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Inactivation of the gene for the nuclear receptor *tailless* in the brain preserving its function in the eye

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Abstract

During embryogenesis, *tailless*, an orphan member of the nuclear receptor family, is expressed in the germinal zones of the brain and the developing retina, and is involved in regulating the cell cycle of progenitor cells. Consequently, a deletion of the *tailless* gene leads to decreased cell number with associated anatomical defects in the limbic system, the cortex and the eye. These structural abnormalities are associated with blindness, increased aggressiveness, poor performance in learning paradigms and reduced anxiousness. In order to assess the contribution of blindness to the behavioural changes, we established *tailless* mutant mice with intact visual abilities. We generated a mouse line in which the second exon of the *tailless* gene is flanked by loxP sites and crossed these animals with a transgenic line expressing the Cre recombinase in the neurogenic area of the developing brain, but not in the eye. The resulting animals have anatomically indistinguishable brains compared with *tailless* germline mutants, but are not blind. They are less anxious and much more aggressive than controls, like *tailless* germline mutants. In contrast to germline mutants, the conditional mutants are not impaired in fear conditioning. Furthermore, they show good performance in the Morris water-maze despite severely reduced hippocampal structures. Thus, the pathological aggressiveness and reduced anxiety found in *tailless* germline mutants are due to malformations caused by inactivation of the *tailless* gene in the brain, but the poor performance of *tailless* null mice in learning and memory paradigms is dependent on the associated blindness.

Introduction

The exact regulation of proliferation and differentiation is essential for correct brain development. One factor involved in this process is the nuclear receptor *tailless* (tlx; Monaghan *et al.*, 1997; Land & Monaghan, 2003; Roy *et al.*, 2004; Shi *et al.*, 2004). Strong tlx expression is detected in forebrain structures including the optic stalk, neural retina and the embryonic germinal zones during development (Monaghan *et al.*, 1995). Previously we showed that mice with a targeted tlx mutation have a reduction of limbic and rhinencephalic structures, including the entorhinal cortex, amygdala and dentate gyrus (Monaghan *et al.*, 1997). The loss of tlx causes a reduction of the thickness of the cerebral cortex, the striatum and other structures due to impaired proliferation of neuronal progenitor cells (Roy *et al.*, 2002; Stenman *et al.*, 2003). Tlx-deficient mice are abnormally aggressive, less anxious and show poor cognition (Monaghan *et al.*, 1997; Roy *et al.*, 2002; Young *et al.*, 2002; Abrahams *et al.*, 2005). This is in agreement with studies showing that hippocampal and amygdala lesions cause learning and memory deficits (Morris *et al.*, 1982; Phillips & LeDoux, 1992), and that the limbic system is involved in anxiety and aggression (Davis, 1992; Guillot *et al.*, 1994). Because tlx is involved in ocular development (Hollemann *et al.*, 1998), tlx knockout mice additionally have a dramatic reduction in retina thickness and optic nerve diameter with associated blindness (Yu *et al.*, 2000; Young *et al.*, 2002; Miyawaki *et al.*, 2004; Zhang *et al.*, 2006). Comprehensive behavioural studies showed that impaired visual abilities severely influence the performance of rodents in the Morris water-maze and contextual fear-conditioning paradigm (Buhot *et al.*, 2001; Garcia *et al.*, 2004; Clapcote *et al.*, 2005), and also have an impact on anxiety (Cook *et al.*, 2001; Garcia *et al.*, 2004). We therefore asked whether the set of behavioural abnormalities found in tlx mutants are caused by the brain defects *per se* or if they are a reflection of the mutation-associated blindness.

To circumvent the disadvantage of the tlx germline deletion leading to blindness, we analysed tlx mutants with intact visual abilities. Therefore, we engineered a floxed tlx allele for conditional inactivation of the tlx gene. Because we have observed expression of the calmodulin-dependent protein kinase II alpha (CaMKIIα) gene during development in the brain, but not in the eye, we investigated if CaMKIIα gene-driven expression of the Cre recombinase (CaMKCre; Casanova *et al.*, 2001) would allow targeting of tlx in the developing brain, sparing the eye. Indeed, mutants obtained by crossing $tlx^{flox/flox}$ and CaMKCre mice show similar brain defects to tlx germline mutants, but show no abnormalities in the retina and are not blind. By comparing results from several behavioural tests of the conditional tlx mutants with tlx null mice, we conclude that the poor performance of tlx germline mutants in fear conditioning (Roy *et al.*, 2002) results from a deficit at the level of vision. In contrast, the increased

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aggressiveness and reduced anxiety appears to be due to the structural brain abnormalities.

Materials and methods

cDNA synthesis and quantitative polymerase chain reaction (PCR)

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) and used to generate cDNA with SuperScriptII (Invitrogen GmbH, Karlsruhe, Germany). Quantitative PCR was performed using TaqMan Gene Expression Assays according to the manufacturer's recommendations (Applied Biosystems, CA, USA). Relative expression was calculated by the ΔC_T method using hypoxanthine guanine phosphoribosyl transferase (hprt) as a housekeeping gene. Each assay was performed in duplicate.

