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Loss of the *tailless* gene affects forebrain development and emotional behavior

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Abstract

We are studying the role of the evolutionarily conserved *tlx* gene in forebrain development in mice. *Tlx* is expressed in the ventricular zone that gives rise to neurons and glia of the forebrain. We have shown by mutating the *tlx* gene in mice, that in the absence of this transcription factor, mutant animals survive, but suffer specific anatomical defects in the limbic system. Because of these developmentally induced structural changes, mice with a mutation in the *tlx* gene can function, but exhibit extreme behavioral pathology. Mice show heightened aggressiveness, excitability, and poor cognition. In this article, we present a summary of our findings on the cellular and behavioral changes in the forebrain of mutant animals. We show that absence of the *tlx* gene leads to abnormal proliferation and differentiation of progenitor cells (PCs) in the forebrain from embryonic day 9 (E9). These abnormalities lead to hypoplasia of superficial cortical layers and subsets of GABAergic interneurons in the neocortex. We examined the behavior of mutant animals in three tests for anxiety/fear: the open field, the elevated plus maze, and fear conditioning. Mutant animals are less anxious and less fearful when assessed in the elevated plus and open-field paradigm. In addition, mutant animals do not condition to either the tone or the context in the fear-conditioning paradigm. These animals, therefore, provide a genetic tool to delineate structure/function relationships in defined regions of the brain and decipher how their disruption leads to behavioral abnormalities.

Keywords

Mouse; *Tailless*; Transcription factor; Limbic system; Neurogenesis; Neuroanatomy; Aggression; Anxiety; Fear conditioning; Elevated plus maze

1. Introduction

The limbic system functions to control emotional and behavioral activities and is required for normal learning and memory. Developmental defects of the limbic system may underlie many behavioral, neuropsychiatric, and cognitive disorders [1]. These disorders have their onset early in life, leading to chronic or recurrent disease. Few genes have been identified that specifically alter the development of areas in the brain that are essential for emotion and cognition. We have identified an evolutionarily conserved gene, the transcription factor *tlx*, that is required for normal cellular proliferation and differentiation of progenitor cells (PCs) in the forebrain [2,3].

Tlx is a member of the orphan nuclear receptor gene superfamily whose expression is restricted to PCs in the developing telencephalon, diencephalon, eye, and nasal placode from embryonic day 8.5 (E8.5). In adult animals, transcripts become localized to a number of areas, including the rostral migratory stream, the hippocampus, the septum, the amygdala, the hypothalamus, the retinal ganglion cell layer and photoreceptor cells, and the olfactory epithelium and nerve [3]. To investigate the role of the *tlx* gene in mouse brain development and function, we generated a targeted disruption of the gene by homologous recombination [2,3]. Disruption of the *tlx* locus in mice leads to impaired development of a specific subset of forebrain-derived limbic structures. Because of these structural alterations, animals exhibit a variety of behavioral abnormalities, including severe aggression, stereotypy, altered maternal instincts, reduced learning abilities, and late onset epilepsy [2]. Our laboratory is interested in defining the molecular and cellular events that contribute to the formation of the limbic system, and how perturbing these events lead to behavioral abnormalities. The studies presented in this article focus on aspects of emotional behavior and the underlying areas of the limbic system altered in *tlx*^{-/-} animals.

2. Results

2.1. *Tlx* is required for the temporal regulation of cortical neurogenesis

Tlx is expressed in both PCs and a subset of cell types in the mature forebrain and, therefore, may play a role in proliferation, differentiation, cell survival, or functioning of the mature nervous system. Mutant animals survive but suffer from hypoplasia in a number of limbic-associated structures. To identify the cell types and functions specifically targeted by loss of the *tlx* gene, brains from wild-type and mutant littermates were histologically examined at different embryonic ages (E9 to P15). Detailed analyses of the proliferation profiles of PCs using bromodeoxyuridine (BrdU) incorporation and birthdating studies, in conjunction with an analysis of the ontogeny of cell-type-specific markers, indicate that the *tlx* gene primarily targets PCs. From E9 to E14, PCs in mutant animals have a shorter cell cycle and exhibit precocious neuronal differentiation (Fig. 1) [4]. After midgestation (E14.5), however, the PC population becomes depleted leading to the production of fewer differentiated cells. Cell death and neuronal migration appear relatively normal in *tlx*^{-/-} animals compared with *tlx*^{+/-} littermates [4]. These findings indicate that the *tlx* gene is required primarily to regulate the timing of cortical neurogenesis in PCs, and that early abnormalities in mutant animals lead to depletion of late developing PC populations. Consequently, structures that are born near the end of neurogenesis, such as superficial cortical layers and the dentate gyrus, are reduced in size.

