-L1 immunohistochemistry virtually impossible. Therefore, we chose an indirect way to check for ectopic L1 expression by looking for GFAP expression in astrocytes and by comparing our results in two independent L1-transgenic mouse lines.

Immunostaining in wild type and L1-transgenic mice for GFAP was performed at ED 18, PD 1 and PD 3 to check for possible differences in the differentiation of astrocytes in the region of the CST overlying the basilar pons (Fig. 5). In wild type mice, first GFAP-positive cells were observed at PD 1. This staining was restricted to glial endfeet in close vicinity to the meningeal sheets, caudal to the basilar pons. No staining was observed within the basilar pons or the CST rostral to the pons. In transgenic mice, the first GFAP-positive cells in the area caudal to the pons were already visible at ED 18. At PD 1, GFAP-positive processes were detected not only caudal to but also within and rostral to the basilar pons, again much more pronounced in the GFAP-L1 mice. At PD 3, subpial GFAP-immunoreactivity extended across the entire pontine region and well beyond in both animal groups. However, the signal was stronger and more diffuse, and reached deeper into the pontine grey in GFAP-L1 mice than in their wild type littermates.

We then used a second mouse line overexpressing L1 under the GFAP promoter to verify our findings. Mouse line 3426 was previously defined as a ‘strong L1 expressor’, while line 3427 was described as an "intermediate" L1 expressor (Mohajeri *et al*., 1996). To rule out the possibility that the observations described in this report were due to nonspecific, positional effects of the transgene insertion, line 3427 was used to analyse collateral development in the basilar pons region, the time-course of formation of cortical neurons, GFAP-expression in the pontine region, as well as arborization of thalamocortical axons in the barrelcortex. Like line 3426, line 3427 also showed an enhanced outgrowth and earlier branching of corticospinal axons and an earlier formation of the thalamocortical projection into the barrelcortex at PD 1 and PD 3 (results not shown). This comparison confirms that the described alterations in fibre tract formation were caused by the ectopic expression of L1 on astrocytes and not by nonspecific effects that may have been caused by the insertion of the transgene.

## Discussion

In the present study we investigated the development of the corticospinal tract in brains of transgenic mice which express L1 ectopically on astrocytes. We found that the diameter of the CST is

enlarged and that the outgrowth of corticospinal axons occurs at earlier ages in the transgenics. In addition, corticospinal axons form collateral branches innervating the basilar pons earlier and at ectopic sites as compared to their littermates. The ectopic branches, however, are only transient, and no longer present at PD 22. These phenomena are accompanied by the premature appearance of GFAP-immunoreactive cells in the area of the basilar pons.

It has been shown before (Mohajeri *et al*., 1996) that neurite outgrowth on cryosections of optic nerves or on astrocyte monolayers from the transgenic mice is significantly enhanced compared to similar preparations from nontransgenic littermates. Moreover, increased neurite outgrowth on these substrates could be inhibited by antibodies against L1 (Mohajeri *et al*., 1996). The ectopic expression of L1 on the surface of astrocytes within the pathway of corticospinal axons could, therefore, account for the accelerated growth in the transgenic mice. A similar accelerated outgrowth of thalamocortical axons suggests that not only corticospinal axons but also other late developing axon tracts respond to ectopic L1 expression. As L1 is widely expressed in the developing nervous system and also found on corticospinal axons (Joosten & Gribnau, 1989), we were unable to detect additional L1 on the surface of astrocytes in the transgenic mice by immonocytochemistry. Therefore**,** we used the marker GFAP to determine the distribution of astrocytes in relation to the pathway of corticospinal axons in the brainstem. As described for the rat (Bastmeyer *et al*., 1998), GFAP immunoreactivity first appears in radial glial endfeet in the pyramidal tract, caudal to the basilar pons. In the transgenics, however, this occurs as early as ED 18. Radial glial cells span the whole distance from the pia to the ventricular surface in the brainstem and immature astrocytes are present throughout the entire developing CST (Bastmeyer *et al*., 1998). As the ectopic L1 is likely to be expressed on the processes of radial glia, that begin to express GFAP in their pial endfeet, as well as in the differentiating astrocytes within the tract overlying the basilar pons, it might interact with the growing axons and enhance their rate of outgrowth. Although known to be primarily membrane-bound, L1 may be released by proteolytic activity (Nayeem *et al*., 1999). Soluble L1 has been shown to stimulate neurite outgrowth *in vitro* (Doherty *et al*., 1995) and might, therefore, also contribute to the enhanced growth of corticospinal axons in the transgenics.