Generation of mice

To generate tlx^{flox} mice, we used homologous recombination in embryonic stem cells. Embryonic stem cells positive for the conditional tlx allele and the selection cassette ($Tlx^{floxFRTneo/+}$) were injected into C57BL6 blastocysts. After crossing chimerics to C57BL6 animals to detect germline transmission, the positive selection marker PGKneo flanked by FRT sites was removed *in vivo* using Flp deleter mice (Rodriguez *et al*., 2000). Tlx^{flox} and CaMKCre mice were backcrossed to C57BL6 for more than 10 generations before use.

X-Gal staining

Embryos were fixed in 4% paraformaldehyde (PFA) for 2 h at 4 °C, incubated in X-Gal staining solution [in mM: $K_3[Fe(CN)_6]$, 4; $K_4[Fe(CN)_6]$, 4; $MgCl_2$, 2; X-Gal in phosphate-buffered saline (PBS), 0.4 mg/mL] at 37 °C overnight. Then, embryos were immersed in 30% sucrose in PBS until they were sunk, embedded in tissue-freezing medium and sectioned at a cryostat. Twenty-micremetre sections were stained again in X-Gal solution overnight and counterstained with eosin.

Immunohistochemistry

Embryonic brains were fixed in 4% PFA overnight at 4 °C, embedded in 1% agarose and processed as described previously (Berger *et al*., 2006).

Animal experiments

All animal procedures were approved by the German animal welfare office of the Regierungspräsidium Karlsruhe, Germany. Animals were anaesthetized with CO_2 and killed by cervical dislocation.

Behavioural studies

All experiments were performed with 3–7-month-old mice in a C57BL6 genetic background. Test procedures were essentially performed as described earlier (Fleischmann *et al*., 2003; Ridder *et al*., 2005).

Morris water-maze

The setup for the visual version of the Morris water-maze is described elsewhere (Fleischmann *et al*., 2003). In the visible platform test, the platform was black, raised slightly above the water surface, and additionally was visualized with an orange and black falcon tube. Mice were trained with a protocol consisting of four training trials per day with 60 min intertrial intervals. After four trials with the visible platform on the first day, the animals were trained for the following 4 days with the hidden platform. The probe trial was performed on Day 5. Recording and analysis of acquisition was done as described previously (Fleischmann *et al*., 2003). Presented here are the acquisition curves of the visual platform data and the probe trials of the invisible platform task (percentage of time spent either in the target quadrant or in the other quadrants, respectively): visible trials (male $tlx^{CaMKII\alpha\text{-}Cre}$ $n = 7$; $tlx^{flox/flox}$ $n = 8$); probe trials (female $tlx^{CaMKII\alpha\text{-}Cre}$ $n = 6$; $tlx^{flox/flox}$ $n = 8$/male $tlx^{CaMKII\alpha\text{-}Cre}$ $n = 7$; $tlx^{flox/flox}$ $n = 8$).

The dark–light box procedure was carried out as described previously (Ridder *et al*., 2005). The latency to the first exit, number of exits and total time in the light compartment were recorded for 5 min (female $tlx^{CaMKII\alpha\text{-}Cre}$ $n = 6$; $tlx^{flox/flox}$ $n = 8$/male $tlx^{CaMKII\alpha\text{-}Cre}$ $n = 8$; $tlx^{flox/flox}$ $n = 9$).

Hotplate

The temperature was set at 53 °C, and a 45 s cut-off was introduced to prevent injury of mice. Latency to first reaction, i.e. licking of paws or jumping, was assessed.

Fear conditioning

Fear conditioning was performed as described previously (Ridder *et al*., 2005): (female $tlx^{CaMKII\alpha\text{-}Cre}$ $n = 6$; $tlx^{flox/flox}$ $n = 7$/male $tlx^{CaMKII\alpha\text{-}Cre}$ $n = 8$; $tlx^{flox/flox}$ $n = 9$).

Statistical analysis

After the Shapiro–Wilk test established that the data were not normally distributed, the analysis was essentially performed with the non-parametric Kruskal–Wallis test followed by the Mann–Whitney U-test to detect genotype effects within the respective gender.

Results

Brain-specific deletion of tlx

To establish a brain-specific tlx mutation we generated a conditional tlx allele in which exon 2 is flanked by loxP sites (tlx^{flox}), using homologous recombination in embryonic stem cells. Thereby, the tlx gene can be inactivated by selective expression of the Cre recombinase. We took advantage of the 'bacterial artificial chromosome' technology guaranteeing faithful expression of the Cre recombinase under the control of the CaMKIIα regulatory sequences (CaMKCre; Casanova *et al*., 2001). By breeding the ROSA26 reporter line (Soriano, 1999) with CaMKCre transgenic mice ($ROSA26^{CaMKCre}$), we followed CaMKIIα gene-specific Cre-dependent recombination. Intensive X-Gal staining is evident at E12.5 in the forebrain around the lateral ventricles, in the ganglionic eminences, in the parietal cortex and the post-optic area, and in the midbrain around the aqueduct of Sylvius in $ROSA26^{CaMKCre}$ mice, whereas no staining was detected in controls (Fig. 1A–C). We found almost no recombination (one out of seven mutant embryos showed blue staining in a few cells of the eye) in the eye of $ROSA26^{CaMKCre}$ mice and no staining at all in controls (Fig. 1D and E).

