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Tlx and *Pax6* co-operate genetically to establish the pallio-subpallial boundary in the embryonic mouse telencephalon

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SUMMARY

We have examined the role of *Tlx*, an orphan nuclear receptor, in dorsal-ventral patterning of the mouse telencephalon. *Tlx* is expressed broadly in the ventricular zone, with the exception of the dorsomedial and ventromedial regions. The expression spans the pallio-subpallial boundary, which separates the dorsal (i.e. pallium) and ventral (i.e. subpallium) telencephalon. Despite being expressed on both sides of the pallio-subpallial boundary, *Tlx* homozygous mutants display alterations in the development of this boundary. These alterations include a dorsal shift in the expression limits of certain genes that abut at the pallio-subpallial boundary as well as the abnormal formation of the radial glial palisade that normally marks this boundary. The *Tlx* mutant phenotype is similar to, but less severe than, that seen in *Small eye* (i.e. *Pax6*) mutants. Interestingly, removal of one allele of *Pax6* on the homozygous *Tlx* mutant background significantly worsens the phenotype. Thus *Tlx* and *Pax6* cooperate genetically to regulate the establishment of the pallio-subpallial boundary. The patterning defects in the *Tlx* mutant telencephalon result in a loss of region-specific gene expression in the ventral-most pallial region. This correlates well with the malformation of the lateral and basolateral amygdala in *Tlx* mutants, both of which have been suggested to derive from ventral portions of the pallium.

Key words: Acetylcholine esterase, Amygdala, DLX, GSH2, MASH1, Neurogenin 2, NR2E1, Pallium, Pallio-subpallial boundary, Subpallium, Tailless, Ventral pallium, Mouse

INTRODUCTION

The embryonic telencephalon can be divided into distinct dorsal and ventral domains. The dorsal domain (i.e. the pallium) generates projection neurons of the neocortex, hippocampus and piriform cortex (Bayer and Altman, 1991). The ventral telencephalon (i.e. the subpallium) gives rise to the striatum and globus pallidus of the basal ganglia as well as to the basal forebrain (Smart and Sturrock, 1979). In addition, the subpallium also contributes GABAergic interneurons to pallial-derived structures via tangential migration (for a review, see Marin and Rubenstein, 2001). Another prominent nuclear complex, the amygdala, has received less attention. It is currently unclear where the different nuclei of the amygdala are generated (Swanson and Petrovich, 1998), however, it has been suggested that both pallial and subpallial regions contribute to its formation (Fernandez et al., 1998; Puelles et al., 1999; Puelles et al., 2000).

The dorsal and ventral portions of the telencephalon are separated by a morphologically identifiable radial glial palisade (Stoykova et al., 1997; Götz et al., 1998; Chapouton et al., 1999). The location of this glial palisade marks the pallio-subpallial boundary and coincides with the juxtaposition of cells expressing distinct developmental control genes. Precursor cells on the subpallial side express the homeobox gene *Gsh2* and the bHLH gene *Mash1* (*Ascl1* – Mouse Genome Informatics), whereas cells on pallial side express the paired homeobox gene *Pax6* as well as the bHLH genes neurogenin 1 (*Ngn1*) and *Ngn2*. Several of these developmental control genes play important roles in the establishment and/or maintenance of this boundary (Stoykova et al., 1997; Stoykova et al., 2000; Chapouton et al., 1999; Chapouton et al., 2001; Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001). For example, *Gsh2* is required to repress pallial gene expression in the subpallium and in its absence ventricular zone cells in the lateral ganglionic eminence (LGE) are misspecified, resulting in a truncation of the LGE-derived striatum (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001). Conversely, in *Small eye* (*Sey*) mutants, which contain a point mutation in the *Pax6* gene (Hill et al., 1991), the pallium is misspecified by ectopic ventral gene expression along with restricted loss of pallial gene expression (Stoykova et al., 1996; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001; Muzio et al., 2002).

The above-mentioned genes are unlikely to be the only molecular players involved in the establishment and/or maintenance of the pallio-subpallial boundary. In the present study, we have examined a role for the orphan nuclear receptor

Tlx (also known as *tailless*; *Nr2e1* – Mouse Genome Informatics) (Yu et al., 1994; Monaghan et al., 1995) in this process. Homozygous *Tlx* mutants have been shown to exhibit altered telencephalic morphology as well as abnormally aggressive behavior (Monaghan et al., 1997; Young et al., 2002). However, the underlying mechanisms that lead to these defects have not been elucidated. We show here that *Tlx* is required for correct dorsal-ventral patterning of the embryonic telencephalon and for the normal differentiation of amygdalar structures in the ventrolateral telencephalon. Moreover, our data demonstrate that *Tlx* and *Pax6* cooperate genetically to establish the pallio-subpallial boundary.

MATERIALS AND METHODS

Animals and genotyping

Tlx mice and embryos (Yu et al., 2000) were genotyped by PCR using the following primers: 3TlxlacZ – ATT CGC GTC TGG CCT TCC TGT AG, 3Tlxwt – ACC CTG GGG AGT ACC TGG TTT CC, and 5Tlxcommon – CTC TTC CCG TCT TTC AGG CCG. The wild-type allele results in a 177 bp band, whereas the targeted allele gives a 453 bp band. *Sey* mice and embryos were typed visually based on eye morphology and eye size as described previously (Hill et al., 1991). *Dlx5/6-cre-IRES-EGFP* mice were genotyped as described in Stenman et al. (Stenman et al., 2003). B6;129-Gtrosa26^{tm1Sho} (*gtROSA*) mice (Mao et al., 1999) were obtained from Jackson laboratories and genotyped as described in Jackson's Gtrosa genotyping protocol available online (http://www.jax.org). Fate mapping studies were performed on mice that were double-transgenic for the *Dlx5/6-cre-IRES-EGFP* and *gtROSA* alleles. For staging of embryos, the morning of the appearance of the vaginal plug was designated as embryonic day 0.5 (E0.5).

Histological analysis

Embryos were fixed and sectioned on a cryostat as previously described (Toresson et al., 2000). Adult brains were removed from 3-week- to 8-month-old animals and fixed overnight by immersion in 4% paraformaldehyde at 4°C before sinking in 30% sucrose and sectioning at 30 μm on a cryostat. Immunohistochemistry was performed on slide-mounted sections for the embryonic tissue, whereas the adult brain sections were immunostained under free-floating conditions. The protocol was as previously described (Olsson et al., 1997) with the modification that 0.3% H_2O_2 was used for 10-15 min instead of 3% H_2O_2. Primary antibodies were used at the following concentrations: rabbit anti-DARPP-32 (1:1000, Chemicon); rabbit anti-distalless (i.e. pan DLX) (1:1000, provided by G. Panganiban); rabbit anti-Er81 (1:5000, provided by S. Morton and T. Jessell); rabbit anti-GSH2 (1:5000) (Toresson et al., 2000); rabbit anti-ISL1/2 (1:500, provided by T. Edlund); rabbit anti-MASH1 (1:1000, provided by J. Johnson); rabbit anti-MEIS2 (1:5000, provided by A. Buchberg); rabbit anti-nestin (1:500, provided by R. McKay); rabbit anti-parvalbumin (1:1000, provided by P. Emson); rabbit anti-PAX6 (1:200, Covance) and goat anti-PAX6 (1:250, Santa Cruz). The secondary antibodies used were biotinylated swine anti-rabbit antibodies (DAKO), FITC-conjugated anti-rabbit (Jackson Immunoresearch) and Cy3-conjugated anti-goat antibodies (Jackson Immunoresearch). The ABC kit (Vector labs) was used to visualize the reaction product for the biotinylated antibodies with diaminobenzidine as the final chromogen. Confocal microscopy was performed on a Zeiss LSM510 confocal microscope.