2.2. Loss of specific cell types in limbic-associated structures in *tlx*^{-/-} animals

Loss of *tlx* during development leads to a reduction in cell number in a subset of limbic structures. *Tlx*^{-/-} animals are similar in size to their littermates at birth but suffer transient postnatal growth retardation. The olfactory bulbs and cerebral cortex are smaller in mature mutant animals compared with littermates. Structures caudal to the cerebral cortex appear normal [2]. The shrunken appearance of the cortex is primarily due to hypoplasia of structures in the rhinencephalon, including the piriform cortex, the islands of Calleja, the anterior commissure, the corticomedial amygdala, and the entorhinal cortex. In the hippocampus, ammons horn appears relatively normal in size and organization; however, the dentate gyrus is smaller in *tlx*^{-/-} animals. Although the normal pattern of cortical lamination is present in mutant animals, the cerebral wall is thinner (Fig. 2A–C). Detailed histological and immunohistochemical analysis of the cortex in mutant animals reveal a reduction in cell number in somatostatin- and calretinin-positive cells throughout the thickness of the cerebral wall [2]. In mutant animals, Layers I and IV to VI are similar in size and organization to wild-type littermates; however, superficial layers (II/III) are attenuated (Fig. 2A) (Monaghan and

Land, unpublished observations; [4]). These findings lead to the prediction that loss of *tlx* will result in malfunction of limbic structures.

2.3. *Tlx*^{-/-} animals are hyperresponsive and aggressive

The limbic system has been implicated in the regulation of emotional behaviors including aggression, social behavior, anxiety, and sexual activity. The most striking phenotype of *tlx*^{-/-} animals is severe aggression. This is consistent with the alterations observed in the limbic system. *Tlx*-deficient animals are relatively difficult to handle from birth, they hypervocalize, jump, and bite handlers more often than do wild-type littermates. Hyperaggressiveness is observed in *tlx*^{-/-} animals irrespective of their background genotype (C57Bl/6J or 129/SVE). Although *tlx*^{-/-} animals are generally smaller than wild-type littermates, males attack and kill their cagemates beginning at puberty. Females can be housed together with littermates but attack conspecifics when stressed. Fig. 3 illustrates increased aggression in a sexual aggression test. Adult *tlx*^{-/-} and *tlx*^{+/+} males ($n = 4/\text{genotype}$) were housed in isolation for 4 weeks and subsequently exposed to a non-estrus female in a neutral arena and monitored. The number of attacks and attempted mounts was recorded for the first 15 min of each hour for 3 h. *Tlx*^{-/-} males assaulted the females significantly more often than did the control males (60 ± 4 , 33 ± 3 , and 35 ± 2 in successive 15-min periods, in contrast to only 4 ± 1 , 3 ± 1 , and 2 ± 1 assaults by *tlx*^{+/+} males in the same time period). The number of scored mounts/attacks by *tlx*^{-/-} males decreased after the first hour of observation because the female hung from the cage top in an apparent effort to escape.

Tlx^{-/-} animals are more aggressive but the origin of this behavior is unknown. They may be more fearful, attacking littermates in anticipation of a confrontation, or they may attack because they have no fear. To begin to distinguish between these alternatives, exploration in two fear-evoking situations was tested.

2.4. *Tlx*^{-/-} animals are less anxious

To determine whether the level of activity in *tlx*^{-/-} mice was increased and to examine the exploratory strategies of these mice compared with those of control littermates, each animal was tested in an open-field chamber [5] (*tlx*^{-/-} and *tlx*^{+/+}, $n = 4/\text{genotype}$). The total number of crossings and the amount of rearing were not significantly different between *tlx*^{-/-} and *tlx*^{+/+} animals. The percent of entrances into the inner squares was increased in *tlx*^{-/-} compared with *tlx*^{+/+} animals, although this difference did not reach significance (Table 1). One type of exploratory behavior was specifically altered by loss of the *tlx* gene: the number of nose-pokes, a measure of novelty-seeking behavior, was significantly reduced in mutant animals compared with wild-type littermates (Table 1). These results suggest that mutant animals are not hyperactive but that novelty-seeking behavior is blunted in mutant mice compared with control littermates.