These findings prompted us to investigate if the CaMKCre transgene could be of use to genetically separate tlx functions in the brain from functions in the eye. We generated mice lacking tlx mRNA in CaMKIIα expression domains by crossing CaMKCre mice with $tlx^{flox/flox}$ animals to obtain $tlx^{flox/flox}$; CaMKCre ($tlx^{CaMKCre}$) mice. Quantification of Cre mRNA by quantitative PCR in $tlx^{CaMKCre}$ mutants showed that Cre mRNA is expressed moderately at E14.5 in the medial and lateral ganglionic eminences, whereas it is hardly detectable in the embryonic eye at this age (ganglionic eminences 0.21, $n = 3$; eye 0.01, $n = 4$). Surprised about the early appearance of CaMKIIα gene-driven Cre mRNA during development, we wished to confirm the embryonic expression by immunohistochemistry. Using a Cre-specific antibody, we detected Cre expression in ganglionic eminences, the ventricular zone, the intermediate zone (neostriatum) and medial/dorsal to the olfactory tract (Fig. 2A–F). These data indicate that the CaMKCre transgene will deplete tlx in the brain, without leading to recombination in the eye.

To verify embryonic expression of endogenous CaMKIIα, we quantified the expression level in wild-type brains from E12.5 to E18.5, and found a relatively low but constant endogenous

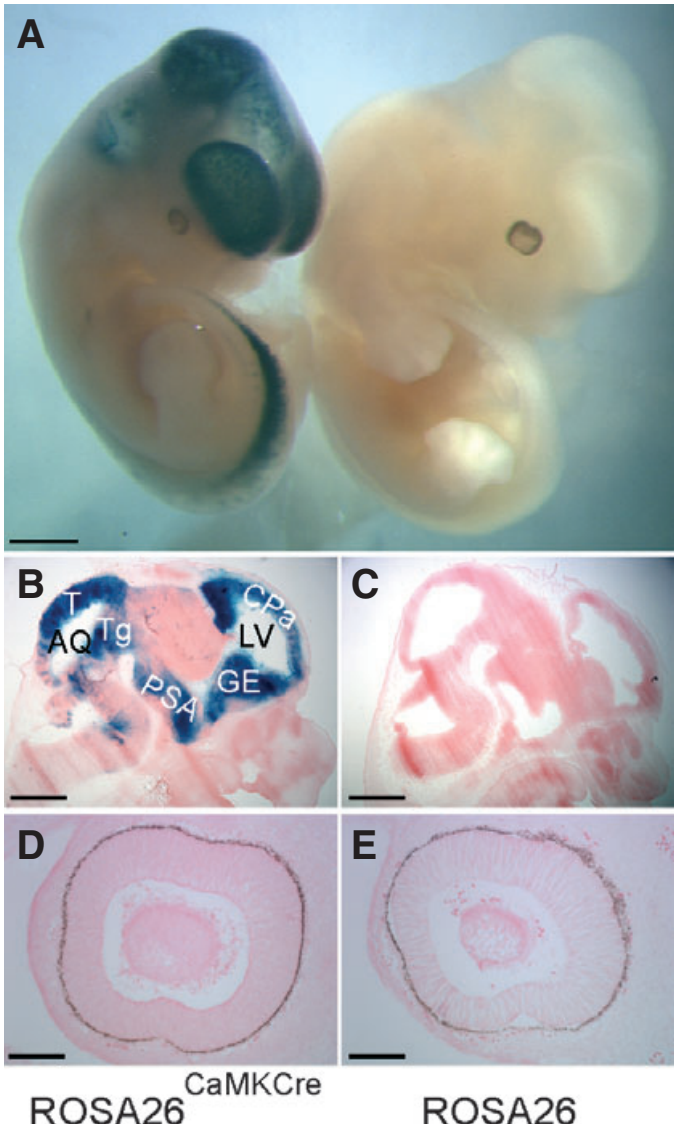

FIG. 1. The CaMKCre transgene leads to recombination in the brain, but not in the eye: (A) Recombination was detected in the hemispheres, midbrain and spinal cord in $ROSA^{CaMKCre}$ embryos (left), whereas no recombination was detected in controls by X-Gal staining (right). (B) Representative sagittal section through an E12.5 $ROSA26^{CaMKCre}$ embryo. Recombination in the brain was detected around the lateral ventricles (LV) in the parietal cortex (CPa), the ganglionic eminences (GE), the post-optic area (PSA), and around the aqueduct of Sylvius (AQ) in the tectum (T) and the tegmentum (Tg). (C–E) No recombination was detected in sagittal control sections (C) and the eyes of E12.5 $ROSA26^{CaMKCre}$ (D) and ROSA26 (E) embryos. Scale bars: 1 mm (A); 200 μm (B and C); 25 μm (D and E).

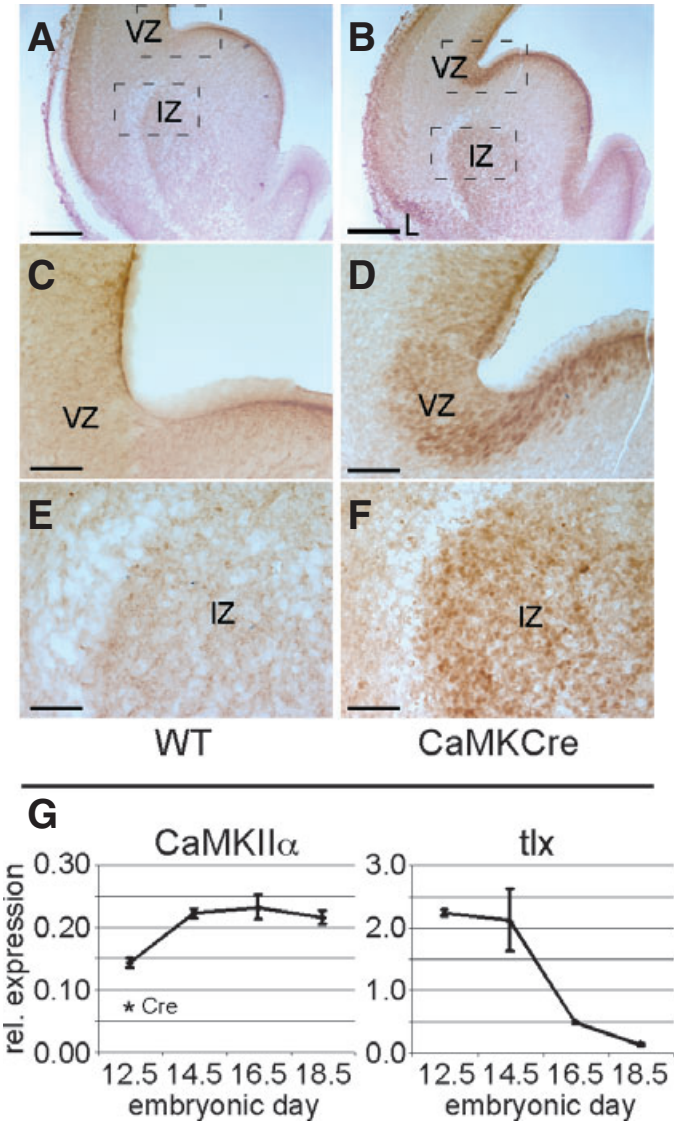

FIG. 2. The CaMKIIα promoter is active in the embryonic mouse brain: boxes in (A) and (B) represent (C and E) and (D and F), respectively. No Cre-specific staining was detected in coronal sections of wild-type (WT) brains (A, C, E), but in the ventricular zone (VZ), the ganglionic eminence, the intermediate zone (IZ; neostriatum) and additionally medial/dorsal to the lateral olfactory tract (L) in E14.5 brains of CaMKCre transgenic embryos on the basis of immunohistochemistry using a Cre-specific antibody (B, D, F). Scale bars: 50 μm (A and B); 12.5 μm (C–F). (G) Quantification of embryonic CaMKIIα (left) and tlx (right) mRNA expression in the mouse WT brain using quantitative PCR. We found CaMKIIα mRNA expressed in all embryonic stages tested. The expression level is low in comparison to tlx, but relatively constant from E12.5 to E18.5. The star (*) represents the level of Cre expression at E12.5 in the brain of $tlx^{CaMKCre}$ mutants, $n = 4$. Error bars represent SEM.

expression level of CaMKIIα mRNA in comparison to tlx mRNA (Fig. 2G). To show a comparable expression level of endogenous CaMKIIα mRNA and transgenic Cre mRNA, driven by CaMKIIα regulatory sequences in $tlx^{CaMKCre}$ mice, we used Cre-specific TaqMan primer (custom-made) in a quantitative PCR approach. We found that the expression level of the mRNA for Cre in $tlx^{CaMKCre}$ mice is in the same range as the mRNA of the endogenous CaMKIIα gene (Fig. 2G, indicated by a star), demonstrating functionality of the BAC transgene.

To investigate the level of recombination in the brain, progenitor cell domains and eyes from $tlx^{CaMKCre}$ embryos and adult mice, we isolated RNA from these tissues and quantified the amount of non-recombined tlx mRNA. We used a quantitative PCR-based approach, in which one TaqMan primer is located in the floxed tlx exon. After successful Cre-mediated deletion of exon 2, only one primer can bind and thereby only residual non-recombined tlx mRNA is detected. Tlx mRNA expression is strongly reduced in mutant brains at E12.5 but not yet completely lost, whereas it is hardly detectable at E14.5 (Table 1). In the medial and lateral ganglionic eminences of E14.5 and E15.5 embryos, almost all tlx mRNA is recombined (Table 1). Additionally, by using mutants expressing a reporter gene under the control of the tlx regulatory regions, we could show that tlx is expressed exclusively in radial glial cells at this stage (our unpublished observations). Almost all cells in the embryonic tlx expression domain (VZ) are radial glial cells (Mori *et al.*, 2005). Because radial glial cells are proven to be the progenitors of the entire adult brain (Anthony *et al.*, 2004), and tlx expression is abolished in embryonic germinal regions in $tlx^{CaMKCre}$ mice (Table 1), we conclude that tlx expression is entirely eliminated in adult $tlx^{CaMKCre}$ brains. As expected from the $ROSA26^{CaMKCre}$ recombination data (Fig. 1), we found no significant difference in the amount of tlx mRNA in embryonic and adult eyes from $tlx^{CaMKCre}$ mutants and controls (Table 1). In summary, by using CaMKIIα gene-driven expression of Cre recombinase, we established a mouse line with depleted expression of the orphan receptor tlx in neurogenic areas of the embryonic brain, whereas expression in the eye is not affected.

TABLE 1. Quantification of tlx mRNA in $tlx^{CaMKCre}$ mutants and controls

	Relative tlx expression	
	Control	Mutant
Total brain E12.5	2.41 ± 0.36	0.42 ± 0.03
Total brain E14.5	1.50 ± 0.1	0.09
LGE/MGE E14.5	3.31	0.01
LGE/MGE E15.5	2.03	0.01
Eye E14.5	1.28	1.18

RNA was isolated from total embryonic brain, the embryonic lateral and medial ganglionic eminences (LGE/MGE), and adult eyes. The RNA was used in a quantitative PCR-based approach to quantify functional (non-recombined) tlx mRNA. Data represent tlx expression (ΔC_T) normalized against the house keeping gene *hprt*. Total brain: E12.5 $n = 2$ for $tlx^{flox/flox}$ and $tlx^{CaMKCre}$; E14.5 $n = 3$ for $tlx^{flox/flox}$, $n = 1$ for $tlx^{CaMKCre}$; LGE/MGE: E14.5 $n = 2$ for $tlx^{flox/flox}$, $n = 3$ for $tlx^{CaMKCre}$, pooled material; E15.5 $n = 2$ for $tlx^{flox/+}$ and $tlx^{CaMKCre}$, pooled material; eye: E14.5 $n = 3$ for $tlx^{flox/flox}$, $n = 4$ for $tlx^{CaMKCre}$, pooled material; adult $n = 5$ for $tlx^{flox/flox}$ and $tlx^{CaMKCre}$. Values are given as mean ± SEM.

Brain-specific tlx mutants show regular visual abilities

Cre-mediated inactivation of the tlx gene leads to similar structural brain abnormalities as reduced brain size, enlarged lateral ventricles and a severely reduced hippocampus (Fig. 3A) and stunted growth parameter (data not shown), as found in tlx germline mutants (Monaghan *et al.*, 1997; Young *et al.*, 2002). Because tlx expression is absent in the brain of $tlx^{CaMKCre}$ mice, but unaltered in the mutant eye (Figs 1 and 2), we analysed if the mutants have normal eye morphology and normal vision.

We found no gross morphological changes in adult retinae of $tlx^{CaMKCre}$ mutants (Fig. 3B) in contrast to adult tlx germline retinae that show disorganization and reduction in thickness of several retinal layers (Yu *et al.*, 2000; Young *et al.*, 2002; Miyawaki *et al.*, 2004; Zhang *et al.*, 2006). To demonstrate that $tlx^{CaMKCre}$ mutants have normal vision, we used two standard tests. The visual placing task is a qualitative test, in which the mice are lowered towards the edge of a table by their tail, resulting in extension of the forepaws prior to touching the table if they can see. Both, control and $tlx^{CaMKCre}$ mice showed the expected behaviour in this task, i.e. extending the forepaws before reaching the table (data not shown). Furthermore, a visual version of the Morris water-maze was carried out in which the animals had to locate a visible platform and climb on it. Using a four trials per day test procedure, $tlx^{CaMKCre}$ mice showed a similar learning curve to find and climb on the platform as control animals (Fig. 3C), indicating that the conditional tlx mutants are not blind in contrast to tlx germline mutants (Zhang *et al.*, 2006).

Reduced anxiety and increased aggressiveness in brain-specific tlx mutants

Because tlx germline mutants are less anxious than controls (Roy *et al.*, 2002; Young *et al.*, 2002), we tested if the $tlx^{CaMKCre}$ mutants show reduced anxiety using the dark–light box. In this test, both female and male mutant mice spent significantly more time in the anxiety-related lit compartment than controls (Fig. 3D). Moreover, mutant mice exhibited a clear reduction of latencies to enter the lit compartment and entered it significantly more often than control littermates (data not shown). Furthermore, similar to tlx germline mutants (Monaghan *et al.*, 1997; Roy *et al.*, 2002; Young *et al.*, 2002; Abrahams *et al.*, 2005), $tlx^{CaMKCre}$ mice are hyperaggressive and frequently attack other mice despite their reduced body size.

Regular contextual, associative and spatial learning in brain-specific tlx mutants

Because it was shown that tlx germline mutants are impaired in fear conditioning (Roy *et al.*, 2002), we investigated if we can find similar defects in this paradigm using conditional tlx mutants with intact visual abilities. The amount of freezing, as reflected by immobility, represents a correlate of fear-associated memory. After excluding an altered pain sensitivity by exposing the animals to the hotplate test (data not shown), we observed no differences between mutants and controls during the 2.5-min training period. Twenty-four hours after training, all groups of animals showed a good freezing response (> 40%) when re-exposed to the training context (Fig. 3E), in contrast to tlx germline mutants (Roy *et al.*, 2002). There were no statistical differences between male or female $tlx^{CaMKCre}$ mice and their respective control groups. The same holds true for cue-dependent fear conditioning that was tested 48 h after the initial training session (Fig. 3F).

