# Expression of the Transcription Factor, *tailless*, Is Required for Formation of Superficial Cortical Layers

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**The gene *tailless* (*tlx*) encodes a forebrain-restricted transcription factor that is robustly expressed in progenitor cells of the ventricular and subventricular zones during neurogenesis. To investigate the role of *tlx* in neocortical development we generated a targeted deletion of *tlx* by homologous recombination. Here we compared the lamination, connectivity and patterning of cortical regions in adult *tlx*–/– mice and their wild-type littermates. We found first that neocortical thickness is reduced by 20% in mutant animals; most of this reduction is due to a diminution of supragranular layers, while layer I and layers IV through VI are relatively intact cytoarchitecturally. Consistent with this, the cross-sectional area of the corpus callosum is reduced by over 40%. Second, thalamocortical and intrinsic excitatory circuits in *tlx*–/– mice exhibit an essentially normal distribution from layer IV to the white matter, but are reduced superficial to layer IV. Finally, within parietal cortex of mutant mice a vibrissa-like pattern of cortical barrels is present in the expected rostro-caudal location. These observations indicate that loss of *tlx* function most severely affects generation and differentiation of neurons destined for superficial cortical layers. Thus, *tlx* may be important in sustaining the progenitor cell population throughout late prenatal development. Establishment of functional cortical areas, and development of basic patterns of thalamocortical and intracortical circuits occurs independently of *tlx* function.**

## Introduction

Normal functioning of the cerebral cortex requires a carefully orchestrated sequence of developmental events. Appropriate numbers of cortical neurons are generated in the ventricular zone (VZ) and subventricular zone (SVZ) of the embryonic telencephalon, and postmitotic cells migrate to establish distinct cortical layers, where they subsequently differentiate and form precise patterns of synaptic connections. A critical, early step in this process is correct timing of progenitor cell proliferation, which gives rise to neurons that will populate successively more superficial cortical layers (Angevine and Sidman, 1961; Rakic, 1974; Takahashi *et al*., 1993, 1995a,b; Brustle *et al*., 1995; Campbell *et al*., 1995; Fischell, 1995; Temple and Qian, 1995; Levitt *et al*., 1997; Kornack and Rakic, 1998; Haydar *et al*., 2000). Proliferation of progenitor cells is regulated by factors that are both extrinsic and intrinsic to the VZ/SVZ (McConnell and Kaznowski, 1991; Ferri and Levitt, 1995; Eagleson *et al*., 1996; Ferri *et al*., 1996; Frantz and McConnell, 1996; Qian *et al*., 1997; Lillien, 1998). One potential intrinsic factor is encoded by the orphan nuclear receptor gene, *tailless* (*tlx*).

*tlx* is the vertebrate homologue of the *Drosophila tailless* gene, *tll*, and is a member of the orphan nuclear receptor gene superfamily that encodes ligand activated transcription factors (Pignoni *et al*., 1990; Monaghan *et al*., 1995). In *Drosophila*, *tll* initially is expressed in the embryonic termini, where it is critical for normal pattern formation including development of the brain (Pignoni *et al*., 1990). In mouse (Monaghan *et al*., 1995, 1997), rat and chick (Yu *et al*., 1994) and human (Jackson *et al*., 1998) transcription of *tlx* is restricted principally to the telencephalon, diencephalon and associated structures including the retina and olfactory placode (Yu *et al*., 1994; Monaghan *et al*., 1995, 1997). *tlx* expression first can be detected ~8.5 days post conception in the VZ of the embryonic mouse forebrain. Beginning around midgestation *tlx* also is expressed in cells of the SVZ and marginal zone (i.e. future layer I) (Lake *et al*., 2000; Roy *et al*., 2001, 2002).

To investigate the role of *tlx* in development of the vertebrate brain we generated a targeted disruption of the gene by homologous recombination (Monaghan *et al*., 1997). Animals that are homozygous for the disrupted gene (*tlx*–/–) are indistinguishable from their normal littermates at birth and are reproductively competent upon reaching sexual maturity (Monaghan *et al*., 1997). Mutant animals nevertheless display pronounced behavioral abnormalities including severe aggression, stereotypy, altered maternal instincts, late onset epilepsy and reduced learning abilities (Monaghan *et al*., 1997; Roy *et al*., 2002). Consistent with these behavioral observations, preliminary analyses have identified structural alterations in the rhinencephalon of *tlx*–/– animals, which exhibit reductions in size of certain nuclei of the amygdala, the islands of Calleja, the piriform and entorhinal cortices, and the hippocampal dentate gyrus (Monaghan *et al*., 1997).

How loss of *tailless* function affects neocortical development is unknown. Robust expression of *tlx* throughout the dorsal and ventral telencephalic VZ and SVZ from E8.5 until birth suggests a possible role in regulating the generation of cortical neurons, which are born during this time (Angevine and Sidman, 1961; Bayer and Altman, 1991; Levison and Goldman, 1993; Luskin and Boone, 1994; Monaghan *et al*., 1995, 1997). Preliminary comparisons of proliferation and differentiation in neocortex of *tlx*–/– mutant versus wild type embryos support this idea. Thus, embryos lacking *tailless* function exhibit an accelerated proliferation rate from embryonic day (E) 9.5 to E14.5, and an enhanced number of neurons in the marginal zone from E9.5 to E12.5. However, after mid-neurogenesis (i.e. E14.5) the number of progenitor cells is significantly diminished, and their proliferation rate is slower than in wild-type littermates of the same age (Roy *et al*., 2001, 2002) (Roy *et al*., submitted).

To identify effects of *tlx* deletion on cortical development we began a series of experiments aimed at comparing the lamination, connectivity and patterning of cortical regions in adult *tlx*–/– mice and their wild-type littermates. We found that neocortical thickness is reduced by ~20% in mutant animals, primarily due to thinning of superficial cortical layers. Concomitantly, the corpus callosum is severely reduced in volume. Despite reduced cortical thickness, thalamocortical and intrinsic excitatory circuits exhibit relatively normal patterns of distribution. Finally, we observed a vibrissa-like pattern of cortical barrels within the parietal cortex of all mutant mice. These

findings suggest that *tlx* expression is important for sustaining the progenitor cell population throughout late prenatal development. Loss of *tlx* function most severely affects the generation and differentiation of neurons destined for the superficial layers of neocortex. The establishment of particular cortical regions, however, occurs independently of *tlx* function.

## Materials and Methods

### *Animals*

Targeted disruption of the *tlx* gene has been described in detail elsewhere (Monaghan *et al*., 1997). Mating heterozygous 129J/C57Bl/6 mice (ninth generation backcrossed into C57Bl/6) generated homozygous and heterozygous animals in the expected ratio. Offspring of these matings were genotyped by PCR as described previously (Monaghan *et al*., 1997). This study is based upon examination of 18 *tlx*–/– mice and 16 wild-type (i.e. *tlx*+/+). Several heterozygous animals also were examined; the brains of these mice appeared identical to wild-type animals in all respects. Animals were born in our breeding colony at the University of Pittsburgh and were 2–6 months of age when they were killed. The care and handling of animals was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and conformed to NIH guidelines.

