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Abnormal development of zinc-containing cortical circuits in the absence of the transcription factor *Tailless*

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Abstract

Absence of the transcription factor *tailless* (*tlx*) leads to premature laminar development and thinning of neocortex. We used zinc autometallography to determine if *tailless* deletion alters the organization of cortical circuits. In *tlx*^{−/−} mice, layer 4 barrels, which normally lack synaptic zinc, are densely innervated by zinc-containing terminals. Furthermore, barrels with zinc inputs are constructed, in part, from zinc-sequestering neurons, a phenotype not normally found in layer 4.

Keywords

Barrel; Vibriss; Whisker; Somatosensory cortex; Lamination

The mammalian *tailless* gene (*tlx*) is a forebrain-restricted transcription factor that is expressed by progenitor cells in the ventricular and subventricular zones during neurogenesis [17,18]. *Tlx* expression is essential for normal development of neocortex. In mice lacking *tlx*, cortical thickness is reduced to about 80% of that seen in wild-type (WT) mice [14]. Interestingly, superficial cortical layers are more adversely affected than deep layers 5 and 6, which appear relatively normal.

Tlx appears to regulate proliferation and timing of differentiation of progenitor cells. Around embryonic day 9.5 in *tlx*^{−/−} mice, the cell cycle is significantly shorter than in WT littermates [21]. The accelerated proliferation leads neurons destined for a particular cortical layer to become postmitotic and differentiate precociously. Subsequently, the late progenitor cell population is prematurely depleted, around mid-gestation, resulting in more severe effects on later-born superficial layers [21]. These may be direct or indirect consequences of loss of *tlx*. Abnormalities in the number and time of differentiation of cortical neurons are likely to profoundly alter cortical circuits and their function.

In the present study, we used histochemical techniques to investigate the organization of zinc-ergic cortical circuits in the somatosensory (S1) barrel cortex of four adult *tlx*^{−/−} mice and four of their WT littermates. We focused on S1 because of the context provided by the layer 4 whisker-related barrels [26], which are visible in mutant animals [14]. Zinc autometallography 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 [3,9]. Zinc-containing synapses arise from intrinsic cortical neurons, mostly pyramidal neurons in layers 2/3 and 6, and are distributed heterogeneously among the cortical layers in a pattern that is highly conserved across mammals [4,5,7,8,10,12,13,23]. Localization of zinc-sequestering neurons also can be determined by autometallography (see below).

To localize zinc-containing synaptic terminals, two mice of each genotype received an intraperitoneal (IP) injection of the zinc chelator sodium selenite (20 mg/kg) from a freshly prepared stock solution and were anesthetized with IsoVet (Isoflurane; Abbot Labs, Abbot Park, IL) and killed by decapitation 1 h later. Each brain was quickly removed, encased in OCT embedding medium (Miles, Elkhart, IN) and rapidly frozen. One series of 20 μ m coronal or tangential sections from each hemisphere was stained for synaptic zinc according to a modification of the Danscher method [6,8,13]. The distribution of zinc-sequestering cortical neurons was assessed in two additional mice of each strain. These mice received 12 mg/kg sodium selenite IP and were killed 24 h later. The longer survival period allows for zinc-specific retrograde transport of chelated zinc back to the neuronal somata of origin of zinc-containing synapses [23]. Sections were cut and stained as above. Sections then were counterstained with 0.1% thionin or were further dehydrated and coverslipped. Series of adjacent sections were stained for cytochrome oxidase (CO) according to previously published methods [15,25]. The care and handling of animals was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and conformed to NIH guidelines.

Zinc-containing terminals are distributed in a layer specific pattern in WT and *tlx*^{-/-} mice (Fig. 1). In both genotypes, density of synaptic zinc is high in layers 1–3 and in layer 5, intermediate in layer 6 and very low in layer 4. Staining of adjacent sections for zinc or CO shows that regions of layer 4 in S1 of WT mice stained lightly for zinc (e.g., Fig. 1A) denote hollows of cortical barrels (Fig. 1C). Many layer 4 barrels in *tlx*^{-/-} mice also stain lightly for zinc (Fig. 1B). However, in S1 of mutant mice, we observed that some barrels, in contrast, are conspicuous by virtue of the high level of synaptic zinc that they contain (e.g., arrows in Figs. 1B and D).

In tangential sections, it is evident that regions of layer 4 in *tlx*^{-/-} mice that stain intensely for zinc are incorporated into the barrelfield proper. In WT mice, layer 4 barrels appear as rows and arcs of lightly stained barrel hollows separated by narrow darkly stained septa (Fig. 1E; [19]). The overall barrel pattern in *tlx*^{-/-} mice (Fig. 1F) is relatively normal (see also [14]), but some barrels are seen to stain darkly while others stain lightly, as in WT mice. We have not examined a sufficiently large number of mutant hemispheres to know if there are predictable patterns of heavy versus light zinc staining. However, it is notable that both small and large barrels may have abnormally high levels of synaptic zinc (e.g., single versus double arrows in Fig. 1F).

We used zinc-specific retrograde transport to investigate potential sources of aberrant zinc-ergic inputs to barrels of mutant mice. In WT mice, zinc-sequestering cortical neurons are located exclusively in layers 2/3 and deep layer 6 (Fig. 2A; [4]); layer 4 barrels are devoid of label (e.g., arrow in Fig. 2A). Layers 2/3 and 6 in *tlx*^{-/-} mice also contain zinc-ergic neurons, although they appear considerably less numerous in layers 2/3 because these layers are markedly thinner in mutant animals (compare Fig. 2B with Fig. 2A; see also [14]). Unexpectedly, whereas many layer 4 barrels in the mutant hemispheres are completely unstained, as in wild-type mice (large arrow in Fig. 2B), other barrels contain zinc-sequestering neurons (e.g., double arrows in Fig. 2B). One such barrel, enclosed by the rectangle in Fig. 2B, is shown at higher magnification in Fig. 2C following Nissl counterstaining. In addition to zinc-labeled neurons in layers 2/3 (e.g., large arrowhead), we observed numerous zinc-sequestering cells in layer 4 barrels (e.g., double arrowhead). These neurons presumably

contribute to the zinc-containing terminals that are seen in mutant barrels with shorter survival times after selenite injection (i.e., Fig. 1, above).

In normal rodents, neurons in the ventrobasal (VB) thalamic nucleus, which do not sequester zinc [4,16], provide a major source of excitatory input to layer 4 barrels [22]. We therefore considered the possibility that the phenotype of thalamic neurons might be altered in mutant mice such that their synaptic terminals now contain zinc. Such a phenotypic switch could further contribute to abnormally high levels of synaptic zinc in some mutant barrels. We found, however, that VB and other principal thalamic nuclei like the lateral geniculate nucleus (LGN) do not contain zinc-ergic neurons in mutant mice that exhibit zinc-stained barrels (Figs. 2D and E).

The present findings show that, in addition to an overall reduced thickness of superficial cortical layers, loss of *tailless* function leads to marked abnormalities in at least one major intracortical circuit. Zinc-containing circuits in *tlx*^{-/-} mutants densely innervate portions of layer 4, a cortical layer largely devoid of zinc inputs in normal animals. More remarkably, a major source of these inputs appears to be zinc-ergic layer 4 neurons—a neuronal phenotype normally not observed in that layer. There are at least two possible explanations for this finding. For example, as a result of the *tlx*^{-/-} mutation part of the normal complement of layer 4 barrel neurons might somehow acquire the capability of sequestering zinc. While we have not ruled out this possibility, we think it is unlikely. Instead, we postulate that in *tlx*^{-/-} mice neurons that, based upon their time of origin, would be destined for layers 2/3 have been recruited into the barrel structure, where they express phenotypic traits typical for neurons in layers 2/3.

During cortical development, formation of barrels is believed to depend critically on the presence and pattern of thalamic input [11]. At the very earliest time that layer 4 neurons are observed to settle from the cortical plate, they aggregate around a highly segregated field of thalamocortical axons to form cytoarchitecturally distinct barrels [20]. In *tlx*^{-/-} mice neurons destined for layers 2/3 become postmitotic and begin to differentiate around embryonic day 14.5—an age when layer 4 neurons are born in normal mice [1,2,24]. We speculate that precocious differentiation of layer 2/3 neurons coupled with the overall diminution of cell numbers in superficial layers creates a scenario wherein thalamocortical axons enlist any available cells – even inappropriate ones – into cytoarchitectonic aggregates that we recognize here as barrels. This would suggest that the as-yet-unknown mechanisms that drive barrel formation are sufficiently robust to override other phenotypic restrictions.

It will be informative to investigate other possible circuit abnormalities in the cortex of *tlx*^{-/-} mice that might include non-zinc-ergic neurons. The data nevertheless indicate that *tlx*^{-/-} mice are a valuable tool for investigating circuit development in the cerebral cortex as well as pattern formation in the mammalian brain.

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Fig. 1.

Barrels in *tlx*^{-/-} mutants contain synaptic zinc. (A, B) Zinc-stained coronal sections through S1 barrel cortex of a wild-type (A) and *tlx*^{-/-} mouse (B). Note heterogeneous staining throughout the cortical layers (labeled 1–6). Barrels in layer 4 normally contain lowest levels of synaptic zinc, but in *tlx*^{-/-} mice some barrels stain darkly (arrows in (B)). (C, D) CO-stained sections adjacent to those shown in panels (A) and (B), respectively. Barrels are clearly visible as dark CO patches in layer 4. Barrels indicated by arrows in panel (B) are seen also in panel (D). (E) Tangential zinc-stained section through layer 4 of a wild-type mouse showing the pattern of barrels, all of which stain lightly for zinc. (F) Tangential zinc-stained section through layer 4 of a *tlx*^{-/-} mouse. Note that many small (arrow) and large (double arrows) barrels stain

darkly; others stain lightly as in wild-type mice. Scale bar in panel (D) = 300 μm for panel (A) through panel (D); scale bar in panel (F) = 300 μm for panels (E) and (F).

Fig. 2.

Barrels in *tlx*^{-/-} mutants are populated by zinc-sequestering neurons. (A) Coronal section through S1 of a wild-type mouse showing that zinc-sequestering neurons (i.e., darkly stained elements) are located within cortical layers 2/3 and 6; layers are indicated by Arabic numerals. Arrow points to a layer 4 barrel that is devoid of zinc staining. (B) Coronal section through S1 of a *tlx*^{-/-} mouse. Layers 2/3 and 6 contain zinc-sequestering neurons as in wild-type mice. Note also that, whereas some layer 4 barrels are free of zinc staining (e.g., single arrow), others stain darkly (e.g., double arrows). Barrel enclosed by rectangle is shown in panel (C) after counterstaining with Nissl. (C) Higher power photomicrograph of region enclosed by rectangle in panel (B). Note zinc containing somata in layer 3 (arrowhead) as well as in a layer 4 barrel (double arrowheads). (D) Zinc-stained section through cortex and thalamus of a *tlx*^{-/-} mouse. Note that principal thalamic nuclei like the LGN and VB are devoid of zinc-sequestering neurons, even though a zinc-containing barrel is visible in the cortex (i.e., arrow); other unstained barrels are visible to the right of the zinc-stained barrel. (E) CO-stained section

adjacent to that in panel (D) confirming location of CO dark thalamic nuclei (i.e., LGN/VB) and cortical barrels. Scale bar in panel (B) = 300 μm for panels (A) and (B); scale bar in panel (C) = 100 μm ; scale bar in panel (E) = 1 mm for panels (D) and (E).