Autoregulation of canonical Wnt signaling controls midbrain development

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Abstract

After the primary anterior–posterior patterning of the neural plate, a subset of wnt signaling molecules including Xwnt-1, Xwnt-2b, Xwnt-3A, Xwnt-8b are still expressed in the developing brain in a region spanning from the posterior part of the diencephalon to the mesencephalon/metencephalon boundary. In this expression field, they are colocalized with the HMG-box transcription factor XTcf-4. Using antisense morpholino loss-of-function strategies, we demonstrate that the expression of this transcription factor depends on Xwnt-2b, which itself is under the control of XTcf-4. Marker gene analyses reveal that this autoregulatory loop is important for proper development of the midbrain and the isthmus. Staining for NCAM reveals a lack of dorsal neural tissue in this area. This reduction is caused by a reduced proliferation rate as shown by staining for PhosphoH3 positive nuclei. In rescue experiments, we demonstrate that individual isoforms of XTcf-4 control the development of different parts of the brain. XTcf-4A restored the expression of the mesencephalon marker genes pax-6 and wnt-2b but not the isthmus marker gene en-2. XTcf-4C, in contrast, restored en-2, but had only weak effects on pax-6 and wnt-2b. Thus, autoregulation of canonical Wnt signaling and alternative expression of different isoforms of XTcf-4 is essential for specifying the developing CNS.

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Introduction

The first steps of neural induction involve complex interactions of several growth factors including fibroblastic growth factors (Fgfs), bone morphogenetic proteins (BMPs), and Wnts (Wilson and Edlund, 2001). In Xenopus, dorsally enriched β-Catenin and the BMP-antagonist Chordin define the preorganizer controlling neural induction. The neural tube is further patterned in dorsoventral direction by gradients of secreted growth factors. A BMP antagonist and Sonic hedgehog (shh) source at the notochord and floor plate form a gradient with ventralizing activity. An opposite gradient derived from a Wnt and BMP source at the roof plate dorsalizes the neuroectoderm (Wessely and De Robertis, 2002; Wilson and Edlund, 2001). As soon as neurulation takes place, a regional specificity in the anterior–posterior direction of the prospective CNS is given by instructive signals of the underlying mesendoderm and mesoderm. An endogenous Wnt/β-Catenin signaling gradient patterns the neuroectoderm. Wnt agonists, including Xwnt-8, Xwnt-3A, and nuclear β-Catenin, posteriorize the neuroectoderm, while Wnt antagonists like FrzB, Cerberus, and Dickkopf induce more anterior neural tissue (Glinka et al., 1998; Kiecker and Niehrs, 2001). These inductive signals during gastrulation and early neurulation result in an embryonic neuroectoderm, which is already prepatterned, both in dorsoventral and anterior–posterior direction. A further subdivision of the developing brain in five secondary brain vesicles, six prosomeres, and eight rhombomeres implicates the presence of additional organizing centers in the developing CNS. Indeed, in several organisms, three organizers have been described: (1) An Fgf-8 and Wnt-1 source at the midbrain/hindbrain boundary (MHB, also termed mesencephalon/metencephalon boundary or isthmus) patterns both the midbrain and the hindbrain (Liu and Joyner, 2001). (2) A shh source between the prosomeres 2 and 3, which
subdivides the prosencephalon into telencephalon and diencephalon (Rubenstein et al., 1994). (3) In the anterior-most region, ectodermal cells of the anterior neural ridge secrete Fgf-8, a growth factor necessary for the subdivision of the telencephalon into prosomeres and for the growth of the optic vesicle (Shimamura and Rubenstein, 1997).

The accumulation of several secreted signaling molecules of the Xwnt family, including Xwnt-1, Xwnt-2b, Xwnt-3A, and Xwnt-8b, in a region spanning from the rostral part of the diencephalon to the isthmus (Cui et al., 1995; König et al., 2000) might reflect an additional organizing center at the forebrain–midbrain boundary controlling anterior–posterior patterning of the brain. These Xwnt molecules activate the Wnt/β-Catenin pathway, resulting in an accumulation of stabilized β-Catenin. Indeed, a striking enrichment of nuclear β-Catenin was observed in mesencephalic nuclei (Schohl and Fagotto, 2002). This area of active Wnt signaling overlaps with the expression pattern of XTcf-4. The other Xenopus Lef/Tcf family members XLef-1, XTcf-1, and XTcf-3 have unique and partially overlapping expression patterns but are not predominantly localized in the developing mesencephalon (Molenaar et al., 1998; Roel et al., 2003).

Many efforts have been undertaken to study the role of the canonical Wnt pathway in early Xenopus embryogenesis. There is no doubt that Wnt/β-Catenin signaling plays a major role in axis formation, mesoderm induction, neural induction, and neural crest induction (Cadigan and Nusse, 1997; Gradl et al., 1999a) but only little is known why so many Xwnts are expressed in the developing mesencephalon. Recently, it has been shown by the use of a synthetic reporter that β-Catenin-driven transcription in the developing murine brain is most prominent in the mesencephalon and the adjacent parts of the diencephalon and metencephalon (Mareto et al., 2003), again overlapping with tcf-4 expression. In Xenopus, we have shown that functionally different isoforms of XTcf-4 are alternatively expressed, starting with XTcf-4C transcription at stage 16, XTcf-4A at stage 18, and XTcf-4B around stage 20. In axis induction assays and Wnt-target promoter analysis, XTcf-4A acts as a repressor and XTcf-4C acts as an activator (Gradl et al., 2002).