### *Histology*

Six wild-type and eight mutant animals were anesthetized with IsoVet (isoflurane; Shering-Plough, Union, NJ) and perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3). Brains were removed, post-fixed overnight then transferred to 30% sucrose in phosphate buffer for cryoprotection. Frozen sections were cut at 50 μm on a sledge microtome and collected in phosphate buffer. Some hemispheres were cut in the coronal plane (i.e. normal to the pial surface). Other hemispheres were flattened by gently pressing them with a microscope slide during the freezing process so that subsequent sections would pass tangential to the pial surface and successively through each cortical layer (Land and Simons, 1985). A 1:2 series of sections was mounted onto chrome-alum gelatinized slides, stained for Nissl with 0.1% thionin, dehydrated in an ascending series of ethanol, cleared in xylene and coverslipped with Permount (Histologic Mounting Media, Fisher Scientific, FairLawn, NJ).

An adjacent series of sections was stained for cytochrome oxidase (CO) activity to reveal the metabolic architecture of the cortex, and in particular to mark the location and arrangement of barrel-like structures denoting the somatosensory cortical area (Woolsey and van der Loos, 1970; Welker and Woolsey, 1974). Briefly, sections were rinsed in 0.1 M phosphate buffer (3 × 5 min) and incubated at 38°C in a CO reaction mixture described previously (Wong-Riley, 1979; Land and Simons, 1985). The CO reaction was monitored visually and terminated by transferring the sections to chilled 0.1 M buffer, after which they were rinsed in buffer, mounted onto slides, dehydrated and coverslipped.

### *Immunohistochemistry*

An additional six wild-type and six mutant mice were perfused as described above. Thirty micron thick coronal sections through the cortices of four animals (two mice of each genotype) were stained immunohistochemically for vesicular glutamate transporter 2 (VGLUT2) (Synaptic Systems, Gottingen, Germany) diluted 1:20 000. Within the neocortex VGLUT2 (previously identified as 'differentiation-associated $Na^+$-dependent inorganic phosphate transporter'; DNPI) (Aihara *et al*., 2000) is localized to the synaptic vesicles of thalamocortical axon terminals, thus marking their location (Fujiyama *et al*., 2001). The pattern of VGLUT2 immunoreactivity therefore provides insight into the overall inter- and intralaminar distribution of thalamocortical axons in wild-type and mutant cortices. To assess possible layer-specific effects of *tailless* deletion, cortices from eight mice (four of each genotype) were sectioned at 30 μm in the sagittal plane. Sections in this plane were stained immunohistochemically with the monoclonal antibody SMI-32 (Sternberger Monoclonals, Lutherville, MD) diluted 1:1000 or with antibodies against calbindin D-28k (Sigma-Aldrich, St Louis, MO) diluted 1:200. SMI-32 recognizes nonphosphorylated epitopes on neurofilament protein (Sternberger and Sternberger, 1983) and is enriched in certain populations of large cortical pyramidal neurons, especially in layer V (van der Gucht *et al*., 2001); calbindin D-28k is a calcium binding protein that, in rodents, is predominantly localized to a subpopulation of cortical nonpyramidal neurons and small pyramidal neurons in layers II/III (Hof *et al*., 1999).

From each brain a 1:3 series of sections was washed in 0.05 M Tris buffered saline (TBS; pH 7.3; 3 × 10 min) and preincubated for 1 h at room temperature in blocking serum consisting of 0.05 M TBS containing 10% normal goat serum, 1% BSA and 0.4% Triton X-100. Sections then were incubated for 60 h at 4°C in primary antibody diluted in blocking serum. Subsequently they were washed in 0.05 M Tris buffer, and processed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. After rinsing in buffer, sections were incubated in 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB) with 0.01% $H_2O_2$. DAB stained sections were washed in buffer, mounted onto slides, dehydrated and coverslipped.

### *Zinc Histochemistry*

Cortices from four wild-type and four mutant mice were stained histochemically for synaptic zinc. Zinc histochemistry reveals a discrete population of glutamatergic, presumably excitatory synaptic terminals that are distinguished by the presence of zinc, along with glutamate, within their synaptic vesicles (Frederickson, 1989; Beaulieu *et al*., 1992; Frederickson *et al*., 2000). Zinc-containing circuits arise from intrinsic cortical neurons and are distributed heterogeneously among the cortical layers in a highly conserved pattern (Slomianka *et al*., 1990; Garret and Slomianka, 1992; Dyck and Cynader, 1993; Dyck *et al*., 1993; Land, 1995; Casanovas-Aguilar *et al*., 1998; Land and Akhtar, 1999). These circuits thus provide a separate index of cortical lamination for comparison with Nissl- and VGLUT2- stained specimens.

Histochemical localization of synaptic zinc was assessed using the Danscher method (Danscher, 1982). Mice received an intraperitoneal injection of the zinc chelator sodium selenite (20 mg/kg) from a freshly prepared stock solution (20 mg/ml in distilled $H_2O$) and were anesthetized with IsoVet and killed by decapitation 1 h later. The brain was quickly removed, encased in OCT embedding medium (Miles, Elkhart, IN) and rapidly frozen by surrounding it with crushed dry ice. Twenty-micron coronal sections were cut on a cryostat, collected onto warm (38°C) microscope slides and stored with a desiccant at –40°C until they were processed. One series of sections from each hemisphere was stained histochemically for synaptic zinc according to a modification of the Danscher method (Dyck *et al*., 1993). Briefly, slides were thawed, allowed to dry at room temperature, and fixed and hydrated in a descending series of ethanol (i.e. 95%, 15 min, 70%, 50%; 2 min each), after which they were rinsed in distilled $H_2O$ (3 × 2 min). Rinsed slides were dipped in 0.5% gelatin and dried with a hair dryer. The gelatin-coated slides were immersed in a physical developer prepared from 50% Acacia Gum (120 ml), 2.0 M sodium citrate buffer (20 ml), 0.5 M hydroquinone (30 ml) and 37 mM silver lactate (30 ml). The latter two reagents were added to the incubation medium in the dark. The slides were incubated at room temperature in the dark for ~120 min with agitation. After development, the slides were washed for 30 min in warm (40°C) running tap water in order to remove gelatin and any adhering precipitates, then rinsed in distilled $H_2O$ (3 × 2 min). The slides were fixed in 5% sodium thiosulfate for 12 min, rinsed in distilled $H_2O$ (4 × 2 min) and postfixed in 70% alcohol for 30 min. The sections then were counterstained with 0.1% thionin or were further dehydrated and coverslipped. Occasional series of adjacent sections were stained for Nissl only or for CO as described above.

### *Quantitative Measurements*

It became obvious early in this study that the mutant cortices were considerably thinner than those of wild-type mice. Regional and laminar specificity of reduced cortical thickness were evaluated quantitatively. To do this we measured the thickness of cortical layers along a radial line extending from the pial surface to the white matter in the frontal, parietal and occipital regions of mutant and wild-type animals. Because mutant cortices are somewhat smaller in overall dimensions than those of wild-type mice and because they have enlarged lateral ventricles (Fig. 1) (Monaghan *et al*., 1997) we could not assume that stereotaxic location would permit sampling of homologous cortical regions in the two groups.

Thus, the measured portions of particular cortical areas were selected as follows from coronal sections. Frontal cortex was defined as a relatively agranular region of cortex immediately lateral to the cingulum bundle at the level of the septal nuclei. In normal mice this corresponds to area 6 of motor cortex (Caviness, 1975). Parietal cortex was defined as a region of granular cortex on the lateral convexity of the hemisphere at the level of the anterior thalamus. In each case measurements were made through the center of one of the cortical barrels, which were plainly visible in layer IV of primary somatosensory cortex (area 3) of mutant and wild-type mice (e.g. see Figs 2, 6 and 7). Occipital cortex was defined as a region of granular cortex caudal to the splenium of the corpus callosum and lateral to its forceps major. We took this to be primary visual cortex (area 17) (Caviness, 1975). The mean value of each of these measures in the mutants ($n$ = 7) was compared with controls ($n$ = 5) and the significance evaluated with $t$-tests.

To investigate a potential effect of reduced cortical thickness on interhemispheric connections, we measured the cross-sectional area of the corpus callosum in sagittal sections from four wild-type and four mutant mice. To avoid possible gender-related differences in callosal area (DeLacoste-Utamsing and Holloway, 1982; Kim and Juraska, 1997) all eight mice used in these analyses were females. We used Neurolucida (Microbrightfield, Inc., Williston, VT) to trace the perimeter of the corpus callosum in the first full parasagittal section through one hemisphere of each mouse (usually ~60–90 µm from the mid-sagittal plane). We then determined the cross-sectional area of each corpus callosum using NeuroExplorer (Microbrightfield) and exported the data into a spreadsheet where we calculated the means and standard deviations for each strain. An unpaired $t$-test was used to compare the resulting values.

## Results

In mice lacking the transcription factor *tailless* the cerebral hemispheres and olfactory bulbs are visibly reduced in size while major components of the brainstem and the cerebellum appear relatively normal (Fig. 1). Previous studies noted that specific regions of the limbic forebrain in *tlx* mutants are smaller than in wild-type littermates, due at least in part to reduction in the number of constituent neurons (Monaghan *et al.*, 1997). Because neurons destined to form neocortex also are generated during the period when *tlx* normally is expressed in the VZ and SVZ we investigated here whether loss of *tlx* function leads to abnormalities in neocortical development in addition to the conspicuous defects in limbic structures.

### *Cytoarchitecture of tlx–/– Neocortex*

The neocortex of *tlx*–/– mice is reduced in thickness compared with wild-type littermates (Fig. 2). It nevertheless is possible to identify individual cortical layers in Nissl-stained sections from mutant mice based upon cell size and packing density. Indeed, the overall inside-out pattern of lamination in *tlx*–/– mice is relatively normal. Compared with the cortical cytoarchitecture of wild-type littermates, however, mutant cortices display a marked attenuation of superficial layers, in particular layers II/III.

Figure 2 illustrates the cytoarchitecture of wild-type and mutant littermates in frontal, parietal and occipital cortical regions. In each location, layer I and layers IV through VI in *tlx*–/– mutants are similar in organization to the pattern observed in wild-type mice. This is particularly evident in the parietal region where barrel-like structures demarcate layer IV (e.g. arrowheads in Fig. 2; see also below) and where layer V is seen to consist of several relatively distinct sublayers.

To quantify the effect of *tlx* deletion on cortical lamination we first measured total cortical thickness (i.e. measured from the pial surface to the white matter) in Nissl-stained sections through frontal, parietal and occipital cortex from five wild-type and seven mutant mice (Materials and Methods). Consistent with qualitative evaluation, the mean total cortical thickness in the mutant mice was significantly reduced compared with wild-type mice in the frontal ($P$ = 0.003), parietal ($P$ < 0.001) and occipital ($P$ = 0.006) regions. On average a particular cortical region in mutant mice is ~80% of normal thickness (frontal, 79.9%; parietal, 80.4%; occipital, 78.6%) (Fig. 3, full columns).

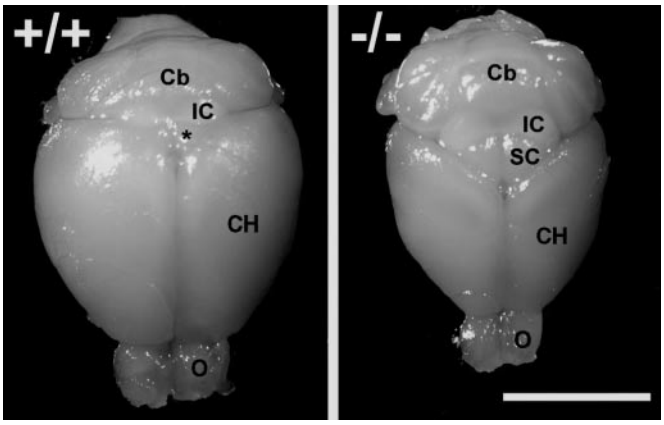


**Figure 1.** Deletion of *tailless* principally affects development of telencephalon. The cerebral hemispheres (CH) and olfactory bulbs (O) of *tailless* mutant mice (–/–) are visibly reduced in size compared with wild-type littermates (+/+). The gross morphology of subcortical structures including the brainstem and cerebellum (Cb) is relatively unaffected. IC = inferior colliculus, SC = superior colliculus, * = caudo-medial aspect of superior colliculus visible between cerebral hemispheres of wild-type mice. Bar = 1 cm.

We then considered separately the superficial versus deep cortical layers (i.e. layers I through IV vs layers V and VI). We chose this parcellation of cortical layers for these measurements because the layer IV/layer V border could be confidently identified in Nissl-stained material from all cortical areas in both strains. We found the thickness of the superficial layers of mutant mice to be significantly reduced compared with wild-type littermates in all regions (frontal $P$ = 0.007, parietal $P$ = 0.003 and occipital $P$ = 0.006, respectively). In contrast, the thickness of the infragranular layers in mutant mice was similar to controls in both the parietal ($P$ = 0.79) and occipital ($P$ = 0.15) regions (e.g. Figure 3, cross-hatched). In the frontal region, however, the thickness of the infragranular layers was significantly less than controls ($P$ = 0.02). The latter finding could indicate a more severe influence of *tlx* deletion on development of frontal cortex, whose neurons are generated earliest in the rostro-caudal wave of cortical neurogenesis. It may, on the other hand, reflect a shortcoming in our criteria for routinely identifying in mutant animals a frontal cortical area for quantitative study (i.e. see Materials and Methods). Nevertheless, these findings suggest that *tlx* deletion results in a decrease in cortical thickness that is expressed throughout the neocortical mantle. The data indicate, further, that much of the decrease in cortical thickness is due to attenuation of superficial layers.

### *Distribution of Cell-type Specific Molecules*

The cytoarchitectural analyses suggest that deep cortical layers in *tlx*–/– mice are less severely affected than superficial ones, which appear to be reduced in thickness. To further investigate a possible layer-specific effect of *tailless* deletion on cortical development we examined the expression of two molecules, SMI-32 and calbindin D-28k, which are predominantly localized to neurons in layer V and layer II/III, respectively. In both mouse strains SMI-32 immunostaining reveals a population of large pyramidal neurons (Fig. 4; left panels). Stained neuronal somata are especially prominent in layer V (white arrows), and their

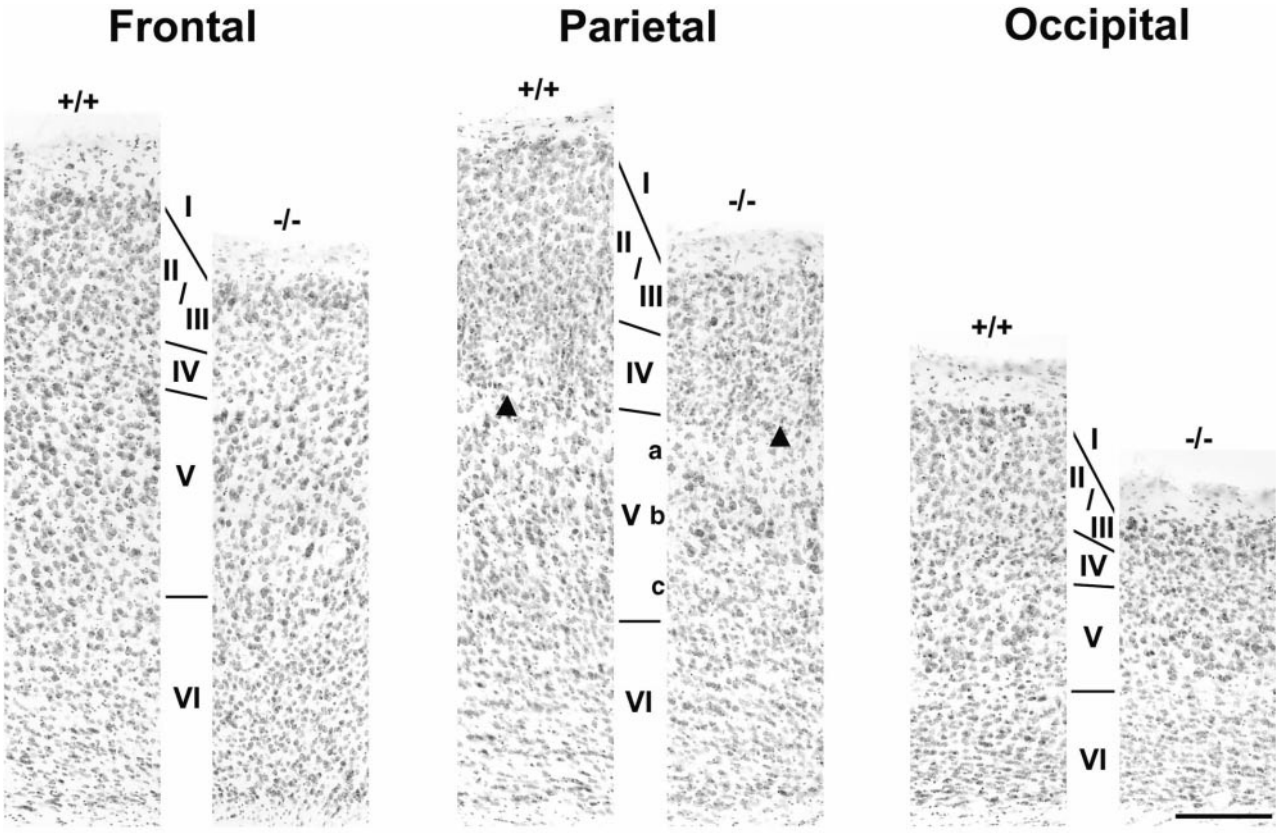


**Figure 2.** Neocortex in *tlx–/–* mice is normally laminated but thinner than in wild-type mice. Panels show pairs of Nissl-stained coronal sections through frontal, parietal and occipital cortical regions of wild-type (+/+) and mutant (–/–) mice. Cortical layers as deduced from cell size and packing density are indicated by Roman numerals I through VI. Individual layers, and in some cases sublayers (e.g. layers Va, Vb and Vc in parietal cortex), are readily recognized in mutant mice. Each cortical region of mutant mice, however, is distinguished by a reduction in overall cortical thickness due principally to abnormally thin layers II/III. Arrowheads = cortical barrel in layer IV of parietal somatosensory region. Bar = 200 μm.

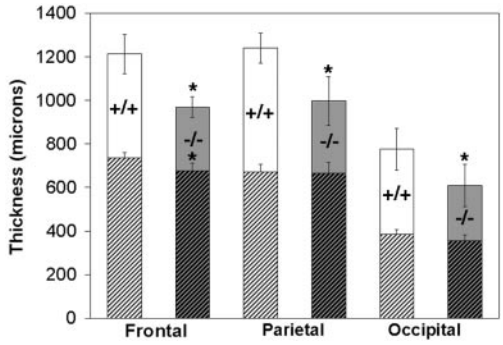


**Figure 3.** Loss of *tailless* function severely affects thickness of superficial cortical layers. Chart compares overall cortical thickness (full columns) or thickness of infragranular layers only (hatched region) in frontal, parietal and occipital cortex of wild-type (+/+; $n = 5$) and mutant (–/–; $n = 7$) mice. Each cortical region in mutant mice is ~80% of the thickness of the corresponding region in wild-type mice (frontal = 79.9%; parietal = 80.4%; occipital = 78.6%). An asterisk indicates that total cortical thickness is significantly thinner than in the corresponding region of wild-type animals (frontal, $P = 0.003$; parietal, $P < 0.001$; occipital, $P = 0.006$). Thickness of the infragranular layers (i.e. layers V and VI) in mutant mice is similar to that observed in wild-type mice in parietal ($P = 0.79$) and occipital regions ($P = 0.15$), but thinner in the frontal region ($P = 0.02$). Bars denote standard deviation.

thick apical dendrites (e.g. black arrowheads) are seen to pass radially through more superficial cortical layers. Thus, neurons in cytoarchitecturally defined layer V of mutant mice can acquire a molecular phenotype and structural characteristics typical for cells in layer V of wild-type mice.

In wild-type mice calbindin D-28k-immunoreactive neurons are scattered diffusely throughout the cortical layers (Fig. 4; right panels; +/+). Stained neurons are quite numerous, however, in layers II/III, where they form a rather uniform stratum that spans the thickness of those layers. A similar pattern is observed in mutant cortex (Fig. 4; –/–). Notably, as predicted by the above cytoarchitectural analyses, the superficial tier of calbindin-immunoreactive neurons is severely attenuated in *tlx–/–* mice. Together these findings indicate that diverse neuronal phenotypes are generated in *tlx–/–* cortex, but mutant mice lack the ability to fully populate superficial cortical layers.

### *Area of the Corpus Callosum*

Late-generated cortical neurons, particularly pyramidal cells residing in layers II/III, are a principal source of interhemispheric axons (Wise and Jones, 1976; Jensen and Altman, 1982); see also (Schwartz and Goldman-Rakic, 1991). The apparent diminution of the superficial layers in *tlx–/–* mice therefore could reduce the overall size of commissural projections. We tested this hypothesis by measuring the cross-sectional area of the corpus callosum in sagitally sectioned hemispheres from four wild-type mice and four mutant mice. In wild-type mice the corpus callosum encompasses 1.992 ± 0.108 $mm^2$, whereas in *tlx–/–* mice the area is reduced to 1.146 ± 0.112 $mm^2$ (Fig. 5). This 42.5% reduction in cross-sectional area is highly significant ($P < 0.0001$). The precise magnitude of axonal loss awaits more detailed ultrastructural studies that are beyond the scope of the present investigation. It nevertheless seems likely that interhemispheric (and perhaps intrahemispheric) projections are

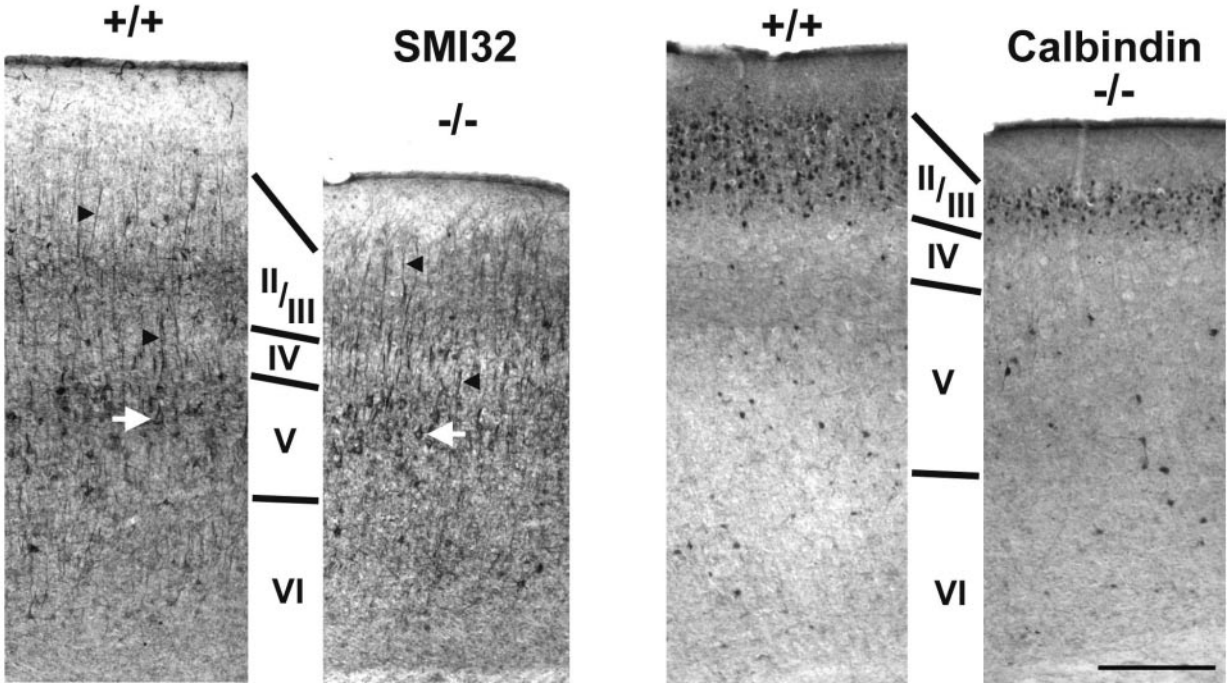


**Figure 4.** Molecular markers highlight layer-specific effects of *tailless* deletion. Panels show pairs of sagittal sections through parietal cortex of wild-type (+/+) and mutant (–/–) mice stained immunohistochemically for SMI-32 (left pair of panels) or for calbindin D-28k (right pair of panels). Large pyramidal cells immunoreactive for SMI-32 are present in deep layers (i.e. layer V) of both mouse strains (e.g. white arrows). Note also that these neurons support prominent apical dendrites (e.g. black arrowheads), exhibiting a relatively normal morphology in *tlx*–/– compared with wild-type cortex. Calbindin-D28k-immunoreactive neurons (right panels) are scattered diffusely throughout deep cortical layers. Their numbers are especially enriched, however, in a superficial stratum that coincides with cytoarchitectonically defined layers II/III. Note that calbindin-immunoreactive neurons in *tlx*–/– mice are distributed in a pattern resembling that observed in wild-type mice, except that the superficial stratum of these cells is abnormally thin. Cortical layers as deduced from nearby sections stained with thionin are indicated by Roman numerals. Bar = 300 μm for all panels.

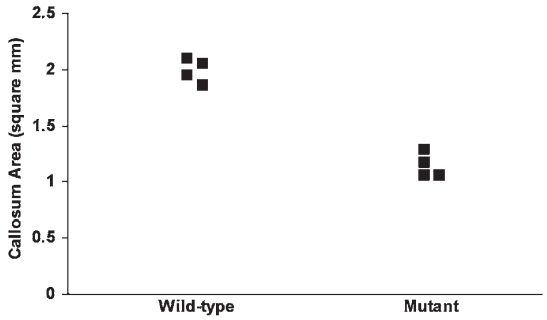


**Figure 5.** Corpus callosum is significantly reduced in *tailless* mutants. Scatter-plot shows cross-sectional area of the corpus callosum from four wild-type and four mutant female mice. Several data points are shifted to the right for clarity. Mean callosal area in wild-type mice is significantly larger than in mutant mice ($1.992 \pm 0.108$ mm$^2$ vs $1.146 \pm 0.112$ mm$^2$; $P < 0.0001$).

substantially compromised in *tailless* mutant mice, commensurate with the reduction in size of superficial cortical layers.

### *Distribution of Cortical Circuits in tlx–/– Mice*

The laminar organization of cortical neurons is reflected in distinct patterns of afferent and intrinsic connections that they receive. This is thought to result from layer-specific expression of molecular cues by cortical neurons that regulate the growth and branching of axons during cortical development (Castellani and Bolz, 1997; Mann *et al*., 2002). We used markers for specific axonal systems to evaluate the influence of *tlx* function on establishment of extrinsic and intrinsic cortical circuits.

The thalamus provides the major afferent input to the cortex. We used antibodies to VGLUT2, a glutamate transporter that specifically labels thalamic axon terminals (Fujiyama *et al*., 2001), to visualize the overall distribution pattern of thalamic projections onto wild-type and mutant cortex. Figure 6 (left panels) shows the pattern of VGLUT2 immunoreactivity in the parietal cortex of wild-type (+/+) and mutant (–/–) mice. As evidenced by the density of VGLUT2-immunoreactive puncta, thalamic axons project heavily onto layer IV, in this case onto a somatosensory cortical barrel, with sparse terminations in the supragranular layers (i.e. layers II/III) and at the layer V/VI border. The overall distribution pattern of thalamic terminals visualized by VGLUT2 staining is virtually identical in normal and mutant mice except that cortex superficial to layer IV is much thinner in *tlx*–/– mice.

To evaluate the distribution of intrinsic cortical circuits we used histochemistry to localize a prominent subclass of glutamatergic axon terminals that also contain zinc within their synaptic vesicles (Beaulieu *et al*., 1992). Zinc-sequestering synapses arise from pyramidal neurons in layers II/III and VI, and are distributed heterogeneously among the cortical layers (Slomianka *et al*., 1990; Garret and Slomianka, 1992; Dyck and Cynader, 1993; Dyck *et al*., 1993; Land, 1995; Casanovas-Aguilar *et al*., 1998; Land and Akhtar, 1999). In *tlx*–/– mice the distribution pattern of zinc-containing circuits is strikingly similar to that observed in wild-type mice. Figure 6 (right panels) shows the distribution of synaptic zinc in the parietal somatosensory cortex. In both *tlx*+/+ and *tlx*–/– mice synaptic zinc is densest in layers II/III and upper layer V, intermediate in density in layer VI and extremely light in layer IV, denoted here by a cortical barrel. The distribution pattern of zinc-containing terminals thus is complementary to that of VGLUT2-stained puncta. As noted

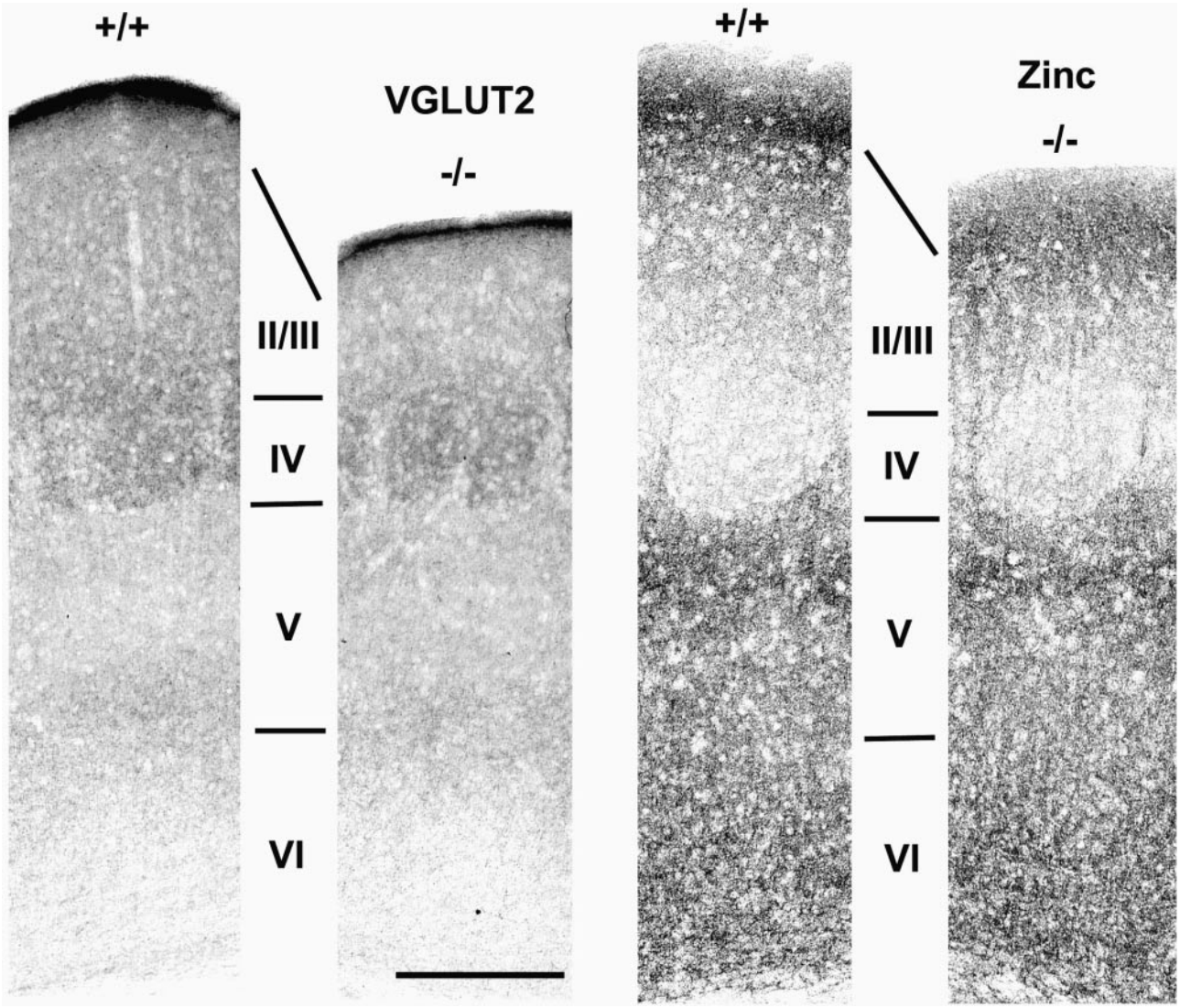


**Figure 6.** Cortical circuits reflect cortical cytoarchitecture. Panels show pairs of coronal sections through barrel cortex of wild-type (+/+) and mutant (–/–) mice stained immunohistochemically for VGLUT2 (left pair of panels) or for synaptic zinc (right pair of panels). Each panel is centered on a layer IV barrel as determined from adjacent Nissl-stained sections. Note the complementary density and distribution of axon terminals stained by these two markers. Also note comparable staining patterns in wild-type and mutant cortex for layers IV through VI, and the attenuated thickness of layers II/III. Bar = 300 μm for all panels.

above for VGLUT2 immunoreactivity, a major distinguishing feature of wild-type vs mutant mice is the thickness of cortical tissue superficial to layer IV. Thus, the overall patterns of afferent and intrinsic cortical circuits match the cortical cytoarchitecture in mutant animals, being similar to wild-type from approximately layer IV to the white matter.

### *Specification of Cortical Maps in tlx–/– Mice*

The neocortex normally contains multiple cytoarchitectonically distinct regions that subserve particular functions. Because the overall size of the cerebrum is reduced in *tlx*–/– mice we wondered whether functional cortical areas might be missing and/or displaced. To obtain clues as to area fate in *tlx*–/– mice we prepared flattened sections through several wild-type and mutant cortices and stained them with cytochrome oxidase. We focused on the somatosensory cortical region that, in rodents, contains a discrete map of the contralateral whisker pad (Woolsey and van der Loos, 1970; Welker and Woolsey, 1974). Whisker-related cortical modules (i.e. the barrels) normally are arranged in a pattern that is homeomorphic with the distribution facial whiskers (Woolsey and van der Loos, 1970), and the 'barrelfield' occupies a central position in the cortical mantle (Woolsey and van der Loos, 1970).

Figure 7*A* illustrates a single section through layer IV of mutant cortex that was stained for cytochrome oxidase. As we observed in all mutant animals, a distinct barrel pattern is visible in the expected location mid-way along the rostro-caudal axis of the cortical sheet. This suggests the (as yet untested) possibility that other functional areas occupy regions of cortex both rostral and caudal to the barrelfield as they do in wild-type mice. The barrelfield from this specimen is shown at higher magnification in Figure 7*B*. For comparison, the barrelfield from a wild-type littermate is shown at the same magnification in Figure 7*C*. Note that the overall arrangement of barrel rows and arcs – a reflection of thalamocortical axon distribution – is similar to that observed in wild-type animals. Thus, it seems likely that loss of *tlx* function does not severely disrupt the partitioning of the neocortex into species-specific area maps.

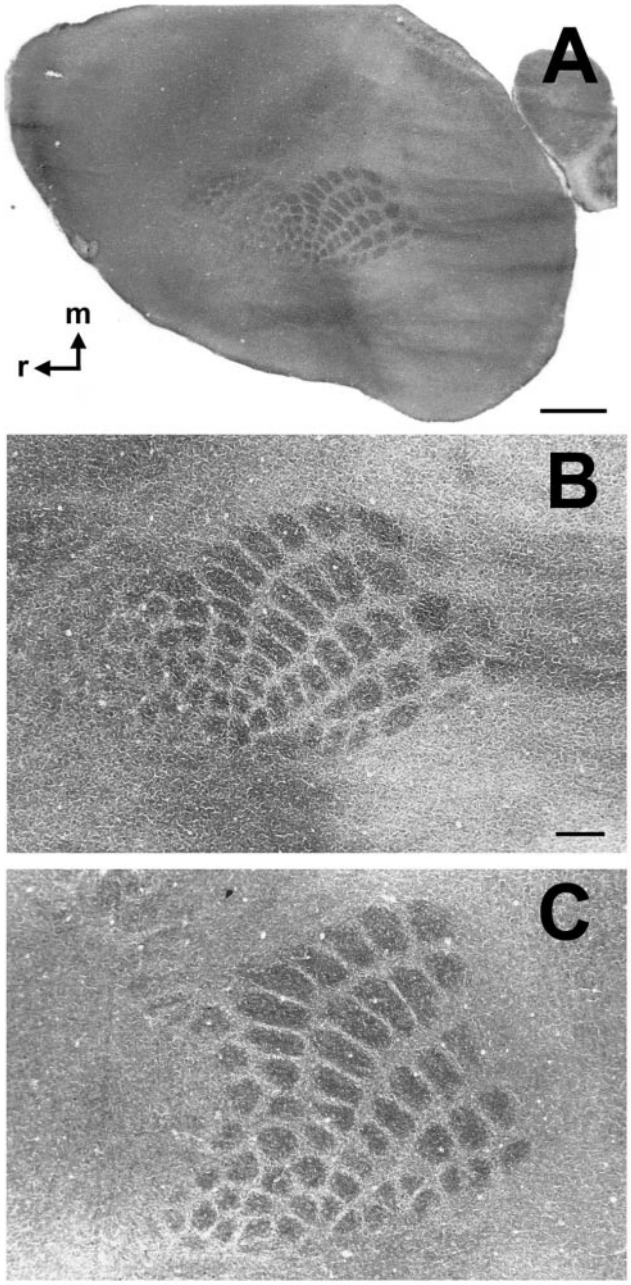


**Figure 7.** Area maps develop normally in *tlx–/–* mice. Panels show single tangential sections through cortical layer IV stained for cytochrome oxidase (CO). (*A*) CO-stained section from *tlx–/–* mouse. Patches of dark CO reactivity denoting somatosensory cortical barrels occupy a central location along the rostrocaudal axis of the neocortex, as they do in wild-type animals. Higher magnification of this barrelfield is shown in *B*. (*B*, *C*) Higher magnification photomicrographs of mutant (*B*) and wild-type barrelfield (*C*). Note that the pattern of barrel rows and arcs is similar in the two mouse lines. Weathervane in *A* points rostrally (r) and medially (m). Bar in *A* = 1 mm; bar in *B* = 300 μm (also applies to *C*).

## Discussion

Our main finding is that the lack of *tailless* function during embryogenesis results in abnormal thinning of the cortical sheet, layers II/III being more severely affected than the deep layers or layer I. The overall distribution pattern of afferent and intrinsic cortical circuits, and the development of cortical area maps in *tlx–/–* mice are relatively normal. Thus, *tlx* deletion affects development of neurons destined for superficial cortical layers but does not seem to alter neuronal migration and differentiation, or the spatial patterning of the developing cortex.

### *Loss of tlx Function Affects Development of Superficial Cortical Layers*

The basic inside-out pattern of cortical lamination is preserved in *tlx–/–* mice, indicating that *tlx* function is not required for radial neuronal migration *per se*. Mutant cortices, however, are ~20% thinner than those of wild-type mice. Quantitative analyses reveal that most of the reduced cortical depth in *tlx–/–* mice is due to attenuation of superficial cell layers. In Nissl-stained sections from all cortical regions of *tlx–/–* mice it is apparent that layers II/III, in particular, are diminished in thickness. This is supported by our observation that a superficial stratum of neurons immunoreactive for calbindin D-28k, which normally marks a population of small pyramidal and non-pyramidal cells in layers II/III (Hof *et al*., 1999), is substantially narrower in *tlx–/–* mice compared with wild-type mice. The extent to which the composition of layer IV (vs II/III) also is affected in mutant animals is less obvious in the absence of a layer-specific marker. However, specialized structures like somatosensory cortical barrels are present in layer IV of all mutant animals (e.g. Figures 2, 6 and 7), suggesting that layer IV may be somewhat less affected by loss of *tailless* function than layers II/III. A less severe effect of *tailless* deletion on layer IV would be consistent with preliminary findings of Roy *et al*. (Roy *et al*., 2001, 2002) (Roy *et al*., submitted) who observed in *tlx–/–* mice that numbers and proliferative capacity of cortical progenitor cells begin to decrease around E16 – an age when most, though perhaps not all neurons destined for cortical layer IV are likely to have left the proliferative epithelium (Polleux *et al*., 1997; Takahashi *et al*., 1999).

The majority of local and long-distance horizontal, and interhemispheric projections in neocortex arise from neurons in layers II/III (Jensen and Altman, 1982; Lund, 1988; Burkhalter, 1989; Schwartz and Goldman-Rakic, 1991). Diminution of superficial layers in *tailless* mutants therefore is likely to substantially impact the organization of intracortical connections (see also below). Indeed, in *tlx–/–* mice we found that the cross-sectional area of the corpus callosum, the largest forebrain commissure, is significantly reduced (i.e. by over 40%) compared with wild-type mice. These quantitative data are consistent with previous qualitative observations that the anterior and hippocampal commissures in mutant mice are smaller than normal (Monaghan *et al*., 1997). Additional studies are required to establish precisely the degree of axon loss in mutant commissures, and the effect, if any, on interhemispheric projection patterns. The data nevertheless support the idea that many projection neurons are missing from the superficial layers of mutant cortex.

By contrast with effects of the *tailless* mutation on layers II/III, infragranular layers appear relatively well-formed and have a cytoarchitectonic organization similar to deep layers of wild-type mice. Consistent with this qualitative observation, cortical thickness measured from the white matter to the bottom of layer IV in *tlx–/–* mice (i.e. layers V and VI) is not significantly different from values obtained in wild-type animals in most areas. There nevertheless is a trend toward slightly thinner layers in mutant animals, which reached significance in the frontal region. There are several possible explanations for the slight but noticeable effect on infragranular layers. For example, it might denote the loss of a small population of late-born neurons that normally settle in the deeper layers (Berry and Rogers, 1965; Hicks and D'Amato, 1968). In addition, it could reflect in part re-

duced numbers of GABAergic neurons that migrate tangentially from the telencephalic medial and lateral ganglionic eminences (de Carlos *et al*., 1996; Anderson *et al*., 1997, 2001; Wichterle *et al*., 2001), where neurogenesis also is affected by loss of *tailless* function (Monaghan *et al*., 1997). Finally, there may be subtle changes in cell size or packing density in deep layers of mutant cortex that could be revealed by more detailed morphometric analyses than those employed in the present investigation. It is interesting to note, nevertheless, that particular subpopulations of deep layer neurons – like large pyramidal neurons expressing SMI-32 identified in this study – are present in mutant cortex, where they assume a normal location and acquire relatively normal morphology.

It is intriguing that loss of *tailless* function in all forebrain progenitor cells, throughout development, has a more profound effect on superficial than on deep cortical layers. Previous studies suggest that distinct subsets of committed progenitor cells exist in the dorsal VZ before neurogenesis begins (Tan and Breen, 1993; Ware *et al*., 1999; McCarthy *et al*., 2001), and give rise to progeny with different laminar fates. The restriction of cortical progenitors to deep versus superficial layers has been investigated by a variety of techniques. For example, the developmental potential of early versus late progenitor cells has been explored by transplanting progenitor cells to hosts of different ages. Such studies demonstrate that laminar fate of early progenitors (i.e. those destined for deep layers) is specified before the last cell division (McConnell and Kaznowski, 1991). Thus, early cortical progenitors transplanted in S-phase of the cell cycle to the cortex of an older host, and allowed to undergo one round of division in the host environment adopted a host (i.e. superficial layer) phenotype, while cells transplanted later in the cell cycle adopted a donor (i.e. deep layer) phenotype. By contrast, progenitor cells from older embryos (i.e. cells destined to form superficial layers) are unable to generate deep layer neurons (Frantz and McConnell 1996). Lineage tracing studies and clonal analyses support these observations (Rakic, 1988; Walsh and Cepko, 1988; Luskin *et al*., 1988) and indicate that early progenitor cells are multipotent whereas later progenitor cells exhibit a progressive restriction in their laminar potential. These studies also show that restriction of developmental potential of progenitor cells occurs early and that, over time, both environmental and intrinsic signals underlie the different behaviors of these populations [(Luskin *et al*., 1988; Barbe and Levitt, 1991, 1992, 1995; Parnavelas *et al*., 1991; Takahashi *et al*., 1993, 1995a,b; Davis and Temple, 1994; Bohner *et al*., 1997; Lim *et al*., 1997; Mayer-Proschel *et al*., 1997; Qian *et al*., 1997, 1998, 2000); reviewed in (Lillien, 1998)].

Mechanisms that regulate a progenitor cell's potential to produce deep- versus superficial-layer progeny are not fully understood. Because *tlx* is absent from progenitor cells throughout development it will be important to determine whether cortical abnormalities observed here result from primary versus secondary effects. Thus, the alterations observed in superficial cortical laminae could be due to a primary requirement for *tlx* expression in late progenitor cells (i.e. those that seed superficial cortical layers). On the other hand, the abnormalities observed in late progenitor cells could be a secondary response to the altered environment present in mutant animals. Our observation that deep cortical layers are relatively well-formed in these mice but superficial ones are attenuated suggests that, despite the accelerated pace of neurogenesis, assignment of laminar fate initially proceeds normally. It remains to be established whether *tailless* is involved directly in promoting neurogenesis (e.g. vs gliogenesis), or whether expression of this gene enables progenitor cells to respond to environmental cues that would signal continued proliferation. Our findings nevertheless suggest that *tailless* may be an essential regulator of a progenitor cell's choice to proliferate or differentiate. The data further demonstrate that small changes in cell cycle kinetics early can have profound consequences over time (Kornack and Rakic, 1998). Late developing structures, like superficial cortical layers, are particularly vulnerable to early changes in the rate of proliferation since such changes will become amplified as development proceeds.

### *Laminar Distribution of Intrinsic and Extrinsic Cortical Circuits Is Preserved in tlx$^{-/-}$ Mice*

VGLUT2 immunoreactivity, a marker of thalamocortical axon terminals (Fujiyama *et al*., 2001), is localized to two tiers in *tlx–/–* mice, as it is in wild-type mice (i.e. Figure 6). The location of these strata in both mouse lines corresponds to cytoarchitecturally defined layer IV and the layer V/layer VI border. These layers normally are principal targets of primary thalamocortical projections. Thalamic axons in mutant mice thus appear to recognize layer-specific signals that guide them to their appropriate cortical locus. Moreover, VGLUT2 staining in the parietal somatosensory region of *tlx–/–* mice clearly delineates the cell-sparse hollows of cortical barrels. This suggests that mutant cortical neurons, in turn, respond to cues provided by the segregated thalamic axons with preferential dendritic growth and orientation, and cell body displacement that creates the barrel structure (Harris and Woolsey, 1981; Erzurumlu and Jhaveri, 1990).

Zinc-containing terminals, which denote a prominent subset of intracortical circuits, are heterogeneously distributed in *tlx–/–* mice in a pattern similar to that of wild-type mice, and that resembles the pattern observed for zinc-containing pathways in other cortical areas and in other species (Garret and Slomianka, 1992; Dyck *et al*., 1993; Land and Akhtar, 1999). Although we have not examined this quantitatively, the overall staining intensity for synaptic zinc in individual layers of mutant cortex appears comparable to that observed in wild-type animals (cf., Fig. 6, above). These findings are particularly interesting because a majority of zinc-sequestering cortical neurons (i.e. neurons whose synaptic terminals bear the neuron-specific zinc transporter, ZnT-3) are pyramidal cells in layers II/III (Garret and Slomianka, 1992; Land, 1995; Palmiter *et al*., 1996). These layers are markedly attenuated in the absence of *tailless* function (see above). This suggests that in mutant mice individual layer II/III neurons might compensate for missing cells by expanding their projections within normal target regions. It would be interesting in this regard to examine in greater detail the horizontal and interlaminar connections of zinc-ergic pyramidal neurons in *tlx–/–* mice. Taken together, these data suggest that molecular cues regulating axo-dendritic growth and the precise matching of presynaptic input with postsynaptic target are expressed in appropriate spatiotemporal patterns by cortical neurons in mutant animals (Castellani and Bolz, 1997; Hevner *et al*., 2002; Mann *et al*., 2002).

### *Tlx–/– Cortex Is Subdivided into Species-specific Area Maps*

Somatosensory cortical barrels can be identified in the expected location on the lateral cortical convexity in all mutant animals. This is true irrespective of the staining method employed in a particular experiment. In tangentially sectioned hemispheres it is evident that the somatotopic distribution of barrels and the rostro-caudal position of the barrelfield in mutant mice resemble

the arrangement observed in wild-type subjects. This suggests the (as yet untested) possibility that other functional areas occupy regions of cortex both rostral and caudal to the barrelfield as they do in wild-type mice. There are two additional important implications of this finding. First, it indicates that clustered thalamocortical axons, which are believed to convey the periphery-related map onto the cerebral cortex (Erzurumlu and Jhaveri, 1990), encounter sufficient cues in mutant forebrain to maintain their normal distribution patterns and to recruit cortical neurons into barrels. Second, correct placement of the barrelfield along the rostro-caudal axis implies that gradients of signaling molecules thought to contribute to parcellation of neocortex into functional areas (Fukuchi-Shimogori and Grove, 2001; Ragsdale and Grove, 2001) are expressed in normal patterns in the absence of *tlx–/–* function. These findings were unexpected given that precocious progenitor cell differentiation in the absence of *tlx* function could alter the environment encountered by ingrowing thalamic axons. In summary, *tailless* expression is critical for the development of late-generated cortical neurons (i.e. those destined for superficial layers) but is not required for cortical patterning *per se* or for the laminar and columnar arrangement of cortical circuits.

### *Manipulating the Tlx Gene Provides a Tool for Dissecting Cortical Neurogenesis*

Our findings indicate that *tlx* deletion does not alter radial neuronal migration or the spatial patterning of the developing cortex. However, preliminary studies indicate that the temporal regulation of proliferation and differentiation are uncoupled in the developing forebrain of *tlx–/–* mice (Roy *et al.*, 2001, 2002) (Roy *et al.*, submitted). The hypoplasia of superficial cortical layers observed in the present study could thus be a consequence of this dysregulation. Interestingly, other proteins that directly regulate proliferation of progenitor cells also have been shown to alter formation of upper cortical layers. For example, absence of the transcription factor Pax-6 in cortical progenitor cells leads to an enhanced rate of cellular proliferation until mid-gestation. Pax-6 also is required for the differentiation and migration of late-born cortical progenitor cells (Stoykova and Gruss, 1994; Stoykova *et al.*, 1996; Gotz *et al.*, 1998; Warren *et al.*, 1999). In addition, both rats and mice with a mutation in the citron-kinase gene (CK) exhibit altered cytokinesis in progenitor cells, massive apoptosis in proliferative regions, and thinning of superficial cortical layers (Di Cunto *et al.*, 2000; Sarkisian *et al.*, 2002). Together with the results of the present study these findings indicate that cells destined for superficial cortical layers are sensitive to upsetting the balance between proliferation and differentiation. This can occur through altered cell cycle kinetics as in the *tlx* mutation, altered migration/differentiation as in the Pax-6 mutation or altered cell survival as in the CK mutation.

Until the development of animal models such as the *tlx* mutation, investigators have relied on pharmacological methods to study the anatomical and behavioral consequences of perturbing proliferation of progenitor cells during development. The cortical abnormalities described here for *tlx–/–* mice are reminiscent of the phenotype observed following fetal exposure to the cytotoxic alkylating agent methylazoxymethanol acetate (MAM) (Marsumoto *et al.*, 1972; Johnston and Coyle, 1979). However, in contrast with MAM treatment, which has widespread systemic effects, genetic manipulation of the *tlx* gene specifically targets progenitor cells in the forebrain. Future experiments aimed at controlling the temporal and spatial expression of *tlx* will enable a dissection of the behavior of both early and late progenitor cell populations. Compared with the above models, the advantage of manipulating the *tlx* gene to gain insights into mechanisms regulating cortical development is that *tlx* is restricted to the forebrain, and mutant animals survive enabling functional and behavioral analyses to be performed in adults.

## Notes

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