To investigate fear-related behaviors, exploratory strategy on the elevated plus maze was monitored [6]. *Tlx*^{-/-} animals spent more time on the open arms of the plus maze than did control littermates [*tlx*^{+/+}, $n = 5$; *tlx*^{+/-}, $n = 3$; *tlx*^{-/-}, $n = 8$; $F(1,15) = 5.696$, $P < .05$] (Fig. 3A). This behavior was at the expense of spending time on the closed arms [$F(1,15) = 7.807$, $P < .05$] (Fig. 3A). Exploration of the center compartment was comparable between genotypes [$F(1,15) = 0.867$] (Fig. 3A). *Tlx*^{-/-} animals made a similar number of total arm entries compared with *tlx*^{+/+} animals, strengthening our aforementioned observations from the open-field apparatus that *tlx*^{-/-} animals are not more active than their wild-type counterparts. The exploratory strategy of *tlx*^{-/-} animals was different from that of *tlx*^{+/+} animals. *Tlx*-deficient animals entered an open arm and explored the distal end whereas wild-type animals traveled back and forth between the closed arms, exploring the proximal open arms only occasionally [percentage of open-arm entries, $F(1,15) = 4.8$, $P < .05$, and percentage of closed-arm entries,

$F(1,15) = 4.8, P < .05]$ (Fig. 3B). The tendency of *tlx*-deficient animals to explore the open arms of the maze suggests that they are less anxious and may be less fearful than *tlx*^{+/+} mice.

2.5. Reduced memory for fear in *tlx*^{-/-} animals

Cued and contextual fear-conditioning involves processing and integration of sensory signals by the hippocampus, amygdala, and cortex [7], three areas known to be affected by loss of *tlx*. *Tlx*-deficient mice and control littermates were tested in a fear-conditioning paradigm [5]. The baseline degree of immobility (freezing) in the conditioning chamber was similar between *tlx*^{-/-} and *tlx*^{+/+} animals before exposure to the tones or shocks (*tlx*^{+/+}, *n*=6; *tlx*^{+/+}, *n*=2; *tlx*^{-/-}, *n*=8) (Fig. 4) or during the tones (data not shown). *Tlx*^{-/-} animals were hyperresponsive to the shock, as indicated by increased jumping and vocalizations compared with wild-type littermates (data not shown).

To determine whether the animals associated the context of the conditioning chamber with the aversive stimulus, animals were returned to the conditioning chamber 24 h after training. *Tlx*^{-/-} animals froze significantly less than *tlx*^{+/+} animals [$F(1,15) = 14.62, P < .05]$ (Fig. 4). Post hoc analyses indicated that the amount of freezing observed in *tlx*^{-/-} mice was not different from baseline levels ($P > .2$). In contrast, control littermates spent more time immobile during testing than during training ($P < .001$). Two hours after testing contextual conditioning, each animal was tested for conditioning to the acoustic cue in a novel context. In the novel environment, *tlx*^{-/-} and *tlx*^{+/+} animals exhibited similar low levels of baseline freezing. Upon tone presentation, freezing in both *tlx*^{-/-} and *tlx*^{+/+} animals increased, but the level of freezing in *tlx*^{-/-} animals was attenuated compared with that of *tlx*^{+/+} animals [$F(1,15) = 7.21, P < .05]$ (Fig. 4). Post hoc analyses indicated that *tlx*^{-/-} animals exhibit impaired conditioning, freezing equally to tone presentations during training and testing (Fig. 4). Control animals froze significantly more to the tone during testing compared with their low levels of freezing to the tone during training ($P < .0001$). The deficits observed in *tlx*^{-/-} animals suggest that these animals are impaired in acquiring, storing or retrieving an association between footshock and the context in which they experienced the footshock as well as between the footshock and a cue that predicts the occurrence of the aversive event. This phenotype may be mediated by the reductions observed in cells of the entorhinal cortex or the amygdala in mutant animals.