Unlike XTcf-1, XTcf-3, and XLef-1, expression of XTcf-4 is restricted to the anterior midbrain and the forebrain–midbrain boundary, where it overlaps with Xwnt-2b. To investigate the role of endogenous canonical Wnt signaling in midbrain development, we knocked down Xwnt-2b and Xwnt-4 by antisense morpholino injections. We demonstrate that an autoregulatory Xwnt-2b/XTcf-4 loop regulates midbrain and isthmus development and that XTcf-4 is essential for cell proliferation in the dorsal part of the developing midbrain. To discriminate the role of individual XTcf-4 isoforms in midbrain development, we coinjected the XTcf-4 morpholino together with XTcf-4A and XTcf-4C. We show that XTcf-4A is essential for midbrain but not for isthmus development. Instead, XTcf-4C is involved in isthmus development, but plays a minor role in midbrain development.

Materials and methods

Plasmids, constructs, in vitro transcription

Capped mRNAs were transcribed from linearized DNA templates using mMESSAGE mMMachine (Ambion). Digoxigenin-labeled antisense probes for in situ hybridization were synthesized with DIG RNA labeling kit (Roche). Probes for in situ hybridization are described elsewhere: Bf-1 (Kiecker and Niehrs, 2001), en-2 (Landesman and Sokol, 1997), fgf-8 (Christen and Slack, 1997), krox-20 (Borchers et al., 2001), nrp-1 (Borchers et al., 2001), otx-2 (Blitz and Cho, 1995), pax-6 (Hirsch and Harris, 1997), tcf-4 (König et al., 2000), wnt-1 (Wolda et al., 1993), wnt-2b (Landesman and Sokol, 1997). Full-length Xwnt-2b, kindly provided by S. Sokol, was C-terminally fused to EGFP and subcloned in pCS2 expression vector using PCR strategies (primers are available upon request). PCMV2-Xwnt-11 was kindly provided by A. Glinka, pRe/CMV-Xwnt-5A and Xwnt-8, pCS2-XTcf-4A and XTcf-4C are as described (Gradl et al., 1999b, 2002). The following antisense morpholinos were used: Xwnt-2b (Wmo): 5′-gca tcc tag agg tgg ctc caa tga t-3′, XTcf-4 (Tmo): 5′-cgc cat tca act ggg gca tgg ctc c-3′, and control morpholino (Como): 5′-ata ttc att cac att gac tcc att ctc c-3′.

Xenopus embryo manipulations

Eggs from HCG-treated females were fertilized by standard methods and staged according to Nieuwkoop and Faber (1967). Morpholino (10 pmol) were coinjected with 100 pg EGFPmyc mRNA into one blastomere of two-cell stage embryos in a total volume of 12 nl. For coinjections, 100 pg cDNA encoding for Xwnt-2b, Xwnt-5A, Xwnt-8, Xwnt-11, XTcf-4A, or XTcf-4C were added. At stage 30, embryos were fixed in MEMFA (0.1 M MOPS pH 7.2, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde). For vibratome sections, fixed embryos were embedded in 3% agarose and sectioned using a Leica VT 1000S vibratome. For visualizing XTcf-4 expression in late tadpole stages, complete brains were explanted from stage 50 embryos.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed according to previously described procedures (Gawantka et al., 1995). Localization of mRNA was visualized using digoxigenin antibodies conjugated to alkaline phosphatase, followed by incubation with nitro blue tetrazolium (NBT) and 5-bromo 4-chloro 3-indolyl phosphate (BCIP). Images were captured on a Leica MZFLIII microscope using a digital camera (Qimaging) and Improvisation software (Openlab).
**Immunostaining**

Embryos, isolated brains, and vibratome sections were permeabilized by washing in APBS/0.1% Triton (103 mM NaCl, 2.7 mM KCl, 0.15 mM KH2PO4, 0.7 mM NH2PO4, 2 mM CaCl2, pH 7.5, 0.1% Triton X-100). After blocking for 1 h in 1% bovine serum albumin in APBS, samples were incubated with primary antibody overnight at 4°C. The following primary antibodies were used: α-TCF-3/4 (1:100), α-NCAM (1:100), α-myc epitope 9E10 (1:100), α-phosphoH3 (1:100), and α-EGFP (1:100). As secondary antibodies, we used Cy2- and Cy3-labeled goat anti-mouse or goat anti-rabbit 1:200. Images were captured on a Leica DMIRE2 microscope using a digital camera (Hamamatsu) and Improvision software.

**Immunoblotting**

NOP lysates (150 mM NaCl, 10 mM Tris–HCl pH 7.8, 1 mM MgCl2, 0.75 mM CaCl2, 2% Nonidet P40) corresponding to one embryo were separated by SDS-PAGE and transferred to nitrocellulose. After blocking, the membrane was incubated with α-myc (9E10) or α-EGFP monoclonal antibody and peroxidase-coupled secondary antibody. As loading control, blots were incubated with α-Gemin antiserum kindly provided by U. