Six3 inactivation causes progressive caudalization and aberrant patterning of the mammalian diencephalon

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The homeobox gene Six3 represses Wnt1 transcription. It is also required in the anterior neural plate for the development of the mammalian rostral forebrain. We have now determined that at the 15- to 17-somite stage, the prospective diencephalon is the most-anterior structure in the Six3-null brain, and Wnt1 expression is anteriorly expanded. Consequently, the brain caudalizes, and at the 22- to 24-somite stage, the prospective thalamic territory is the most-anterior structure. At around E11.0, the pretectum replaces this structure. Analysis of Six3;Wnt1 double-null mice revealed that Six3-mediated repression of Wnt1 is necessary for the formation of the rostral diencephalon and that Six3 activity is required for the formation of the telencephalon. These results provide insight into the mechanisms that establish anteroposterior identity in the developing mammalian brain.

KEY WORDS: Mouse, Six3, Wnt1, Forebrain, Diencephalon, Zona limitans intrathalamica (ZLI)

INTRODUCTION

The forebrain arises from the anterior neuroectoderm (ANE) during gastrulation, and different forebrain identities arise within the rostral neural plate (Shimamura and Rubenstein, 1997; Stern, 2002; Wilson and Houart, 2004). Graded posteriorizing signals specify posterior identity to the ANE (Lumsden and Krumlauf, 1996; Rhinn et al., 2005); among others, Fgfs, retinoic acid, BMPs and Wnts have been proposed as posteriorizing signals in this process (Gamse and Silve, 2005); among others, Fgfs, retinoic acid, BMPs and Wnts have been proposed as posteriorizing signals in this process (Gamse and Silve, 2005). Once the initial anteroposterior (AP) pattern of the neural plate is established, further local regionalization is required. One signaling center that influences neural plate patterning is the zona limitans intrathalamica (ZLI), located in the boundary between prethalamus and thalamus. In chicken, the mutual repression of Six3 and Irx3 may control ZLI positioning (Kobayashi et al., 2002). Later, basal plate signals induce ZLI expansion through the alar plate, and signals from the dorsal diencephalon midline oppose its formation (Larsen et al., 2001; Zeltser, 2005; Vieira and Martinez, 2006). The ZLI expresses Shh (Echelard et al., 1993).

To better understand how antagonists of caudalizing signals function during early mammalian forebrain development, how ectopic anterior expansion of Wnt signaling affects rostral forebrain patterning, and which aspects of the Six3 brain phenotype were caused by abnormal ectopic expansion of Wnt1 and which were caused by other Six3 functions, we performed a detailed temporal characterization of the developing Six3-null and Six3;Wnt1 double-null brains.

We found that the abnormal posteriorization of the Six3-mutant brain is gradual and starts in the roof plate at around the 3- to 6-somite stage, continuing until around E11.0. At the 22- to 24-somite stage, the prospective prethalamus is replaced by the prospective thalamus and tegmentum. We also determined that the ZLI is defective in the Six3-null brain; therefore, as late as E11.0, the prospective thalamus acquires a pretectum identity. Finally, we conclude that Six3-mediated repression of Wnt1 is required to avoid alar plate posteriorization and to allow ZLI formation, thereby ensuring proper AP patterning of the diencephalon. In addition, Six3 activity is required for the formation of the telencephalon.

MATERIALS AND METHODS

Mice
Six3F/F mice (Lagutin et al., 2003), Six3-Cre mice (Furuta et al., 2000) and Six3F/F mice (Liu et al., 2006) were previously described. CAGG-CreERT2 mice (Hayashi and McMahon, 2002) and Wnt1F/F mice (McMahon and
Bradley, 1990) were provided by A. McMahon (Harvard University, Cambridge, MA). Six3<sup>F/F</sup> mice were crossed to Six3<sup>Cre</sup>- and Six3<sup>F/F</sup>-CAGG-CreERT2 mice to produce Six3<sup>3/–</sup>-Six3-Cre and Six3<sup>0/0</sup>-CAGG-CreERT2 embryos. For the induction of Cre recombination, pregnant dams were injected intraperitoneally with tamoxifen (TM; 1 mg/15 g body weight) dissolved in safflower oil at various embryonic stages. Genotypes were determined by PCR analysis. Wild-type and Six3<sup>–</sup>-embryos were used as controls. For embryonic staging, the following standardized nomenclature was used: E8.5, 10- to 15-somite stage; E9.0, 15- to 20-somite stage; E9.5, 20- to 25-somite stage; E10.0, 25- to 30-somite stage; E10.5, 30- to 35-somite stage; E11.0, 35- to 40-somite stage.