Reduced pain sensitivity would decrease the salience of the footshock and, consequently, might lead to less robust associations between the footshock and the context in which it occurred. *Tlx*^{-/-} animals responded to the shock presentations with jumping and vocalizations to a greater extent than did wild-type animals. These observations render it unlikely that impaired fear conditioning resulted from reduced pain sensitivity. Nevertheless, to confirm the ability of *tlx*^{-/-} animals to feel pain, animals previously tested in the fear-conditioning paradigm were placed on a 52 °C hotplate, and their pain threshold was assessed using previously described procedures [5]. The latency to paw lick or jump was comparable between genotypes (Table 1), which suggests that *tlx*^{-/-} animals do not have an increased threshold for pain. As previously shown, *tlx*^{-/-} animals are not hyperactive (open field, Table 1). Impaired spatial and cue conditioning in *tlx*^{-/-} animals, therefore, cannot be attributed to an inability to feel pain or to an increase in general activity levels.

3. Discussion

We have identified a novel forebrain restricted transcription factor, *tlx*, whose loss alters maturation of limbic regions. These defects arise during the ontogeny of specific cell types in the limbic system. We have found that loss of *tlx* leads to an altered cell proliferation profile in PCs and to enhanced neuronal differentiation of early generated neurons coupled with a loss of cell types specified for late generated structures. *Tlx* is therefore a critical factor required for the temporal regulation of neurogenesis in the forebrain.

Structurally, mutant adult brains exhibit microencephaly primarily due to hypoplasia in the olfactory bulbs, entorhinal cortex, amygdala, hippocampus, and associated structures of the medial temporal lobe. In addition, cortical thickness is reduced due to decreased cell number in upper cortical layers. It is therefore not surprising that *tlx*^{-/-} animals show deficiencies in a number of limbic-mediated behaviors. The most striking behavior observed in *tlx*-deficient animals is the extreme aggression directed toward both conspecifics and handlers. Several regions in the brain have been implicated in the manifestation of aggressive behaviors when altered. The septal complex in rats has been implicated in regulation of aggressive behavior, and lesions of the septal region are associated with rage-like behaviors [8]. In cats, the limbic–amygdala–hypothalamic–periaqueductal gray axis has been shown to mediate the two main types of aggression, i.e., defensive rage and offensive/predatory rage (reviewed in Ref. [9]). Whether the aggressive behavior observed in *tlx*^{-/-} animals is offensive or defensive has yet to be determined but regions implicated in the manifestation and/or modulation of aggression such as the amygdala are altered in mutant animals. Alterations in subcortical or limbic afferents to the hypothalamus and/or olfactory structures, or decreased GABAergic transmission [10], all of which are also affected in *tlx*^{-/-} mice, could also be responsible for the increased aggression in the mutants.

In addition to increased aggression, *tlx*^{-/-} animals exhibit a distinct decrease in anxiety. For instance, *tlx*^{-/-} animals spend more time on the unprotected arms and make more open-arm entries in the elevated plus maze than do control littermates [11,12]. This finding is consistent with observations from the open-field assay. *Tlx*^{-/-} animals tended to make more entries into the inner squares of the chamber than did wild-type animals, although this difference failed to reach significance. Arm choice in the elevated plus maze is influenced both by visual and thigmotactic abilities [13]. Diminished visual acuity in *tlx*^{-/-} animals may contribute to the increased exploration of open arms compared with controls. Interestingly, one test of novelty-seeking behavior (nose pokes), which has been shown to be associated with reduced anxiety when elevated, was, in contrast, reduced significantly in mutant animals compared with wild-type littermates.

Reductions in anxiety could lead to impaired conditioning of emotional responses. Investigations into the neural basis of conditioned emotional responses have demonstrated that inactivation of the amygdala prevents fear conditioning to both cue and context whereas hippocampal dysfunction prevents fear conditioning to the context only [14–17]. We observed that *tlx*^{-/-} animals, unlike controls, did not respond with increased fear to either the context or the cue when each was paired with footshock. Alterations in factors unrelated to learning, including hyperactivity and decreased pain sensitivity, could not account for the deficits in fear conditioning. The structural organization defects detected in the hippocampus, amygdala, and cortex are the most likely substrates for the poor conditioning of *tlx*^{-/-} animals. The exact cellular basis of the poor conditioning is not clear.