For acetylcholine esterase (AChE) staining, adult brain sections were mounted on slides and dried at 37°C for 1 hour before placing in incubation medium (3 mM cupric sulfate, 10 mM glycine, 15 mM acetic acid, 35 mM sodium acetate, 0.08 mM tetraisopropyl pyrophosphoramide, 1.5 mM acetylthiocholine iodide, adjusted to pH 5.0) at 37°C for 4 hours. They were then developed in a 40 mM sodium sulphide solution (pH 7.5).

Non-radioactive in situ hybridization was performed as described in Toresson et al. (Toresson et al., 1999) using the following probes: *Dbx1 (*IMAGE clone AA003371) (Yun et al., 2001), *Sfrp2* (Kim et al., 2001), *Ngn2* (Sommer et al., 1996) and *Tlx* (Yu et al., 1994; Monaghan et al., 1995).

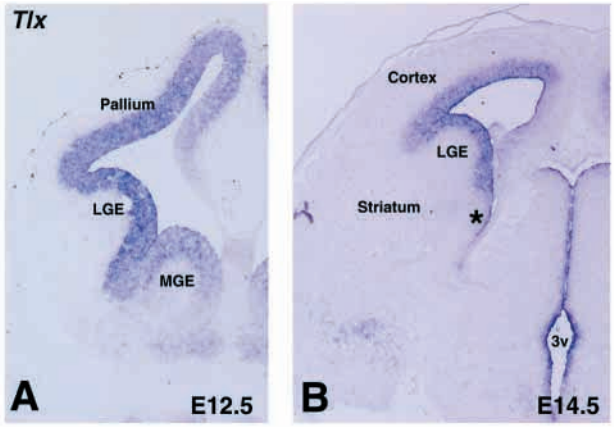

Fig. 1. Forebrain expression of *Tlx*. (A) At E12.5, *Tlx* is expressed at high levels in the ventricular zone of the lateral telencephalon, whereas lower levels are present in the dorsal-medial telencephalon and in the medial ganglionic eminence (MGE). (B) The *Tlx* expression pattern is similar at E14.5. The asterisk indicates the remnant of the MGE. Note also that *Tlx* is also expressed in the ventricular zone of the diencephalon surrounding the third ventricle (3v).

RESULTS

Altered patterning of the lateral telencephalon in *Tlx* mutants

Tlx is expressed in the mouse forebrain as early as the five-somite stage (approximately E8.5) (Monaghan et al., 1995). The telencephalic and diencephalic expression of this gene is restricted to the ventricular zone (Fig. 1). At E12.5, *Tlx* is highly expressed in the lateral portion of the telencephalon, including both the dorsal and lateral pallium and the lateral ganglionic eminence (LGE) (Fig. 1A). Low but detectable levels are found in the dorsomedial pallium and in the medial ganglionic eminence (MGE) (Fig. 1A). This expression pattern is maintained at both E14.5 (Fig. 1B) and E16.5 (data not shown).

At E12.5, *Tlx* mutants appear morphologically normal, however, at the molecular level they exhibit defects in the patterning of the lateral telencephalon. The pallio-subpallial boundary is marked by the juxtaposition of GSH2- and PAX6-expressing cells of the subpallial and pallial ventricular zones, respectively (Fig. 2A-C). Confocal micrographs show only a minimal overlap (i.e. 2-3 cell diameters) of cells expressing these two proteins in the wild-type telencephalon (Fig. 2C,D). In *Tlx* mutants, GSH2-expressing cells are found dorsal to their normal limit (i.e. up to and in the LGE-pallium angle, Fig. 2E). In these mutants, the overlap of GSH2 and PAX6 in ventricular zone cells is much broader than in the wild types (Fig. 2E-H).

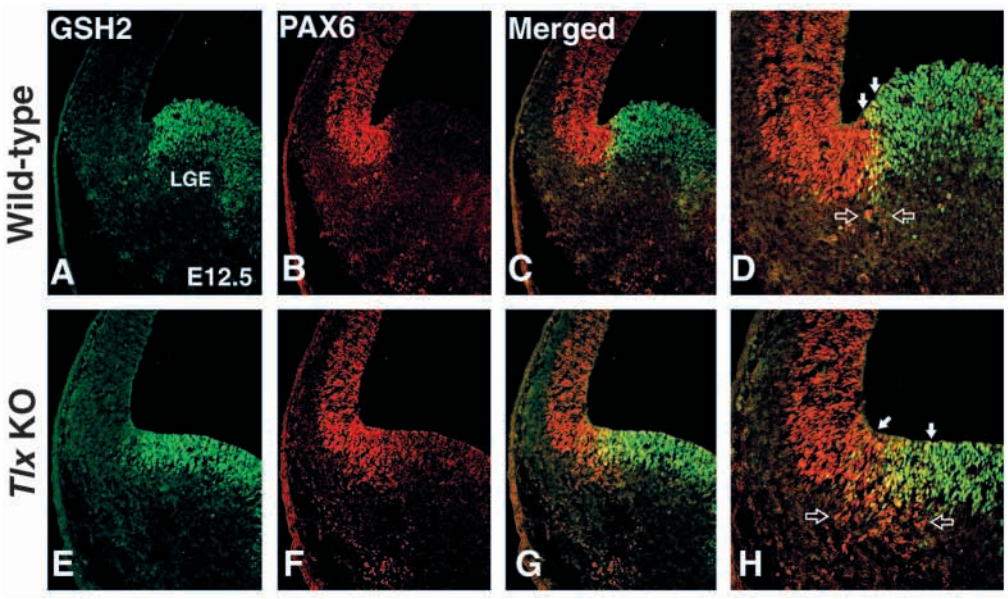

Fig. 2. Dorsal expansion of GSH2 expression in the *Tlx* mutant telencephalon. (A,E) GSH2 expression in the E12.5 wild-type (A) and *Tlx* mutant (E) LGE. (B,F) PAX6 expression in wild type (B) and *Tlx* mutants (F). (C,D,G,H) Merged confocal images of GSH2 and PAX6 expression; D and H are higher magnification of C and G, respectively. Note that in wild type (C,D) a small overlap of cells (approximately 2-3 cell diameters) expressing GSH2 and PAX6 is evident (yellows cells). In the *Tlx* mutant (G,H), this overlap is broader than in wild type (C,D). In D and H the filled arrows point to the domain of overlap; unfilled arrows point to the broader domain of PAX6-positive cells in the SVZ of the mutant (H) compared to the wild type (D).

Interestingly, this broader overlap correlates with the altered organization of PAX6-positive cells in the subventricular region. In wild types, PAX6-positive cells are seen to emanate from the region where GSH2 and PAX6 overlap in the ventricular zone and to form a stream from the subventricular zone (SVZ) out to the pial surface (Fig. 2B-D) (Puelles et al., 1999). In *Tlx* mutants, however, the PAX6-positive cells form a broader domain in the SVZ (Fig. 2H), which corresponds well with the wider overlap of GSH2 and PAX6 expression. The dorsal shift of GSH2 expression in the *Tlx* mutants is still observed at E14.5 (Fig. 3E), however, high-level PAX6 expression appears to have retracted dorsally by this stage (Fig. 3H). Moreover, in the *Tlx* mutants, the position of the PAX6-expressing cells in the SVZ region has shifted dorsally and there are 2.2-fold more of these cells (Fig. 3H) than in wild types (Fig. 3D; 514±89 versus 235±90 cells, respectively, $P<0.02$, $n=3$).

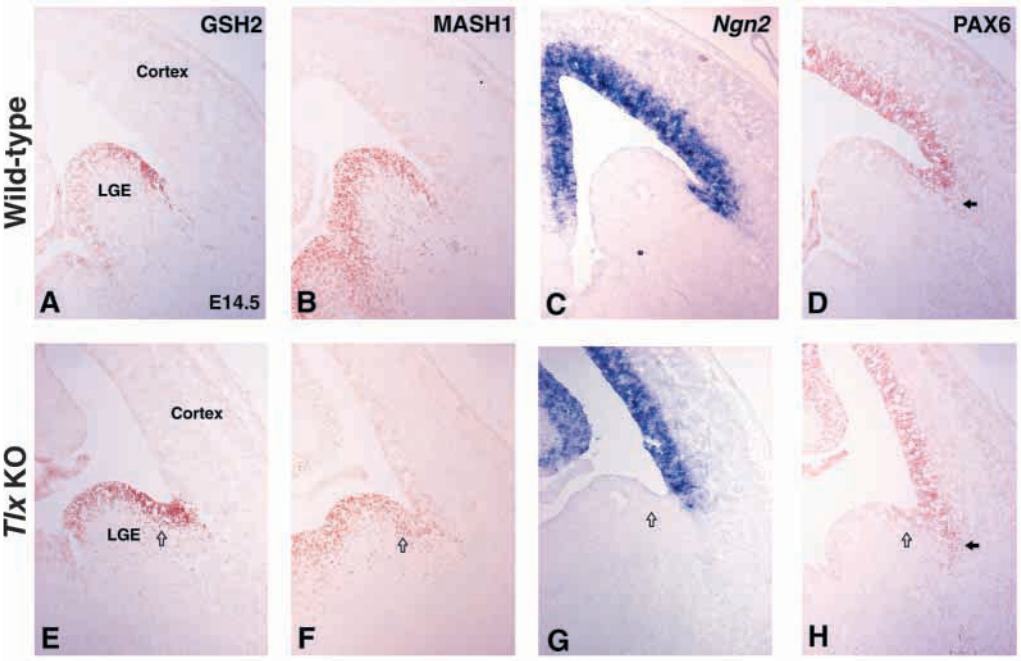

Fig. 3. Alterations in gene expression at the pallio-subpallial boundary of *Tlx* mutants at E14.5. In the wild-type telencephalon, GSH2 (A) and MASH1 (B) expression stops short of the LGE-cortex angle (i.e. ventral pallium). Expression of these subpallial markers in the *Tlx* mutant is shifted dorsally (arrows in E and F) into the LGE-cortex angle. *Ngn2* (C) and PAX6 (D) are normally expressed throughout the ventricular zone of the pallium with a ventral limit of expression in the LGE-cortex angle. However, in the *Tlx* mutant expression of these markers is retracted from this region (G,H). Unfilled arrows in E-H point to the approximate position where the pallio-subpallial boundary would be in a wild type. Note that the PAX6-expressing SVZ cells (marked by filled arrows), which normally emanate from the pallio-subpallial boundary, are shifted dorsally and are more numerous in the *Tlx* mutant (H) than in the wild type (D).

The bHLH genes *Mash1* and *Ngn2* also mark cells on the pallial and subpallial sides of the pallio-subpallial boundary, respectively. In addition to the dorsal expansion of GSH2 expression in *Tlx* mutants (Fig. 2E, Fig. 3E), MASH1-positive cells can also be found in a more dorsal position in the mutant telencephalon (Fig. 3F). Concomitant with this, *Ngn2* expression in *Tlx* mutants is retracted from its normal ventral limit (Fig. 3G), as is the case for PAX6 (Fig. 3H). These alterations in gene expression are seen at both rostal and caudal levels. Taken together, these results

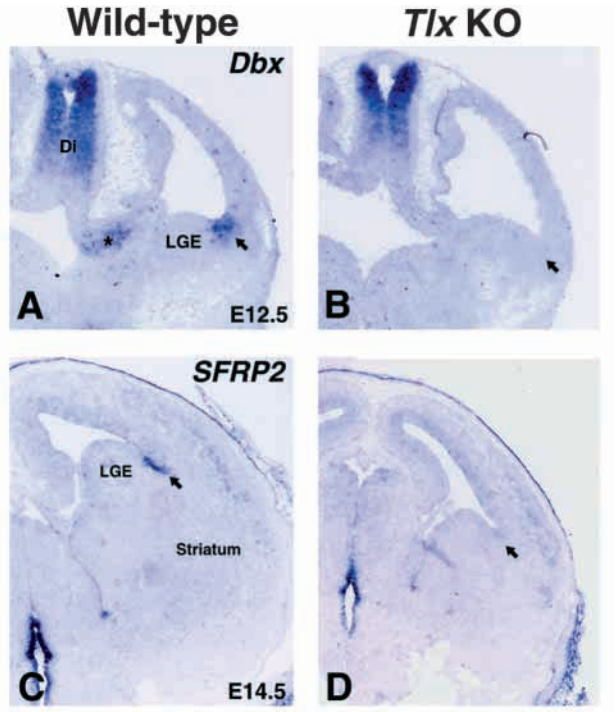

Fig. 4. Loss of ventral pallial markers in the *Tlx* mutant telencephalon. (A) *Dbx1* is normally expressed in the LGE-cortex angle (i.e. ventral pallium, arrows in A and B). (B) In the*Tlx* mutant, however, *Dbx1* expression is lost in this region but not in the diencephalon (Di). Asterisk in A marks *Dbx1* expression in a portion of the diencephalon that is in close contact with the ventral telencephalon. (C) *Sfrp2* is also expressed in the LGE-cortex angle (arrows in C and D). Expression of this gene is also missing in this region of the *Tlx* mutant (D). Note that *Sfrp2* expression remains in the *Tlx* mutant diencephalon around the third ventricle, which also normally expresses *Tlx* (see Fig. 1B).