In situ hybridization and S-Gal staining

S-Gal staining was performed as described (Kishigami et al., 2006). S-Gal-stained embryos were washed in PBS, fixed with 4% paraformaldehyde (PFA) for 1 hour on ice and used for whole-mount in situ hybridization. For whole-mount in situ hybridization analysis, embryos were fixed for 2 hours in 4% PFA on ice and processed as described (Belo et al., 1997). In situ hybridization in sections was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993). Double in situ hybridization/immunohistochemistry was performed as described (Lavado and Oliver, 2007). Images were obtained on a Leica MZFLIII stereomicroscope equipped with a Hamamatsu C5810 camera.

TUNEL and proliferation assays

TUNEL assay of whole-mount embryos was performed as described (Smith and Cartwright, 1997). TUNEL assay on tissue sections was performed using the ApoTag Plus Peroxidase Kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions. For proliferation assays, phosphohistone H3 (PH3) immunohistochemistry was performed as described (Lavado and Oliver, 2007). Time-mated female mice were injected with BrdU (40 μg/g body weight, intraperitoneally), and embryos were harvested 1 hour later. Anti-BrdU monoclonal antibody (1:10; BD Biosciences, San Jose, CA) was used. Immunohistochemistry images were acquired with an Axiovert 1.0 microscope (Zeiss, Jena, Germany) equipped with a Progres C14 camera (Jenoptic, Jena, Germany).

RESULTS

Establishment of brain identity is delayed in the anterior Six3-null head

Six3-null embryos lack the telencephalon, and around the 6- to 8-somite stage the Wnt1 expression domain ectopically expands into the ANE, resulting in posteriorization of the mutant roof plate (Lagutin et al., 2003). We have now determined that the expression of the midbrain and diencephalic markers Otx1 (Simeone et al., 1992a) and Irx1 (Bosse et al., 1997) is also anteriorly expanded in 6- to 8-somite stage Six3-null brains (see Fig. S1A’,B’ in the supplementary material). However, expression of the prethalamic markers Lhx5 (Sheng et al., 1997), Axr (Miura et al., 1997), Fezf1 and Fezf2 (Hirata et al., 2004; Hirata et al., 2006a) (see Fig. S1C’-F’ in the supplementary material) suggested that the ventral prospective prethalamus is present in the Six3-null brain. Moreover, the reduced expression of Nkx2.1 (Shimamura et al., 1995) and the absence of Six6 (Jean et al., 1999) suggested that the anterior hypothalamus is absent or very small at this stage (see Fig. S1G’,H’ in the supplementary material). This is the first evidence that the Six3-null brain is abnormally caudalized as early as the 6- to 8-somite stage.

Next, we analyzed the caudalization process during later stages. At around the 16-somite stage, Irx1 expression was detected in the mesencephalon and caudal diencephalon of the wild-type brain (Fig. 1A), and expression of Lhx5 was observed in the prethalamus and telencephalon (Fig. 1B). Axr was detected in the prethalamus and dorsal telencephalon (Fig. 1C). Fezf1 and Fezf2 were detected in the ventral prethalamus and telencephalon (Fig. 1D,E). Nkx2.1 was detected in the prethalamus and posterior hypothalamus (Fig. 1F), and Six6 was found in the anterior hypothalamus and Rathke’s pouch (Fig. 1G). At this somitic stage, Irx3 was detected in the mesencephalon, pretectum and thalamus of the wild-type brain (Bosse et al., 1997) (Fig. 1H), and Tcf4 was observed in the alar plate of the pretectum, thalamus and ventral prethalamus (Cho and Dressler, 1998) (Fig. 1J).

Analysis of these same molecular markers in Six3-null littermates detected Irx1 in a restricted portion of the most-antrodorsal region of the Six3-null brain but not in most of the alar plate (Fig. 1A’). By contrast, Lhx5 was widely expressed in the anterior basal and alar plates (Fig. 1B’) and Arx (Fig. 1C’), Fezf1 (Fig. 1D’) and Fezf2 (Fig. 1E’) were restricted to a small ventral portion of the Lhx5-expression domain. The expression of Nkx2.1 (Fig. 1F’) and Six6 (Fig. 1G’) suggested that the anterior hypothalamus is reduced or absent in the Six3-null brain. In contrast to the ectopic anterior expansion of Wnt1, Irx1, Pax3 or Otx1 detected at the 6- to 8-somite stage, expression of Irx3 and Tcf4 was not expanded, even at the 17-somite stage (Fig. 1H’ J’). These results suggest that at the 17-somite stage, the ventral part of the anterior Six3-null brain has a prospective prethalamic identity, and that the Irx1-expressing dorsal portion has not yet acquired a prospective thalamic identity.

Analysis of the Six3-null brain at later stages revealed graded, anterior expansion of the caudal diencephalic markers Irx3 (Fig. 1I’) and Tcf4 (Fig. 1K’ and see Fig. S2 in the supplementary material), indicating that although anterior expansion of Wnt1 started early, the resulting abnormal posteriorization of the Six3-null brain was not complete until the 22- to 24-somite stage. The presence of an Irx3- and Tcf4-free territory rostral to the posterior diencephalon at the 17-somite stage, and the later anterior expansion of these markers, could have been caused by cell death in the anterior brain territory, and replacement of those cells by posterior diencephalon cells and/or surviving rostral cells acquiring a posterior diencephalic identity.

To analyze these possibilities, TUNEL assay was performed in embryos at the 17-somite stage, when posteriorization of the mutant brain starts. Few apoptotic cells were identified in the rostral diencephalon of wild-type brains (mean, 9.29/100 cells; s.d., 5.88) (Fig. 1M), and the mean number of apoptotic cells was reduced in the anterior expansion of the caudal diencephalic markers (mean, 6.58/100 cells; s.d., 3.08; P=0.057×10<sup>–7</sup>) (Fig. 1M’N). The percentage of apoptotic cells was reduced in the anterior Six3-null brain at the 21-somite stage (mean, 6.58/100 cells; s.d., 3.08; n=3) (Fig. 1N’). Analysis of cell proliferation using PH3 immunostaining revealed no differences between the wild-type anterior diencephalon (mean, 9.17/100 cells; s.d., 0.62) and the Six3-null rostral brain (mean, 11.51/100 cells; s.d., 1.46; P=0.0146) (data not shown); also, no differences were detected in the posterior diencephalon (data not shown).

These results indicated that abnormal caudalization of the Six3-null rostral brain is a gradual process that starts at the 2- to 3-somite stage in the ANE, continues into the roof plate a few somites later, and posteriorizes the alar plate by the 22- to 24-somite stage. The alar plate (prospective pretectum and thalamus) was abnormally displaced toward the most-anterior region of the mutant brain, and the establishment of brain territory identities was delayed in that region. The increase in cell death detected in the 17-somite stage Six3-null brain probably contributed to the gradual posteriorization identified at the 17- to 24-somite stage. Furthermore, the lack of proliferation differences between wild-type and mutant posterior diencephalon suggests that cell fate change could also contribute to caudalization of the Six3-null brain at these stages.
Prospective thalamus is the most-anterior structure of the alar plate in the 22- to 24-somite stage Six3-null brain

We analyzed the expression of additional alar and basal plate markers at later stages. At the 22- to 24-somite stage, Wnt3a (Parr et al., 1993) was expressed in the mesencephalon, roof and alar plates of the thalamus and roof plate of the prethalamus (Fig. 2A). Fig15 (McWhirter et al., 1997) was expressed in the posterior mesencephalon and alar plate of the thalamus (Fig. 2B) and Otx1 was localized in the mesencephalon and alar plate of the diencephalon (Fig. 2C). Thus, in the 22- to 24-somite stage Six3-null littersmates, the prospective thalamus (Fig. 2A') is the most-anterior structure in the alar plate. These findings made us question whether these alterations affect the prethalamus.

In wild-type controls, Fezf1 and Fezf2 are expressed in the prethalamus (Fig. 2D) and Dlx2 (Porteus et al., 1991) in the ventral-most part of the prethalamus (Fig. 2E). In Six3-null littersmates, the expression of Fezf1 (Fig. 2D) and Fezf2 (data not shown) was greatly reduced, and few Dlx2-expressing cells were detected (Fig. 2E). We conclude that only a small portion of the ventral prethalamus is present in the Six3-null brain at this stage.

Next, we analyzed how this posteriorized phenotype affects the mutant basal plate. Nkx2.2 is normally expressed at the boundary between the alar and basal plates (Fig. 2F) (Shimamura et al., 1995). In the Six3-null brain, its expression in this region was mostly unaffected (Fig. 2F'), the exception being the truncated anterior-most domain (Fig. 2F'). This finding indicated that an alar-basal boundary is present in the mutant head. However, truncated expression of Nkx2.2 in the anterior basal plate suggested that the hypothalamus is defective. This proposal was supported by the observation that Bmp4 (Jones et al., 1991), a marker for the basal plate of the posterior hypothalamus (Fig. 2G), was not detected in the Six3-null brain at any stage analyzed (Fig. 2G' and data not shown).