Like tlx germline mutants, conditional $tlx^{CaMKCre}$ mutants have a reduced brain size and, in particular, dramatically reduced hippocam-

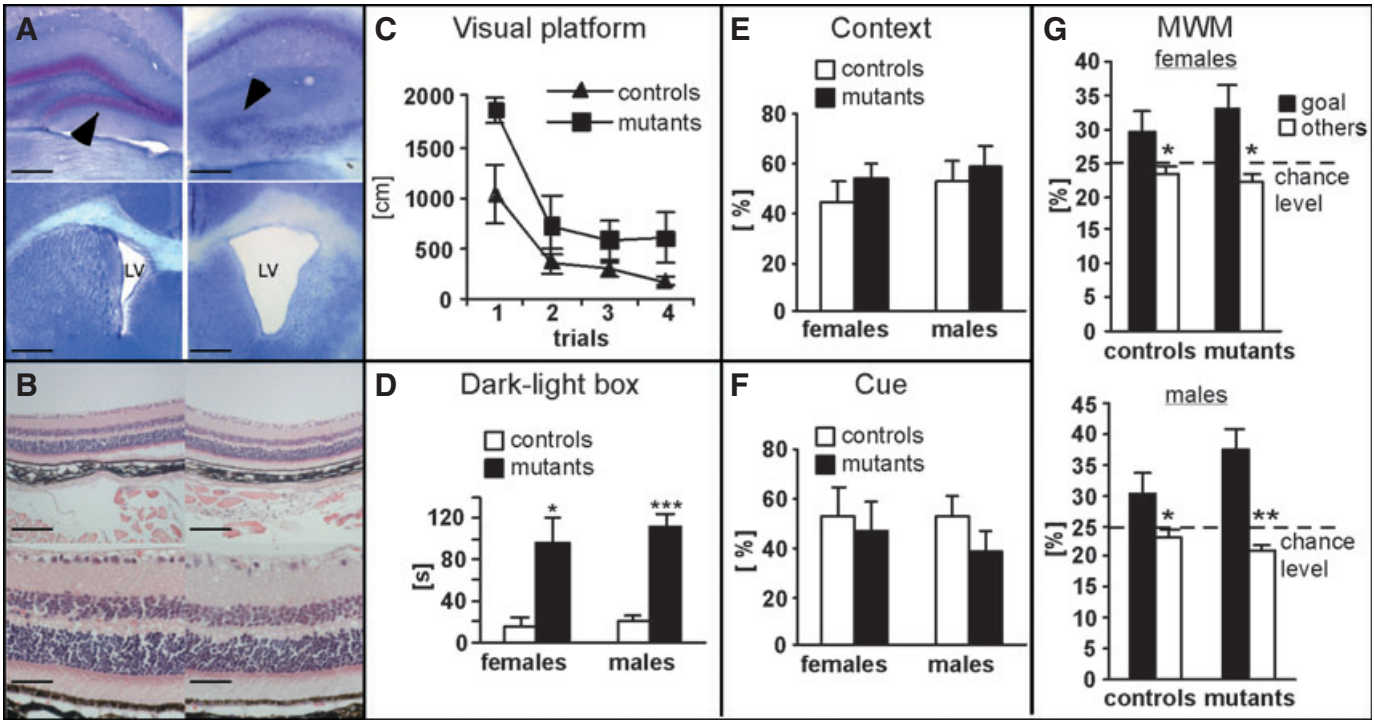

FIG. 3. Structural and functional abnormalities in adult $tlx^{CaMKCre}$ mutants: (A) Nissl-stained coronal sections from $tlx^{flox/flox}$ control animals show the normal structured dentate gyrus (arrowhead) and lateral ventricle (LV; left). Mutant littermates show a reduced dentate gyrus (arrowhead) and enlarged LV (right). Scale bars: dentate gyrus 100 μm; LV 50 μm. (B) No morphological difference between wild-type (left) and mutant (right) H&E-stained retinas were found. Scale bars: upper 25 μm; lower 8 μm. (C) $Tlx^{CaMKCre}$ mutants were able to localize the platform in the visual version of the Morris water-maze (MWM; only males shown). The *y*-axis represents the total distance moved. (D) The time spent in the aversive light compartment was significantly increased in mutants in the dark–light box. $H = 19.145$, $P = 0.000$; U(females) $= 4.5$, $P = 0.011$; U(males) $= 0.0$, $P = 0.001$. (E and F) $Tlx^{CaMKCre}$ mutants and their respective controls show no statistical difference in freezing response in contextual (E) and cued fear conditioning (F). The *y*-axis represents percentage freezing. (G) All groups tested in the Morris water-maze show significant memory retention for the platform position in the probe trial, as shown by the time spent in the goal quadrant (%). U(female controls) $= 50$, $P = 0.029$; U(female mutants) $= 31$, $P = 0.019$; U(male controls) $= 48$, $P = 0.046$; U(male mutants) $= 45$, $P = 0.004$. All error bars represent SEM. $*P < 0.05$; $**P < 0.01$; $***P \leq 0.001$.

pal structures (Fig. 3A; Monaghan *et al.*, 1997). There is a strong correlation between hippocampal damage and impaired spatial memory, therefore we wished to find out if $tlx^{CaMKCre}$ mutants could solve the hidden platform version of the Morris water-maze, a hippocampus-dependent learning paradigm (Morris *et al.*, 1982). Both mutant and control mice showed a significant memory retention for the former location of the platform in the probe trial, a stringent measure of spatial selectivity (Fig. 3G).