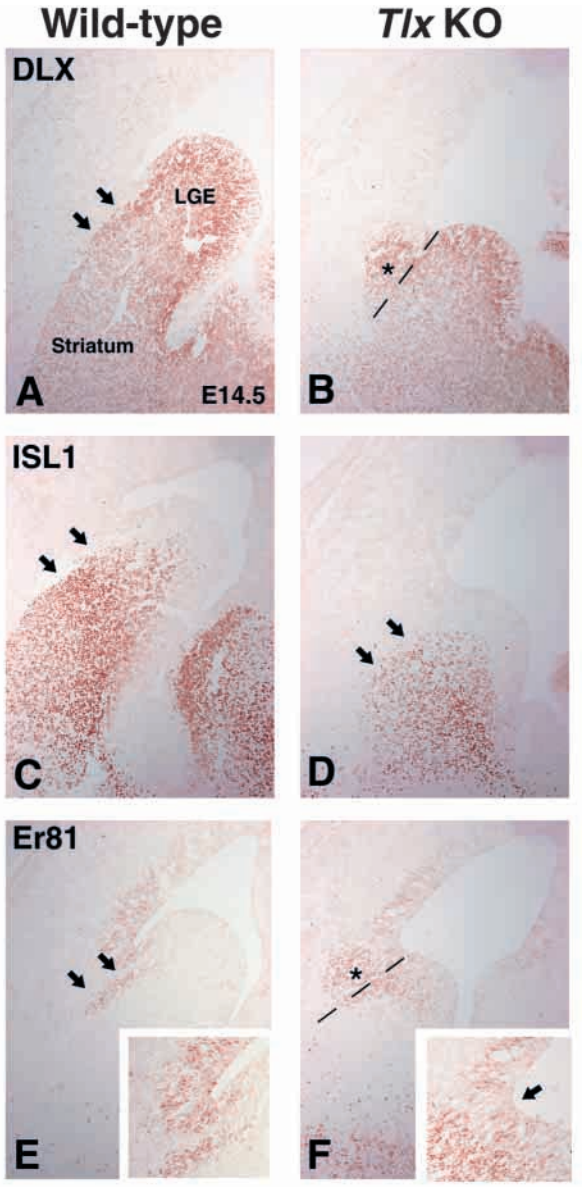

Fig. 5. Dorsal shift in SVZ and mantle markers in the *Tlx* mutant telencephalon at E14.5. (A) DLX proteins are normally expressed in the germinal zones and mantle regions of the ventral telencephalon, including the LGE. Arrows in A show the dorsal limit of DLX expression in the LGE SVZ. (B) In the *Tlx* mutant, the LGE is smaller, which is reflected in the smaller DLX-expressing domain. In addition, the normal limit of DLX expression (indicated by broken line) is shifted dorsally in the mutant (asterisk in B). (C) Islet1 (ISL1) is expressed in the LGE SVZ and developing striatum (arrows point to the dorsal limit of expression). (D) Although this domain is smaller in the *Tlx* mutant, it is not shifted dorsally (arrows) as is the case with DLX (B). (E) Er81 is normally expressed in a small domain of the LGE SVZ at the dorsal limit of DLX expression, as well as in the pallial ventricular zone (see also inset of pallio-LGE angle in E), excluding the medial pallium and part of the dorsal pallium. (F) In the *Tlx* mutant, the LGE SVZ domain of Er81 appears to be selectively expanded (normal limit marked by broken line) and shifted dorsally (asterisk) similar to that for DLX (B). Note the strong reduction of Er81 expression in the LGE-cortex angle (i.e. ventral pallium) of the *Tlx* mutant (arrow in F inset). Er81 expression in the lower portion of E and F reflect cells in the globus pallidus and developing striatum.

demonstrate a dorsal shift in the expression limits of genes, which normally abut at the pallio-subpallial boundary.

Based on gene expression patterns, the pallium has recently been divided into medial, dorsal, lateral and ventral portions (Puelles et al., 1999; Puelles et al., 2000; Yun et al., 2001). The ventral pallium is a small domain located immediately dorsal to the pallio-subpallial boundary. This pallial region is normally marked, at least in part by the expression of *Dbx1*, a homeobox gene (Yun et al., 2001), and *Sfrp2* (secreted frizzled related protein 2), which encodes a putative Wnt inhibitor (Kim et al., 2001). In addition to the dorsal shift in the expression limits of GSH2, MASH1, *Ngn2* and PAX6 in the lateral telencephalon, the ventral pallial region of the *Tlx* mutants lacks expression of both *Dbx1* (Fig. 4B) and *Sfrp2* (Fig. 4D). This loss in expression is specific to the ventral pallium as both genes continue to be expressed in the mutant diencephalon (Fig. 4B and Fig. 4D).

Unlike the case at E12.5, by E14.5 and onwards the *Tlx* mutant forebrains do not appear normal in that there is a significant reduction in the size of the LGE (see Figs 3-5). Despite this reduction, the dorsal limit of the DLX-expressing SVZ appears to have shifted dorsally in accordance with the observed shift of GSH2 and MASH1 (Fig. 5B). Notably, this is not the case for the dorsal limit of Islet1 expression (Fig.

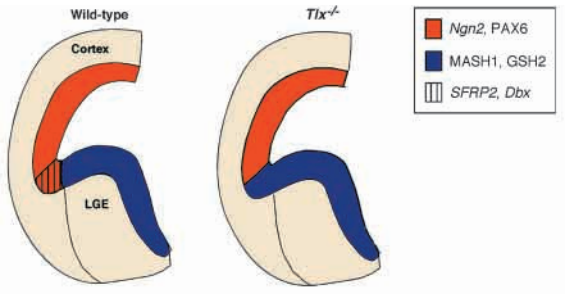

Fig. 6. Schematic diagram of patterning defects in the ventricular zone of the *Tlx* mutant telencephalon. These mutants lack ventral pallial markers and exhibit a dorsal shift in the expression limits of genes that normally abut at the pallio-subpallial boundary.

5D). However, we observed a dorsal expansion in the LGE SVZ-expression domain of the ETS transcription factor Er81 (Fig. 5F). In the wild type, Er81 is also expressed in the ventricular zone of the pallium, including at least the ventral and lateral pallium (Fig. 5E). Commensurate with the dorsally expanded subpallial SVZ expression of Er81, the pallial ventricular zone expression is greatly reduced in at least the ventral pallium (Fig. 5F). We have recently shown that Er81 and Islet1 mark separate dorsal and ventral progenitor pools in the DLX-expressing SVZ of the LGE, respectively (Stenman et al., 2003). These findings indicate that the patterning defects around the pallio-subpallial boundary in *Tlx* mutants result in the selective expansion of certain dorsal LGE characteristics (i.e. DLX and Er81 expression) at the expense of those normally marking the ventral pallium. Thus the data presented above show that the loss of *Tlx* gene function results in a misspecification of the ventral pallium (Fig. 6).

In addition to gene expression patterns, the pallio-subpallial boundary is also marked by a palisade of radial glial fibers originating in the ventricular zone near the LGE-pallium angle and coursing to the pial surface. In the wild-type telencephalon, this glial palisade can be visualized by Nestin staining (Fig. 7A). *Tlx* mutants show fewer nestin-positive radial glial fibers in the region of the pallio-subpallial boundary. Moreover, these fibers do not appear to fasciculate to form the palisade (Fig. 7B). The calcium-binding protein parvalbumin also marks radial glia in the pallio-subpallial boundary (Fig. 7C). Again, the parvalbumin-positive radial glial fibers in the mutant telencephalon fail to fasciculate (Fig. 7D). Thus, in addition to regulating gene expression at the pallio-subpallial boundary, *Tlx* gene function is also required for the normal formation of the radial glial palisade. Indeed, the altered patterning, described above, may contribute significantly to the abnormal formation of this radial glial palisade.

Gene dosage of *Tlx* and *Pax6* regulates the patterning of the lateral telencephalon

Several studies have shown that *Pax6* is required for the correct patterning at the pallio-subpallial boundary (Stoykova et al., 1996; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001) as well as the formation of the radial glial palisade (Stoykova et al., 1997; Götz et al., 1998; Chapouton et al., 1999). Homozygous *Sey* mutants show ectopic ventral (i.e. *Gsh2*, *Mash1* and Dlx) gene expression in the pallium and a loss of dorsal (i.e. *Ngn1* and *Ngn2*) gene expression in the

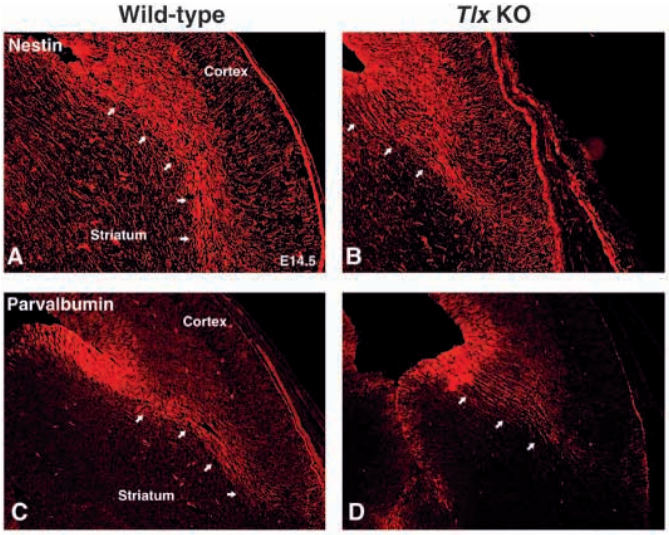

Fig. 7. Alterations in the radial glial palisade of *Tlx* mutants. (A,B) Nestin staining of radial glial fibers in the lateral telencephalon of E14.5 wild type (A) and *Tlx* mutant (B). In the wild type (A) the radial glial fibers fasciculate to form the radial glial palisade characteristic of the pallio-subpallial boundary (arrows). (B) The *Tlx* mutant shows fewer stained fibers and a lack of fasciculation of these fibers. (C,D) Parvalbumin-labeled radial glial fibers in the lateral telencephalon. Again, the parvalbumin-positive radial fibers fasciculate to form the glial palisade in wild type (arrows in C), whereas this does not occur in the mutant (D).

corresponding domain. Moreover, the ventral pallium markers *Dbx1* (Yun et al., 2001) and *Sfrp2* (Kim et al., 2001; Muzio et al., 2002) are both lost in the *Sey/Sey* mutants. The fact that *Tlx* mutants exhibit a similar, but much less severe, phenotype to *Sey* homozygotes could be because of *Pax6* directly or indirectly regulating *Tlx* expression. This does not seem to be the case, however, because *Tlx* is expressed in *Sey/Sey* mutants both at E12.5 and E14.5 (data not shown). Furthermore, the data presented above demonstrate that *Tlx* is not a general regulator of *Pax6* expression.

Tlx and *Pax6* may, therefore, co-operate genetically to establish the pallio-subpallial boundary. To analyze this we generated a series of compound *Sey* and *Tlx* alleles at E14.5 and E16.5. Mice heterozygous for either the *Sey* or *Tlx* mutations do not show alterations in gene expression at the pallio-subpallial boundary with respect to any of the markers we have employed here (data not shown). However, in $Tlx^{+/-}$;*Sey/+* compound heterozygotes, a few scattered GSH2- and MASH1-positive cells are seen in the region of the ventral pallium (data not shown). Furthermore, the expression of *Sfrp2* is reduced, and at some levels missing, in $Tlx^{+/-}$;*Sey/+* compound heterozygotes (data not shown). The double heterozygous phenotype, however, is much less severe than that observed in *Tlx* homozygous mutants (described above). We also analyzed $Tlx^{-/-}$;*Sey/+* mutants and in all cases the limit of GSH2 (Fig. 8D), MASH1 (Fig. 8E) and DLX (data not shown) expression extends further dorsally into the pallium than in *Tlx* homozygous mutants (Fig. 8A,B). Furthermore, *Ngn2* is downregulated in the pallial domain containing ectopic GSH2 and MASH1 cells (Fig. 8F). These findings demonstrate that removal of one allele of *Pax6* on the $Tlx^{-/-}$ mutant background results in a significant dorsal shift in the expression

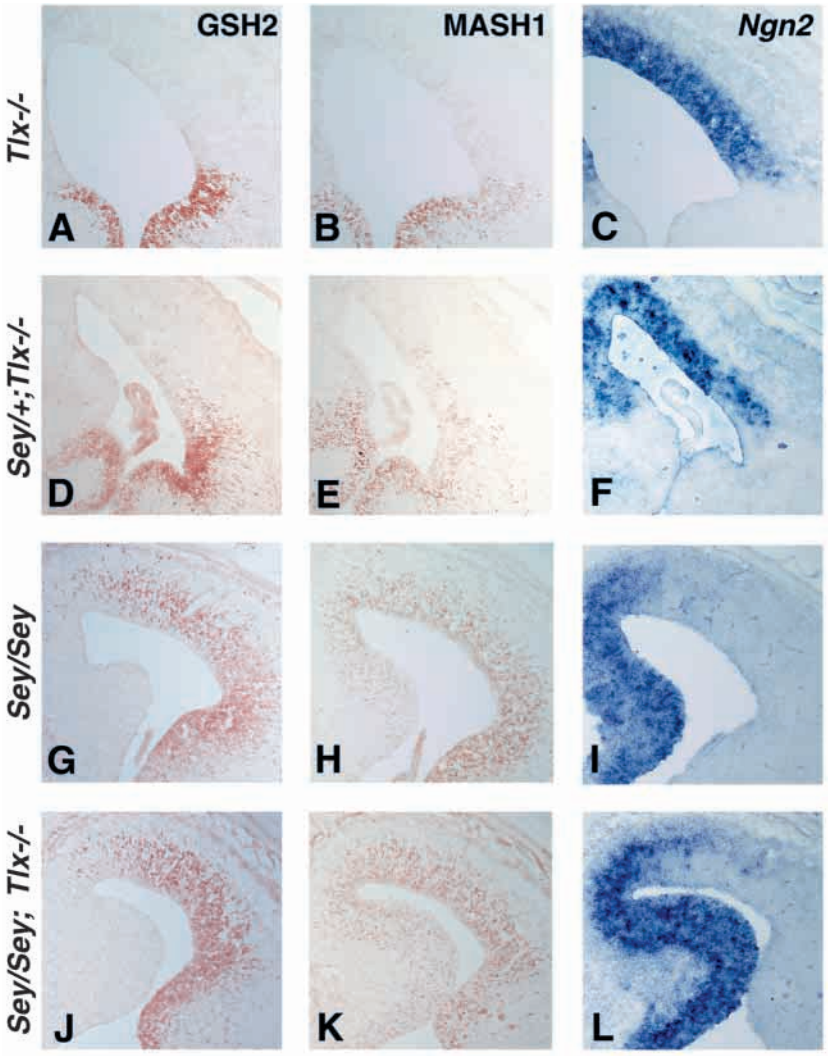

Fig. 8. *Tlx* and *Pax6* genetically interact to pattern the pallio-subpallial boundary. GSH2 (A,D,G,J), MASH1 (B,E,H,K) and *Ngn2* (C,F,I,L) in the telencephalon of E14.5 $Tlx^{-/-}$ (A-C), *Sey/+;*$Tlx^{-/-}$ (D-F), *Sey/Sey* (G-I) and *Sey/Sey;* $Tlx^{-/-}$ mutants (J-L). Note the dorsal shift of GSH2 and MASH1 expression in the *Sey/+;*$Tlx^{-/-}$ (D,E) mutant as compared to the $Tlx^{-/-}$ mutant (A,B). This dorsal shift in subpallial markers also results in a greater retraction of *Ngn2* expression in the *Sey/+;*$Tlx^{-/-}$ mutant (F) as compared to the $Tlx^{-/-}$ mutant (C). The *Sey/Sey* mutant displays a more significant dorsal shift in GSH2 (G) and MASH1 (H) expression as well as retraction of *Ngn2* expression (I) than in the *Sey/+;*$Tlx^{-/-}$ mutant (D-F). Interestingly, removal of both TLX alleles on the *Sey/Sey* background (i.e. *Sey/Sey;* $Tlx^{-/-}$) does not further exacerbate the pallial misspecification (J-L) over that seen in the *Sey/Sey* mutant alone (G-I).

limits of GSH2, MASH1, DLX and *Ngn2*, as compared to the $Tlx^{-/-}$ mutants alone. It is interesting to note that the patterning defects in the *Sey/Sey* mutants (Fig. 8G-I) are more severe than those observed in $Tlx^{-/-}$;*Sey/+* mutants. Moreover, the patterning defects observed in $Tlx^{+/-}$; *Sey/Sey* (data not shown) and $Tlx^{-/-}$;*Sey/Sey* mutants (Fig. 8J-L) were not noticeably different from those seen in the *Sey/Sey* mutants. Thus *Pax6* appears to be required for correct patterning in broader portions of the pallium (i.e. lateral and dorsal pallium) than *Tlx*. Both, however, are important for the correct patterning of gene expression around the pallio-subpallial boundary. Moreover, our findings show that correct gene dosages of both *Tlx* and *Pax6* are required to properly establish this boundary (Fig. 9).

Altered amygdalar development in *Tlx* mutants

Tlx mutants have smaller than normal brains, which exhibit gross morphological defects in numerous telencephalic regions, including the amygdalar region (Monaghan et al., 1997). However, specific defects in the amygdala of these mutants have not, as yet, been described. Previous studies have suggested that the basolateral amygdala derives from the ventral pallium (Fernandez et al., 1998; Puelles et al., 1999; Puelles et al., 2000). Given the molecular misspecification of the ventral pallial region of *Tlx* mutants (described above), alterations in the development of this amygdalar nucleus would be predicted. We analyzed the amygdalar region in perinatal animals, however, because *Tlx* mutants are viable, we were also able to analyze this region in mature brains (i.e. three weeks to eight months old).

In wild-type animals, the basolateral amygdala is marked by the expression of Er81 from late-embryonic stages into adulthood (Fig. 10A). *Tlx* mutant brains stained for Er81 reveal little evidence of a normal basolateral nucleus in either perinatal or mature brains (Fig. 10B). In addition to marking the basolateral nucleus, acteylcholine esterase (AChE) staining also reveals the lateral nucleus and the central nucleus of the amygdala (Fig. 10C). In the *Tlx* mutants, some AChE staining is found in the presumptive region of the basolateral and lateral amygdala (Fig. 10D), however, the amount of staining is drastically reduced as compared to the wild type. Despite this, the size of the central nucleus appears rather similar to that in wild types (Fig. 10C,D). Staining for the phosphoprotein DARRP-32 outlines the lateral and basolateral amygdala in wild types, revealing the 'teardrop'-shaped nuclei (Fig. 10E). This morphology is not apparent in *Tlx* mutants (Fig. 10F). DARPP-32 marks the interstitial nucleus of the amygdala (Fig. 1E), as does MEIS2 (Fig. 10G). This nucleus also seems to be present in *Tlx* mutants and although its morphology is changed, it appears to be somewhat similar in size to that in wild types (Fig. 10G,H). Thus the alterations in the *Tlx* mutant amygdala seem to be rather specific to the basolateral and lateral amygdala.

In order to strengthen the correlation between the misspecification of the ventral pallium and the alterations in the development of the basolateral and lateral amygdalar nuclei, we have performed fate-mapping studies that, by exclusion, support a pallial origin for these nuclei. Using a *Dlx5/6* enhancer (Zerucha et al., 2000) to drive cre recombinase in the subpallial SVZ (Stenman et al., 2003) of *gtROSA* reporter mice (Mao et al., 1999), we have found that the lateral and basolateral amygdala contain very few cells

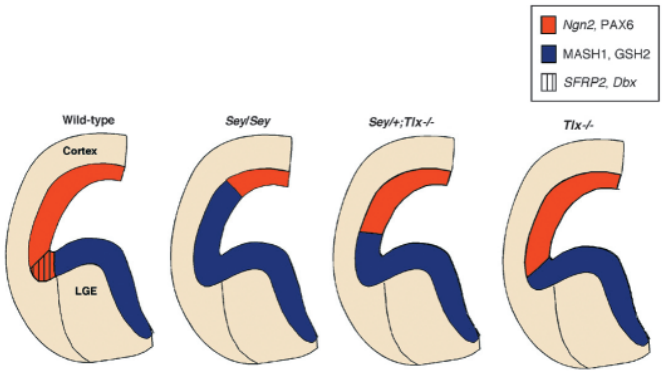

Fig. 9. Schematic diagram illustrating gene dosage requirements for *Tlx* and *Pax6* in regulating gene expression at the pallio-subpallial boundary. Removal of one allele of *Pax6* (i.e. *Sey*/+) results in a dorsal shift of the expression limits of the genes that normally abut at the pallio-subpallial boundary, which is more severe than that seen in homozygous *Tlx* mutants but less so than in *Sey/Sey* mutants.

originating in the subpallium (Fig. 10I). In fact, few, if any, of the subpallium-derived cells in the basolateral amygdala express Er81 (Fig. 10J). Interestingly, the normal expression of Er81 in the ventricular zone of the ventral pallium is lost in *Tlx* mutants (Fig. 5F). Thus, this data, together with the data presented above, indicate that the Er81-expressing cells in the basolateral amygdala are probably derived from the ventral pallium and may represent the glutamatergic projection neurons characteristic of this nucleus (Swanson and Petrovich, 1998). The small population of subpallium-derived cells (Fig. 10J) might represent the GABAergic interneuron population in this nucleus (e.g. Smith et al., 2000). In addition, our data show that the central and medial nuclei of the amygdala are largely derived from the subpallium (Fig. 10I). In summary, the present findings suggest that the patterning defects around the pallio-subpallial boundary in *Tlx* mutants have severe consequences for the development of basolateral and lateral nuclei in the amygdalar complex.