Normally, Wnt1 is expressed in the basal plate at the level of the cephalic flexure (Fig. 2H) (Prakash et al., 2006); its expression was also expanded anteriorly in the mutant brain (Fig. 2H'). Ngn2 (also known as Neurog2 – Mouse Genome Informatics) and Foxa2 are normally expressed in the ventral neural tube, from the caudal spinal cord to the tegmentum (Fig. 2J) (Gradwohl et al., 1996; Sasaki and Hogan, 1993). In the Six3-null brain, their expression in the basal plate was anteriorly expanded (Fig. 2J'). Together, these results indicated that the tegmentum expands into the anterior basal plate of the Six3-null brain at the 22- to 24-somite stage. Moreover, the hypothalamic
region was reduced or absent, possibly owing to this expansion. As indicated by the expression of Fgf15, En1 and Pax6, no alterations were detected in the size or positioning of the mesencephalon in the Six3-null brain (Fig. 2B, K). In conclusion, the mutant alar plate is posteriorized at the 22- to 24-somite stage, and the prospective thalamus is the most-anterior alar plate boundary (Fig. 2L′). In the basal plate, the tegmentum and a severely reduced ventral prethalamus are the most-anterior structures remaining (Fig. 2L′).

The prospective thalamus is replaced by an expanded prepectum in the Six3-null brain during late development

To determine whether caudalization of the Six3-null brain continues beyond the 22- to 24-somite stage, we analyzed the expression of markers for the thalamus and pretectum at later stages. At E14.5, Tcf4 is normally expressed in the pretectum and thalamus (Fig. 3B). In the Six3-null brain, Tcf4 expression was displaced toward the most-anterodorsal region (Fig. 3B′). At E13.5, Lhx2 was detected in the thalamus and at the tegmentum-pretectum boundary (Fig. 3C) (Nakagawa and O'Leary, 2001). Lhx2 thalamic expression was not detected in the anterior territory of the Six3-null brain (Fig. 3C′ and see Fig. S3F in the supplementary material); thus, thalamic formation was probably arrested in the mutant brain at these later stages. Expression of other thalamic markers, such as Gbx2, Ngn2 (Nakagawa and O'Leary, 2001) and Prox1 (Lavado and Oliver, 2007), was not detected in this brain region (see Fig. S4 in the supplementary material). These results suggested that the prospective thalamus identified in the Six3-null brain during early development was later replaced by the prepectum.

To confirm this possibility, we analyzed the expression of additional markers. In the E14.5 wild-type brain, the pretectum marker Lim1 (also known as Lhx1 – Mouse Genome Informatics) is expressed in the tegmentum, pretectum and prethalamus (Fig. 3D) (Fujii et al., 1994), and Ebf1 in the rostral pretectum (Fig. 3E) (Garel et al., 1997). In the Six3-null littermates, Lim1 (Fig. 3D′) and Ebf1 (Fig. 3E′) were found in the anterodorsal region, and the
subcomissural organ was enlarged (see Fig. S5 in the supplementary material). These results confirmed that at later stages, the Six3-null alar plate posteriorizes further, and the anterior pretectum replaces the prospective thalamus.

To determine the developmental stage at which the anterior mutant brain acquires pretectum identity, we analyzed the expression of Lim1. Normally, Lim1 expression in the pretectum starts at E10.0; however, it was not detected in the anterior region of the mutant brain until E11.0 (see Fig. S6 in the supplementary material). The lack of obvious alterations in proliferation or cell death in the Six3-null brain at these later stages (see Figs S7, S8 in the supplementary material), suggested that the prospective thalamus was transformed into a pretectum.

In addition, the lack of expression of the prethalamic and hypothalamic markers Dlx2 and Nkx2.1 (Rinkwitz-Brandt et al., 1995) (Fig. 3F′,G′) at E14.5 confirmed that the hypothalamus is not present in the mutant brain at this stage. As shown by Lim1 (Fig. 3D′) and Nkx6.1 (Fig. 3H′) (Qiu et al., 1998) expression, the tegmentum is the most-anterior ventral structure in the basal plate of the Six3-null brain. A scheme representing the mutant brain is shown in Fig. 3J′.

**Six3 activity is not required for positioning or expansion of the ZLI**

As described above, the caudalization of the anterior mutant diencephalon appears to be caused by the gradual replacement of the prospective thalamus by the pretectum. Abnormal anterior expansion of the pretectum has been reported in animal models in which signaling from the ZLI is defective (Hirata et al., 2006b).