Dysfunction of the limbic system has been implicated in a number of psychiatric disorders. Systemic use of the cytotoxic alkylating agent methylazoxymethanol acetate (MAM) during prenatal rat development is one widely used and well-characterized animal model of schizophrenia [18–20]. Our findings mimic results obtained by exposing prenatal rats to this agent. MAM-treated animals also show reduced functioning of the limbic system, including abnormal social interaction and enhanced aggression in the resident–intruder assays [19,21]. Like *tlx*^{-/-} mice, rats injected with MAM early in gestation are hyperresponsive to stressful situations and are abnormally reactive to external stimuli [21].

The *tlx* mutation may be considered the genetic equivalent of the global administration of the cytotoxic drug MAM. However, systemic MAM treatment does not target the forebrain specifically but kills all dividing cells in the body for a period of 2–24 h from administration

[22]. In contrast, the *tlx* mutation targets dividing cells in the forebrain only and leaves other brain regions intact. Controlling the temporal and spatial expression of *tlx* will enable a dissection of the behavioral consequences of subtle anatomical defects due to loss of specific PC populations. The fact that *tlx* is restricted to the forebrain, and that mutant animals survive, will allow a detailed structure/function analysis in specific forebrain regions of adults. Thus, we may gain insights into mechanisms regulating the development of specific behavioral abnormalities.

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Fig 1. Abnormal proliferation and precocious neuronal differentiation in *tlx*-deficient embryos. DAPI- (A, B) and toluidine blue-stained (C, D) coronal sections through the dorsolateral neocortex on E9.5 (A, B) and E14.5 (C, D). More MAP2-positive cells (red) are observed in *tlx*^{-/-} animals (B) compared with *tlx*^{+/+} littermates (A). (C, D) More BrdU-positive cells (brown) are detected in *tlx*^{-/-} embryos (D).

Fig 2.

Cortical abnormalities in *tlx*-deficient mice. (A) Staining for zinc-containing terminals [4] in the adult neocortex demonstrates that superficial cortical layers (I, II, III) are reduced in thickness whereas deep layers (IV, V, VI) are spared in *tlx*^{-/-} compared with control mice. (B, C) Specific interneuron populations are affected in *tlx* mutant animals, whereas others are spared. GABAergic interneurons labeled by parvalbumin (B) are preserved but (C) those expressing calretinin are reduced by approximately 90%.

Fig 3.

Mice lacking the *tlx* gene are less anxious than their wild-type littermates. (A) $tlx^{-/-}$ mice spend more time on the open arms and less time on the enclosed arms of an elevated plus maze when compared with $tlx^{+/+}$ animals during a 5-min test. (B) $Tlx^{-/-}$ animals make more entries into the open arms and fewer into the protected arms. * $P < .05$.

Fig 4.

Tlx-deficient animals are impaired in fear-based associative conditioning relative to control animals. *Tlx*^{-/-} mice froze significantly less than wild-type littermates in the same context and in a novel context in the presence of the tone after receiving two footshocks paired with an auditory cue in the training test. Baseline measurements were taken before mice received the first footshock and the training tones measurement during the tone presentation on the training day. Context and tone measurements were taken during testing 24 h later. * $P < .05$.

Table 1
Comparison of behavioral responses of *tlx*^{+/+}, *tlx*^{+/-}, and *tlx*^{-/-} in open-field and hotplate assays

	Open field			Hotplate		
	Total crosses	Percentage of center crosses	Number of rears	Number of nose pokes [*]	Paw lick latency(s)	Jump latency (s)
+/+ , +/-	258.0 ± 14.0	16.0 ± 2.5	48.0 ± 8.5	85.5 ± 12.0	14.3 ± 1.0	143.8 ± 19.0
-/-	390.0 ± 79.0	19.0 ± 5.4	84.3 ± 4.0	2.3 ± 0.6	15.0 ± 4.0	172.0 ± 70.0

Numbers represented are ± S.E.M.

^{*} *P* < .05.