DISCUSSION

Tlx is required for patterning of the lateral telencephalon

Our results demonstrate that the orphan nuclear receptor *Tlx* plays an important role in the patterning of the lateral telencephalon. Specifically, *Tlx* mutants do not correctly establish the pallio-subpallial boundary. This is evidenced in both altered gene expression at the pallio-subpallial boundary as well as abnormal formation of the radial glial palisade. In *Sey* mutants, the formation of the pallio-subpallial boundary is also disturbed, which results in an increased migration of subpallial-derived GABAergic neurons into cortical regions (Stoykova et al., 1997; Stoykova et al., 2000; Götz et al., 1998; Chapouton et al., 1999; Toresson et al., 2000; Yun et al., 2001). It will be interesting to determine whether the alterations in the establishment of the pallio-subpalial boundary observed in *Tlx* mutants also leads to an increased ventral to dorsal migration of subpallial neurons.

A stream of PAX6-positive cells, which emanate from the ventricular zone and course downward towards the pial surface, is found at the pallio-subpallial boundary (Puelles et al., 1999). Interestingly, the point at which these cells emerge from the ventricular zone seems to correlate with a slight overlap of GSH2- and PAX6-expressing cells (see Fig. 2), which is also the case in the embryonic chick telencephalon (von Frowein et al., 2002). *Gsh2* appears to be important for the development of this stream of cells because the number of PAX6-positive cells is reduced (but not missing) in *Gsh2* mutants (Toresson et al., 2000). Although the PAX6-positive stream of cells has been suggested to belong to the subpallium (Puelles et al., 1999; Puelles et al., 2000), it may, in fact, represent a population of transitional cells between the pallial and subpallial compartments. Unlike the case in *Gsh2* mutants, the subventricular domain of PAX6-positive cells appears to be broader in *Tlx* mutants as compared to that in wild types. This correlates well, at least at early stages, with the increased overlap of the GSH2- and PAX6-expression domains in the *Tlx* mutant ventricular zone.

In *Tlx* mutants, the dorsal shift in the expression limits of genes that abut at the pallio-subpallial boundary, is accompanied by a loss of *Dbx1* and *Sfrp2* expression (i.e. ventral pallial identity). It is unclear, however, whether this indicates a direct role for *Tlx* in the development of the ventral pallium or if the effect is indirect. Because *Tlx* is not required for the diencephalic expression of *Dbx1* and *Sfrp2*, a direct role for this gene in the regulation of these ventral pallial markers seems unlikely. Alternatively, the dorsal expansion of GSH2 and MASH1 in the mutants might suggest a role for *Tlx* in the repression of these factors within the ventral pallium. The loss of ventral pallial identity in the mutants could therefore be because of the ectopic expression of subpallial genes. In support of this, the ventral pallium marker *Dbx1* is up-regulated in the LGE of homozygous *Gsh2* mutants (Yun et al., 2001), suggesting a role for *Gsh2* in the repression of *Dbx1*. Homozygous *Sey* mutants (which display ectopic *Gsh2* gene expression in the pallium) also exhibit a loss of ventral pallial identity (Kim et al., 2001; Yun et al., 2001; Muzio et al., 2002). However, *Sey*/*Sey* mutants display more severe patterning defects, including both the lateral and the dorsal pallium as well (Toresson et al., 2000; Yun et al., 2001).

The origins of different amygdalar nuclei has previously been unclear. Although it has been suggested that its nuclear components derive from both the dorsal and ventral halves of the embryonic telencephalon (Swanson and Petrovich, 1998). In particular, the basolateral amygdala and the lateral amygdala have been suggested to derive from the ventral pallium and the lateral pallium, respectively (Fernandez et al., 1998; Puelles et al., 1999; Puelles et al., 2000). However no direct evidence for this has been provided thus far. A recent study (Gorski et al., 2002) has fate mapped the *Emx1*-expression region of the dorsal telencephalon. This study showed that, in addition to the neocortex and hippocampus, many structures in the ventrolateral cortical region are also derived from the *Emx1*-expression domain, including both the basolateral and lateral amygdala. A defining feature of the ventral pallium is the lack of *Emx1* gene expression, along with the expression of *Pax6*, *Tbr1*, *Dbx1* and *Sfrp2* (Puelles et al., 1999; Yun et al., 2001; Kim et al., 2001). Thus the *Emx1*-expressing pallial regions should not contribute neurons to the basolateral and lateral amygdala. However, it is possible that low levels of *Emx1* are

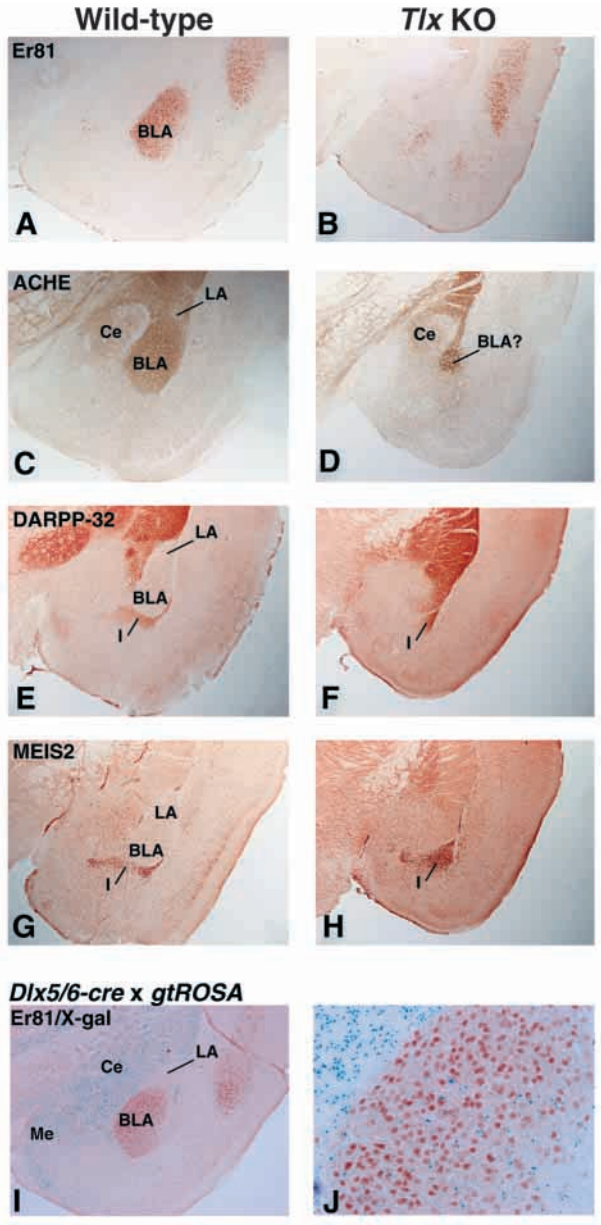

Fig. 10. Amygdalar defects in adult *Tlx* mutant brains. (A,B) Er81 is expressed in cells of the basolateral amygdala (BLA) in the wild type (A), whereas only scattered Er81-expressing cells are found in the amygdalar region of the *Tlx* mutant (B). (C) Acetylcholine esterase (AChE) staining reveals the lateral (LA), BLA and the central (Ce) nuclei of the wild type. (D) Although the staining in the Ce of *Tlx* mutants appears similar to that of wild type, the staining in the BLA and particularly the LA is greatly reduced. (E) DARPP-32 expression delineates the LA and BLA as a 'tear drop' shape in the wild type, by virtue of its expression in portions of the Ce and in the interstitial nucleus (I). (F) No evidence of the BLA or LA is apparent in the DARPP-32-stained *Tlx* mutant brain, however, the interstitial nucleus (I) seems to be present. (G,H) MEIS2 expression is also seen in the interstitial nucleus (I) in both the wild type (G) and mutant (H). (I,J) Fate mapping the subpallial contribution to the LA and BLA using a *Dlx5/6-cre* crossed with the *gtROSA* reporter mouse shows that only a few subpallial cells (i.e. blue X-gal-positive cells) contribute to these amygdalar nuclei. Few, if any, of the Er81-positive cells in the BLA are X-gal positive, supporting the notion that the BLA and LA are largely derived from the pallium. Conversely, many cells in the Ce and medial (Me) amygdalar nuclei are labeled, supporting a largely subpallial origin for these nuclei.