In chicken, Six3 activity is required at the neural plate stage to position the ZLI (Kobayashi et al., 2002); however, in the 30- to 35-somite stage Six3-null mouse brain, the Shh-expressing ZLI primordium was positioned normally (see Fig. S9 in the supplementary material). Therefore, Six3 does not mediate the positioning of the ZLI in mice. Whether later ZLI signaling was defective and promoted caudalization of the anterior region of the mutant brain cannot be ruled out.

Normally, ZLI expansion starts in a region between the thalamus and prethalamus at around E9.5 (Shimamura et al., 1995). As shown above, at around E9.5, the expression of prospective thalamic markers, such as Tcf4, Wnt3a and Fgf15, was abnormally expanded in the Six3-null brain prior to ZLI expansion; this phenotypic alteration could have affected the competence of the ZLI-forming territory.

To determine whether the ZLI expanded dorsally, we analyzed Shh expression at E12.5 (Echelard et al., 1993). Shh expression was detected in the wild-type ZLI (Fig. 3I), but not in the corresponding mutant territory (Fig. 3I′). Moreover, the expression of other genes normally detected in or around the ZLI (e.g. Lim1, Nkx2.2) was also absent (Fig. 3D′ and data not shown). These data demonstrate that the ZLI is not present in the E12.5 Six3-null brain.
Although the lack of ZLI was probably caused by the abnormal posteriorization of the rostral mutant brain, we cannot exclude the possibility that Six3 activity is also required to maintain and/or expand Shh expression through the alar plate. To investigate these alternative possibilities, we used a Six3F/– conditional mouse strain (Liu et al., 2006) to selectively delete Six3 at several time points before or during dorsal ZLI expansion. Six3-Cre (Furuta et al., 2000) or the ubiquitous TM-inducible CAGG-CreERT2 strain (Hayashi and McMahon, 2002) were used to delete Six3 prior to ZLI expansion (E9.0 and E9.5). During ZLI expansion, Six3 was deleted by administering TM at E10.5. Six3 conditional-mutation embryos were analyzed at E12.5. Six3 activity was efficiently deleted from most brain regions (Fig. 4) and the telencephalon was severely reduced when Six3 was deleted before E10.5 (Fig. 4F-O).

At E12.5, the expression of Shh, Lim1 and Ngn2 is normally detected at the prethalamus-thalamus boundary (Fig. 4B-D). Shh expression revealed the position of the ZLI (Fig. 4B); Lim1 expression revealed the pretectum (Fig. 4C); Ngn2 expression, the thalamus (Fig. 4D); and Dlx2 expression, the prethalamus (Fig. 4E).

No obvious alterations in the ZLI or AP patterning of the diencephalon were identified when Six3 activity was conditionally deleted using any of the strategies described (Fig. 4G-J,L-O,Q-T).

These results support the hypothesis that Six3 activity is not directly required for the dorsal expansion or maintenance of the ZLI, and suggest that posteriorization of the alar plate establishes a non-permissive territory for the expansion of Shh expression, which arrests ZLI formation. During later development, this arrest affects the induction of thalamic fate in the Six3-null brain.

**Six3 is required for telencephalon formation**

During normal forebrain development, Six3 directly represses Wnt1 expression in the ANE fated to become forebrain (Lagutin et al., 2003). However, we could not discern the aspects of the brain phenotype caused secondarily by the abnormal ectopic expansion of Wnt1 from those caused by other Six3 functions in this process. To discriminate between these possibilities, we generated Six3;Wnt1 double-null embryos. Similar to each single mutant (McMahon and Bradley, 1990; McMahon et al., 1992; Lagutin et al., 2003), Six3F/–;Wnt1–/– newborn pups died at birth. Like Six3F/–;Wnt1–/– mutants, Six3F/–;Wnt1–/– pups had no eyes and exhibited severe craniofacial abnormalities (data not shown). Similar to Wnt1–/– brains, Six3F/–;Wnt1–/– brains lacked the cerebellum and colliculus and had a severely reduced midbrain (Fig. 6A′).

Foxg1 (Tao and Lai, 1992) expression was detected in the telencephalon of E10.5 control (Fig. 5A) and Wnt1–/– embryos (Fig. 5A′). At this stage, Emx1 (Simeone et al., 1992b) was observed in the dorsal telencephalon of wild-type (Fig. 5B) and Wnt1–/– (Fig. 5B′) embryos, and Tbr1 (Bulfone et al., 1995) was found in the telencephalon and eminentia thalami of control (Fig. 5C) and Wnt1–/– (Fig. 5C′) embryos. However, their expression was not detected in the prospective telencephalic region of Six3-null (Fig. 5A″,B″,C″) or Six3F/–;Wnt1–/– brains (Fig. 5A‴,B‴,C‴). The few Tbr1-expressing cells found in the ventral part of the Six3F/–;Wnt1–/– brain (Fig. 5C‴, arrowhead) probably correspond to the eminentia thalami, a diencephalic structure present in the Six3F/–;Wnt1–/– mutant brain (data not shown). These results demonstrate that Six3 activity is required for the formation of the mammalian telencephalon.

**The posteriorization of the Six3-null diencephalon is Wnt1-dependent**

Additional analyses of Six3F/–;Wnt1–/– embryos were performed to determine whether the prospective thalamus was anteriorly expanded and the prethalamus was lost. Unlike the E10.0 Six3-null brain, Wnt1–/– brains showed no ectopic expansion of Irx1 or Tcf4 expression (Fig. 5D′,E′). The Irx1- and Tcf4-free anterior brain territory (Fig. 5D″,E″) indicated that the pretectum and prospective thalamus were not anteriorly expanded into the anterior alar plate of the Six3F/–;Wnt1–/– brain. Therefore, the Irx1- and Tcf4-free anterior brain territory most likely corresponds to the prethalamus. This possibility was confirmed by the expression of the prethalamic marker Fst/1 (Fig. 5F″). These results confirm that the absence of Six3-mediated Wnt1 repression causes the posteriorization of the alar plate of the Six3-null brain. A schematic representation of each of these brains is shown in Fig. 5G-G‴.

**Six3 repression of Wnt1 activity is required for proper AP patterning of the diencephalon**

We examined whether in the non-caudalized diencephalon of the Six3F/–;Wnt1–/– brains, the Shh expression domain in the ZLI and the formation of the thalamus and prethalamus were normal. The lack of ZLI in E12.5 Six3-null embryos (Fig. 3I′) was rescued in the Six3F/–;Wnt1–/– brain (Fig. 6B′). In addition, the thalamus, as indicated by Lhx2 and Tcf4 expression (Fig. 6C′,D′), and prethalamus, as shown by BF-2 (also known as Foxd1 – Mouse Genome Informatics) (Hatini et al., 1994) and Dlx2 expression (Fig.
The developing Six3
null brain progressively
caudalizes

Early AP patterning of the forebrain partially results from a balance between caudalizing signals and their anterior antagonists (Lumsden and Krumlauf, 1996; Kudoh et al., 2002; Wilson and Houart, 2004; Rhinn et al., 2005). Wnt signaling molecules comprise a well-characterized family of caudalizing factors (Niehrs, 1999; Yamaguchi, 2001; Houart et al., 2002). For example, in chicken and Xenopus embryos, Wnt signaling inhibits anterior forebrain markers and promotes posterior fates (Kiecker and Niehrs, 2001; Braun et al., 2003). In zebrafish, forebrain formation is affected in headless (hdl) mutants, in which tcf3 (also known as tcf7l1a – ZFIN), a component of the Wnt pathway, is mutated (Kim et al., 2000), and loss of wnt8 function results in zebrafish embryos with a reduced midbrain and a larger forebrain (Lekven et al., 2001). Six3 is expressed in the ANE during early development and, as shown in chicken and mouse, it represses Wnt1 signaling in the developing brain (Braun et al., 2003; Lagutin et al., 2003). Similar to Six3-null mouse embryos (Lagutin et al., 2003), in hdl-mutant zebrafish embryos, Wnt signaling ectopically expands, and six3 expression is drastically reduced (Kim et al., 2000).

In mice, the lack of Six3 activity results in ectopic anterior expansion of Wnt1 expression in the developing brain and, similar to zebrafish, frog and chicken embryos, the roof plate posteriorizes (Lagutin et al., 2003). Data from these organisms lead to the proposal that telencephalic development requires suppression of Wnt signaling from the anterior region; defective suppression results in the prospective forebrain acquiring a more-posterior diencephalic identity.

Here, we determined that the anterior part of the Six3-null mouse brain gradually caudalizes; this process starts in the ANE, continues later into the roof and alar plates, and ends once the prospective thalamus is replaced by the pretectum at around E11.0. We also determined that the ectopic anterior expansion of Irx3 and Tcf4 expression is a gradual process that is not completed in the alar plate until the 22- to 24-somite stage. These data indicate that although the ectopic anterior expansion of Wnt1 expression starts at an early somitic stage, the resulting abnormal posteriorization of the Six3-null brain is delayed. A possible explanation for this delay is that the anterior-most mutant brain cannot respond to the ectopic Wnt signaling until later embryonic stages.