From the experiments presented here we conclude that $tlx^{CaMKCre}$ mutants pass the learning and memory paradigms due to their intact visual abilities, in contrast to tlx germline mutants that fail because of blindness (Yu *et al.*, 2000; Roy *et al.*, 2002; Young *et al.*, 2002; Miyawaki *et al.*, 2004; Zhang *et al.*, 2006). The violent aggressiveness and reduced anxiety seen in tlx germline mutants is present in $tlx^{CaMKCre}$ mutants.

Discussion

By deleting tlx in a restricted cell population we established a mouse line with defects similar to tlx germline mutants in the structure of the brain, but with normal visual abilities. On the basis of Northern blot analysis in mice, CaMKIIα mRNA expression was not detected until postnatal day 1 (Bayer *et al.*, 1999). However, due to the higher sensitivity of the quantitative PCR approach, we detected endogenous CaMKIIα expression prenatally in neurogenic areas of the embryonic brain. Accordingly, we found deletion of tlx mRNA in $tlx^{CaMKCre}$ mutants in the lateral and medial ganglionic eminences during development, leading to similar malformations of the brain as found in tlx germline mutants (Monaghan *et al.*, 1997). In $tlx^{CaMKCre}$ mutant eyes on the other hand, we found no reduction of tlx mRNA by quantitative PCR. Consequently, we could demonstrate that these animals have a normal retina morphology and are not blind like tlx germline mutants.

In contrast to tlx germline mutants that show impaired learning (Roy *et al.*, 2002), we found a regular performance in fear conditioning and spatial learning in $tlx^{CaMKCre}$ mutants, despite the severely reduced hippocampus. In line with the idea that disconnecting rather than destroying parts of the brain may impair spatial learning (D'Hooge & De Deyn, 2001), it is possible that despite the reduced size of the hippocampus, connections relevant for this kind of learning might be intact. Blindness leads to impairment in contextual fear conditioning despite the presence of non-visual cues in this paradigm (Clapcote *et al.*, 2005). This might explain the bad performance of blind tlx germline mutants in the tone-dependent fear conditioning paradigm.

In summary, our data show that the reduced anxiety and the most striking tlx phenotype, severe aggression, is intrinsic to the lack of tlx in the brain, but that the reduced cognitive performance in learning and memory is a secondary effect due to blindness associated with the tlx germline mutation.

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Abbreviations

CaMKIIα, calmodulin-dependent protein kinase II alpha; CaMKCre, construct driving Cre recombinase expression under the control of the CaMKIIα gene; E, embryonic age; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; $ROSA26^{CaMKCre}$, transgenic mice carrying the modified ROSA26 and CaMKCre allele; tlx, *tailless*; $tlx^{CaMKCre}$, mice carrying $tlx^{flox/flox}$ alleles and one CaMKCre allele; tlx^{flox}, tlx allele in which exon 2 is flanked by loxP sites; $tlx^{flox/flox}$, mice carrying two tlx^{flox} alleles.

References

Abrahams, B.S., Kwok, M.C., Trinh, E., Budaghzadeh, S., Hossain, S.M. & Simpson, E.M. (2005) Pathological aggression in 'fierce' mice corrected by human nuclear receptor 2E1. *J. Neurosci.*, **25**, 6263–6270.

Anthony, T.E., Klein, C., Fishell, G. & Heintz, N. (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron*, **41**, 881–890.

Bayer, K.U., Lohler, J., Schulman, H. & Harbers, K. (1999) Developmental expression of the CaM kinase II isoforms: ubiquitous gamma- and delta-CaM kinase II are the early isoforms and most abundant in the developing nervous system. *Brain Res. Mol. Brain Res.*, **70**, 147–154.

Berger, S., Wolfer, D.P., Selbach, O., Alter, H., Erdmann, G., Reichardt, H.M., Chepkova, A.N., Welzl, H., Haas, H.L., Lipp, H.P. & Schutz, G. (2006) Loss of the limbic mineralocorticoid receptor impairs behavioral plasticity. *Proc. Natl Acad. Sci. USA*, **103**, 195–200.

Buhot, M.C., Dubayle, D., Malleret, G., Javerzat, S. & Segu, L. (2001) Exploration, anxiety, and spatial memory in transgenic anophthalmic mice. *Behav. Neurosci.*, **115**, 455–467.

Casanova, E., Fehsenfeld, S., Mantamadiotis, T., Lemberger, T., Greiner, E., Stewart, A.F. & Schutz, G. (2001) A CamKIIalpha iCre BAC allows brain-specific gene inactivation. *Genesis*, **31**, 37–42.

Clapcote, S.J., Lazar, N.L., Bechard, A.R., Wood, G.A. & Roder, J.C. (2005) NIH Swiss and Black Swiss mice have retinal degeneration and performance deficits in cognitive tests. *Comp. Med.*, **55**, 310–316.

Cook, M.N., Williams, R.W. & Flaherty, L. (2001) Anxiety-related behaviors in the elevated zero-maze are affected by genetic factors and retinal degeneration. *Behav. Neurosci.*, **115**, 468–476.