normally expressed in the ventral pallium. This expression might not be easily detected by in situ hybridization but could drive sufficient levels of *cre recombinase* to mark cells derived from the ventral pallium (i.e. neurons in the basolateral and lateral amygdala). Our data support a ventral pallial origin for both of these amygdalar nuclei because the patterning defects observed in the *Tlx* mutants appear to be restricted to the ventral pallium. However, it is difficult to determine whether a portion of the lateral pallium is also affected in these mutants because of a lack of specific markers for this pallial region. These amygdalar nuclei are largely generated between E11 and E14 in the mouse (McConnell and Angevine, 1983), which correlates well with the timing of the observed patterning defects in *Tlx* mutants. Moreover, our fate-mapping studies, using the subpallial *Dlx5/6* expression domain, demonstrate a largely pallial origin for the basolateral and lateral amygdala because only a few cells are labeled in these nuclei. In contrast, the central and medial amygdalar nuclei do appear to derive from subpallial sources. Taken together, these fate-mapping studies provide convincing evidence for both pallial and subpallial contributions to the amygdalar complex.

Homozygous *Tlx* mutants can survive after birth and have been reported to display abnormally aggressive behavior (Monaghan et al., 1997; Young et al., 2002). Because the lateral and basolateral amygdala are both thought to be involved in the regulation of fear rather than aggression (Oakes and Coover, 1997; Nader et al., 2001), it is unlikely that the amygdalar defects in these mutants are responsible for the aggressive behavior. It should be noted, however, that other morphological defects are present in the *Tlx* mutant forebrain (Monaghan et al., 1997), which may contribute more or less to their aggressive behavior. Notably, the LGE in *Tlx* mutants is considerably more reduced in size than other telencephalic structures such as the cortex. This reduction in the size of the LGE is unlikely to contribute significantly to the observed defects at the pallio-subpallial boundary in the *Tlx* mutants because the opposite phenotype would be predicted, at least with respect to the gene expression. Indeed, it is probable that a smaller LGE will result in a ventral shift of the expression limits of genes that abut at the pallio-subpallial boundary. We are currently investigating the mechanisms that underlie the reduced LGE size in the *Tlx* mutants, which may include either, or a combination of, cell death, lack of proliferation or a patterning defect.

Tlx and *Pax6* interactions

As mentioned above, the pallial phenotype of homozygous *Tlx* and *Sey* mutants share several similarities, specifically the alteration in gene expression around the pallio-subpallial boundary as well as altered development of the radial glial palisade. This motivated us to further examine the relationship between these two genes in the process of telencephalic dorsal-ventral patterning. Our findings show that the correct gene dosage of both *Tlx* and *Pax6* is crucial for the establishment of the pallio-subpallial boundary. Although the loss of one allele of *Pax6* on the homozygous *Tlx* mutant background results in a significant worsening of the pallial phenotype, removal of either one or both of the *Tlx* alleles on the homozygous *Sey*

background does not further exacerbate the phenotype as compared to *Sey/Sey* mutants alone. Therefore *Tlx* is required to augment *Pax6* gene function in the ventral-most portions of the pallium and thereby to correctly position the pallio-subpallial boundary. This genetic interaction with *Pax6* provides an explanation for why *Tlx*, despite its broad expression pattern, is crucial for the establishment of the pallio-subpallial boundary. It seems that *Tlx* is not the only gene that is expressed across the pallio-subpallial boundary and regulates correct gene expression at this boundary. The zinc finger gene *Gli3*, which is expressed on both sides of the pallio-subpallial boundary, is also required in this process (Tole et al., 2000; Rallu et al., 2002). As is the case with *Tlx*, this is not through the direct regulation of *Pax6* expression.

The fact that *Tlx* and *Pax6* interact genetically in the establishment of telencephalic dorsal-ventral identity suggests that their protein products might do so through direct molecular interactions. PAX6 is known to physically interact with the HMG box protein SOX2 in the regulation of eye development (Kamachi et al., 2001). *Sox2* is expressed in the telencephalic ventricular zone in regions overlapping with *Pax6* expression (Zappone et al., 2000), suggesting that similar interactions may be involved in telencephalic patterning. No interacting partners for TLX have, as yet, been identified in vertebrates, not even retinoid X receptors (RXRs), which are known to interact with many orphan nuclear receptors (for a review, see Blumberg and Evans, 1998). Furthermore, we have not been able to detect a physical interaction between TLX and PAX6 (unpublished data). It seems therefore that these two genes regulate telencephalic patterning through independent but convergent pathways. The convergence of *Tlx* and *Pax6* to pattern the pallio-subpallial boundary could be mediated through the regulation of common gene targets. *Pax6* has been implicated in the regulation of *Ngn2* expression in the pallium (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). Recently, an enhancer, which is capable of driving *Ngn2* expression in the pallium, including the ventral pallium, has been identified (Scardigli et al., 2001). This enhancer was shown to require *Pax6* gene function for its correct expression. Interestingly, this enhancer element contains a putative TLX binding site (J. S., K. C. and F. Guillemot, unpublished data), suggesting that TLX as well as PAX6 may be involved in directly regulating *Ngn2* gene expression in the ventral pallial region. Such a regulation may explain, at least in part, the *Tlx* mutant phenotype, because *Neurogenins* have previously been shown to negatively regulate subpallial (e.g. *Mash1* and Dlx genes) gene expression (Fode et al., 2000). Interestingly, co-regulation of a common gene by TLX and PAX6 has been shown to occur in eye development. The paired homeobox genes *Pax6* and *Pax2* are expressed in the developing retina and optic stalk, respectively. These factors regulate the development of these two eye regions, in part, through direct mutual repression (i.e. PAX6 represses *Pax2* gene expression in the retina and vice versa) (Schwarz et al., 2000). Moreover, the *Pax2* promoter contains a functional TLX binding site, which, when bound by TLX, results in the repression of promoter activity (Yu et al., 2000). It is interesting to note that *Tlx* is expressed in both the retina and the optic stalk (Yu et al., 1994; Monaghan et al., 1995; Yu et al., 2000), and yet it is proposed to participate in patterning the retina-optic stalk transition (Yu et al., 2000). This is similar to the data presented here in which *Tlx* is expressed on both sides of the pallio-subpallial boundary but is involved in the establishment of this boundary. The present results indicate that this function is dependent on a genetic interaction with the pallial-enriched *Pax6* gene.

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