6E', F'), were rescued in the E14.5 Six3+/–;Wnt1+/– brain. A partial rescue of these structures was observed in E14.5 Six3+/–;Wnt1+/– brains (see Fig. S10 in the supplementary material).

These results suggest that the posteriorization of the anterior alar plate in the Six3-null brain is a Wnt1-dependent process that interferes with the normal dorsal expansion of the ZLI. Therefore, nearly all of the prospective prethalamus territory was lost and replaced by the thalamus at the 22- to 24-somite stage; by E11.0, the mesencephalon is drastically reduced (Kim et al., 2000). Six3–/–;Wnt1–/– mice were rescued in the E14.5 Six3–/–;Wnt1–/– brains (also known as tcf7l1a – ZFIN), a gene that is expressed in the alar plate of the pretectum and in the thalamus of wild-type. Wnt1+/– embryos, Wnt signaling ectopically expands, and Six3–/–;Wnt1+/– brain (D). Similar results were obtained with Tcf4, a gene that is expressed in the alar plate of the pretectum and in the thalamus of wild-type. Wnt1+/– embryos, Wnt signaling ectopically expands, and Six3–/–;Wnt1+/– brain (E). These results suggest that the posteriorization of the anterior alar plate in the Six3-null brain is a Wnt1-dependent process that interferes with the normal dorsal expansion of the ZLI. Therefore, nearly all of the prospective prethalamus territory was lost and replaced by the thalamus at the 22- to 24-somite stage; by E11.0, the mesencephalon is drastically reduced (Kim et al., 2000). Six3–/–;Wnt1–/– mouse brains (see Fig. S10 in the supplementary material).

Fig. 5. Prethalamus but not telencephalon is rescued in Six3–/–;Wnt1–/– embryos. Foxg1 is expressed in the telencephalon of wild-type (A) and Wnt1+/– (A’) mouse brains at E10.5. No telencephalic expression of Foxg1 was observed in Six3+/– (A”) or Six3+/–;Wnt1+/– brains (A’’). Emx1 is expressed in the dorsal telencephalon of wild-type (arrow, B) and Wnt1+/– (arrow, B’) brains. Tbr1 is expressed in the telencephalon (arrow) and eminentia thalami (arrowhead) of wild-type (C) and Wnt1+/– (C’) brains. No telencephalic expression of Emx1 and Tbr1 was observed in the Six3+/– (B”, C”) and Six3+/–;Wnt1+/– (B”, C”) brains. In E14.5 Six3–/–;Wnt1–/– brains, Wnt1+/– (D) and Six3-null (E) brains at E10.5. In the Six3-null brain (D’), Wnt1+/– (E’) and Six3-null (E”) embryos (E”). The prethalamus (arrow), as indicated by Fezf1 expression in the wild-type (F) and Wnt1+/– (F’) brain, is extremely reduced in the Six3-null brain (F”), however, it appears normal in the Six3+/–;Wnt1+/– brain (F”). A model for the Six3+/–;Wnt1+/– brain at this stage is shown (G”). In the Wnt1+/– brain (G”), the mesencephalon is smaller than in the wild-type brain (G). The prospective thalamus is the most anterior structure in the Six3-null brain (G”), and the prethalamic remains in the Six3+/–;Wnt1+/– brain (G”) at this stage. The basal plate is indicated in blue. Lines delimit the prospective regions. Anterior is to the right. H, hindbrain; HY, hypothalamus; M, mesencephalon; PT, prethalamus; PTC, pretectum; T, thalamus; TEL, telencephalon.
The lack of telencephalon and eyes reported in medaka fish after Six3 inactivation using morpholinos has been associated with increased cell death in the ANE of late-gastrula embryos (Carl et al., 2002). The lack of telencephalon in the Six3-null mouse brain was detected much earlier than the 17-somite stage, when we observed increased cell death in the anterior brain. Therefore, in mammals, cell death is not responsible for the lack of telencephalon. However, the presence of an Irx3 and Tcf4-free territory rostral to the posterior diencephalon at the 17-somite stage and their later anterior expansion argues that the increased cell death identified in the anterior brain territory of 17-somite stage embryos is responsible for the caudalized brain phenotype of Six3-null embryos. Nevertheless, it could also be argued that a possible change in cell fate could also contribute to this phenotype, or, that similar to what has been described in chicken embryos (Arnold-Aldeia and Cepko, 1996; Golden and Cepko, 1996; Larsen et al., 2001), cell dispersion between the posterior and anterior diencephalon might have also contributed to caudalization at this stage.