The transient increase in the cross-section diameter of the CST may have several reasons: fibre defasciculation, increased formation of collaterals, contribution of ectopic fibres from other projection systems, decreased rate of neuronal cell death in neocortical layer V and increased formation of pyramidal neurons for layer V. The first three mechanisms seem plausible due to the presence of ectopic L1 in the transgenics which could very well exert a disturbing influence on the orderly outgrowth and pathfinding of axons. As we never saw axons branching off the main fibre stream other than those towards the pons and never detected corticospinal fibres attempting to reach ectopic targets, a contribution of ectopic fibres seem to be unlikely. Therefore, we focused on the numbers of large pyramidal neurons present at PD 2 ($NeuN^+$ cells with diameters larger that 10 μm) and their formation in layer V in motor area 6. It has been shown in retrograde tracing experiments using spinal Fluorogold injections that, in the adult, the distribution of large neocortical neurons (diameter 16–21 μm) correlates well with the main population of the large pyramidal cells in layer V which are the origin of the CST (Polleux *et al*., 1998). Again, neither were their number or the layer thickness increased at a time when CST cross-section diameters were larger in the L1 transgenics. From these data it appears most likely that the transient and minor increase in CST thickness in the L1-transgenics is caused by defasciculation of the growing CST axons, resulting from the increased interaction of L1 exposed on their membranes with the ectopic L1 on the surrounding astrocytes. High-resolution tracing experiments, using fast blue or Fluorogold, placed at the pyramidal decussation or into the spinal cord and studies at ultrastructural levels using electron microscopy should provide more compelling proof.

A surprising finding of this study was the earlier appearance of collateral branches along axon segments overlying the basilar pons. Corticospinal axons innervate their targets by a process termed 'interstitial axon branching' (O'Leary & Terashima, 1988). Instead of elaborating an axon that grows into the target, these axons grow past their targets and remain within the axon tract. Subsequently, collateral branches arise *de novo* along the axon shaft well behind the growth cone, exit the axon tract, and innervate their target (Bastmeyer & O'Leary, 1996). In principle, the location of collateral branches along the axon shaft innervating specific targets could be intrinsic to the corticospinal axon itself. In this scenario, the earlier arrival of corticospinal axons in the tract overlying the basilar pons could account for the earlier appearance of collateral branches. Other studies, however, strongly indicate a role for the basilar pons itself in regulating branching (O'Leary *et al*., 1991). *In vitro* assays have shown that pontine tissue releases a chemotropic substance that can attract cortical axons (Heffner *et al*., 1990) and enhance the formation of branches from the shaft of corticospinal axons (Sato *et al*., 1994). The nature of this chemotropic activity is still unknown, but as we did not observe ectopic collateral branches in parts of the tract rostral and caudal to the basilar pons (where the axons are also in contact with radial glial cells and astrocytes and do not form collaterals), it is unlikely that ectopically expressed or soluble L1 accounts for this activity. Alternatively, a chemotropic factor inducing collateral branching might be produced and released by a subpopulation of astrocytes within the basilar pons. As astrocytic differentiation is significantly shifted to earlier developmental stages in the trangenic mice, it is possible that this subpopulation of astrocytes not only expresses L1 but also produces a factor that induces branching at earlier ages. The mechanism underlying the accelerated differentiation of a $GFAP^+$ astrocytic phenotype in the transgenics is unclear, but might involve *cis* interactions of the ectopic L1 molecules with growth factor receptors such as FGF-R and leukaemia inhibitory factor receptor (LIF-R) (Saffell *et al*., 1997; Koblar *et al*., 1998).

Previous analyses of L1-deficient mice (Dahme *et al*., 1997; Cohen *et al*., 1998) have confirmed an essential role for L1 in CST development. These mice have a size reduction of the CST and an altered pyramidal decussation. We did not observe any obvious pathfinding errors in the L1-overexpressing transgenics, suggesting that the ectopic expression of L1 on astrocytes is not involved in guidance of corticospinal axons. However, we did observe a disturbed pattern of collateral branches innervating the basilar pons. Axons originating from motor cortex usually form interstitial branches innervating rostral and caudal parts of the basilar pons (O'Leary & Terashima, 1988). In the rat, the two components are usually separated by a collateral-free axon segment 400–500 μm in length. In the transgenics, however, these collaterals branched off the axon segments continuously in rostrocaudal direction along the entire region of the CST overlying the basilar pons. This abnormality in the transgenics could be explained in two ways: either the basilar pons releases a chemotropic factor not only earlier in development but also at ectopic sites, or the ordered arrangement of corticospinal axons within the tract is altered. The corticopontine projection is topographically organized (Leergaard *et al*., 1995) and axons within the tract are ordered according to their position in the cortex. The

rostro-caudal axis of the cortex is represented in a medio-lateral distribution of axons within the corticospinal tract overlying the basilar pons with the axons originating from motorcortex running closest to the midline. Since the fasciculation pattern of corticospinal axons is altered in the transgenics, the topographic organization within the tract might also be changed and consequently cause branching at ectopic positions. More experiments, using precise mapping of the tract topography with antero and/or retrograde labelling techniques, will be necessary to clarify this point.

In summary, our findings show that ectopically expressed L1 (on the surface of astrocytes) can alter the temporal outgrowth of corticospinal axons, their fasciculation pattern and the topographic organization of collateral branches innervating the basilar pons. That the observed defects are only transient and disappear at later ages suggests that additional mechanisms, like neuronal activity, are involved in establishing the corticopontine projection.

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## Abbreviations

BrdU, 5′-bromodeoxyuridine; CST, corticospinal tract; DAB, diaminobenzidine; DiI, 1,1′-dioctodecyl-3′,3′,3′,3′,-tetramethylindocarbocyanine; ED, embryonic day; GFAP, glial fibrillary acidic protein; (mo)Ab, (monoclonal) antibody; PBS, phosphate buffered saline; PD, postnatal day; RT, room temperature.

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