D'Hooge, R. & De Deyn, P.P. (2001) Applications of the Morris water maze in the study of learning and memory. *Brain Res. Brain Res. Rev.*, **36**, 60–90.

Davis, M. (1992) The role of the amygdala in fear and anxiety. *Annu. Rev. Neurosci.*, **15**, 353–375.

Fleischmann, A., Hvalby, O., Jensen, V., Strekalova, T., Zacher, C., Layer, L.E., Kvello, A., Reschke, M., Spanagel, R., Sprengel, R., Wagner, E.F. & Gass, P. (2003) Impaired long-term memory and NR2A-type NMDA receptor-dependent synaptic plasticity in mice lacking c-Fos in the CNS. *J. Neurosci.*, **23**, 9116–9122.

Garcia, M.F., Gordon, M.N., Hutton, M., Lewis, J., McGowan, E., Dickey, C.A., Morgan, D. & Arendash, G.W. (2004) The retinal degeneration (rd) gene seriously impairs spatial cognitive performance in normal and Alzheimer's transgenic mice. *Neuroreport*, **15**, 73–77.

Guillot, P.V., Roubertoux, P.L. & Crusio, W.E. (1994) Hippocampal mossy fiber distributions and intermale aggression in seven inbred mouse strains. *Brain Res.*, **660**, 167–169.

Hollemann, T., Bellefroid, E. & Pieler, T. (1998) The Xenopus homologue of the Drosophila gene tailless has a function in early eye development. *Development*, **125**, 2425–2432.

Land, P.W. & Monaghan, A.P. (2003) Expression of the transcription factor, tailless, is required for formation of superficial cortical layers. *Cereb. Cortex*, **13**, 921–931.

Miyawaki, T., Uemura, A. & Dezawa, M., Yu, R.T., Ide, C., Nishikawa, S., Honda, Y., Tanabe, Y. & Tanabe, T. (2004) Tlx, an orphan nuclear receptor, regulates cell numbers and astrocyte development in the developing retina. *J. Neurosci.*, **24**, 8124–8134.

Monaghan, A.P., Bock, D., Gass, P., Schwager, A., Wolfer, D.P., Lipp, H.P. & Schutz, G. (1997) Defective limbic system in mice lacking the tailless gene. *Nature*, **390**, 515–517.

Monaghan, A.P., Grau, E., Bock, D. & Schutz, G. (1995) The mouse homolog of the orphan nuclear receptor tailless is expressed in the developing forebrain. *Development*, **121**, 839–853.

Mori, T., Buffo, A. & Gotz, M. (2005) The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis. *Curr. Top Dev. Biol.*, **69**, 67–99.

Morris, R.G., Garrud, P., Rawlins, J.N. & O'Keefe, J. (1982) Place navigation impaired in rats with hippocampal lesions. *Nature*, **297**, 681–683.

Phillips, R.G. & LeDoux, J.E. (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav. Neurosci.*, **106**, 274–285.

Ridder, S., Chourbaji, S., Hellweg, R., Urani, A., Zacher, C., Schmid, W., Zink, M., Hortnagl, H., Flor, H., Henn, F.A., Schutz, G. & Gass, P. (2005) Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. *J. Neurosci.*, **25**, 6243–6250.

Rodriguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F. & Dymecki, S.M. (2000) High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat. Genet.*, **25**, 139–140.

Roy, K., Kuznicki, K., Wu, Q., Sun, Z., Bock, D., Schutz, G., Vranich, N. & Monaghan, A.P. (2004) The Tlx gene regulates the timing of neurogenesis in the cortex. *J. Neurosci.*, **24**, 8333–8345.

Roy, K., Thiels, E. & Monaghan, A.P. (2002) Loss of the tailless gene affects forebrain development and emotional behavior. *Physiol. Behav.*, **77**, 595–600.

Shi, Y., Chichung Lie, D., Taupin, P., Nakashima, K., Ray, J., Yu, R.T., Gage, F.H. & Evans, R.M. (2004) Expression and function of orphan nuclear receptor TLX in adult neural stem cells. *Nature*, **427**, 78–83.

Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.*, **21**, 70–71.

Stenman, J.M., Wang, B. & Campbell, K. (2003) Tlx controls proliferation and patterning of lateral telencephalic progenitor domains. *J. Neurosci.*, **23**, 10568–10576.

Young, K.A., Berry, M.L., Mahaffey, C.L., Saionz, J.R., Hawes, N.L., Chang, B., Zheng, Q.Y., Smith, R.S., Bronson, R.T., Nelson, R.J. & Simpson, E.M. (2002) Fierce: a new mouse deletion of Nr2e1; violent behaviour and ocular abnormalities are background-dependent. *Behav. Brain Res.*, **132**, 145–158.

Yu, R.T., Chiang, M.Y., Tanabe, T., Kobayashi, M., Yasuda, K., Evans, R.M. & Umesono, K. (2000) The orphan nuclear receptor Tlx regulates Pax2 and is essential for vision. *Proc. Natl Acad. Sci. USA*, **97**, 2621–2625.

Zhang, C.L., Zou, Y., Yu, R.T., Gage, F.H. & Evans, R.M. (2006) Nuclear receptor TLX prevents retinal dystrophy and recruits the corepressor atrophin1. *Genes Dev.*, **20**, 1308–1320.