**Six3 activity is not required for the formation or maintenance of the mammalian ZLI**

The interaction between prospective thalamus and prethalamus specifies the position of the ZLI in chicken (Kobayashi et al., 2002; Braun et al., 2003; Echevarria et al., 2003; Vieira et al., 2005) and zebrafish (Scholpp et al., 2006) embryos. In chicken, it has been proposed that the abutting expression of Six3 rostrally and Irx3 caudally marks the position where the ZLI will form (Kobayashi et al., 2002). However, in mouse, the Irx1 expression domain, a gene whose expression pattern is similar to that of chicken Irx3 (Cohen et al., 2000; Hirata et al., 2006b), does not overlap with that of Six3 (data not shown). Furthermore, the absence of Six3 activity does not affect the establishment of the initial domain of Shh expression in the prospective ZLI. These data support the proposal that unlike in chicken, in mammals Six3 does not directly affect ZLI localization; instead, it indirectly maintains the anterior character of the developing rostral brain.

In mammals, other genes expressed in the prospective prethalamus have a role in ZLI localization. Fezf1 and Fezf2 are expressed in this region and their deficit inhibits the formation of the prospective prethalamus and ZLI (Hirata et al., 2004; Hirata et al., 2006b). Here we showed that in Six3-null embryos the ZLI is not present at late developmental stages, and the expression domains of Fezf1 and Fezf2 are greatly reduced. Fezf1 expression and the presence of the ZLI were recovered in the Six3;Wnt1 double-null embryos. These results suggest that at the early stages of ZLI localization, Six3 acts upstream of Fezf1/Fezf2 and maintains the anterior character of the tissue rostral to the future ZLI by repressing dorsal Wnt signaling. By taking advantage of the available Six3 conditional strain, we determined that Shh expression in the ZLI is independent of Six3 activity; therefore, the establishment and maintenance of the ZLI in mammals do not require Six3 activity.

**Six3 is required for the formation of the telencephalon**

The lack of telencephalon in Six3-null embryos may result from the ectopic anterior expansion of Wnt1 (Lagutin et al., 2003). However, ectopic expression of Wnt1 in the mouse ANE was not sufficient to affect the formation of the telencephalon and rostral diencephalon (Ligon et al., 2003). These results suggest that the ectopic anterior expansion of Wnt1 expression in Six3-null embryos is not responsible for the brain truncation; instead, this aberration could be directly related to the lack of Six3 activity. This possibility was supported by our concurrent inactivation of Six3 and Wnt1 in mice, which showed that Wnt1 removal is not sufficient to rescue telencephalic formation.

In mice, other Wnt family members such as Wnt8b and Wnt3a are expressed in the ANE and roof plate (Parr et al., 1993). Wnt8b antagonizes telencephalon formation in zebrafish, and reduced Wnt signaling in mbl–/– zebrafish embryos by the abrogation of wnt8b activity restores the telencephalon (Houart et al., 2002). Wnt3a;Wnt1 double-null mouse embryos exhibit a more-severe brain phenotype.
Rostral diencephalic development depends on Six3 repression of Wnt1 posteriorizing signals

Conditional inactivation of Six3 confirmed not only that Six3 activity does not maintain Shh expression in the ZLI, but also that Six3 activity prior to E9.0 is necessary for rostral diencephalon development. Patterning of the forebrain into telencephalic, eye and diencephalic regions is the result of graded expression of Wnt signaling in the anterior neural plate (Houart et al., 2002; Wilson and Houart, 2004). Accordingly, we argue that anterior neural tissue is more susceptible to subtle changes in Wnt signaling.

In the Six3-null brain, early ectopic expansion of Wnt1 expression is not sufficient to block rostral diencephalic formation. However, at around the 17-somite stage, the abnormal anterior expansion of Wnt activity causes the mutant brain to posteriorize. As a consequence, nearly all the prospective prethalamus is lost at the 22-to-24-somite stage and is later replaced by the pretectum. Because the prethalamus is not maintained, the ZLI and the boundary between thalamus and prethalamus will not form. Defects in ZLI formation or suppression of Shh expression in the ZLI promote caudalization of the mouse diencephalon (Hirata et al., 2006b) and defectivethalamic and prethalamic induction in chicken (Kiecker and Lumsden, 2004; Vieira and Martinez, 2006) and zebrafish (Scholpp et al., 2006; Scholpp et al., 2007) embryos. Therefore, absence of the ZLI is probably at least partially responsible for the late-caudalization phenotype of the Six3-mutant brain.

The generation of Six3; Wnt1 double-null mutants revealed that repression of Wnt1 activity by Six3 in the early ANE is required to protect the rostral diencephalon from posteriorizing signals, to allow the expansion of the ZLI and to pattern the diencephalon.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/9/441/DC1

References


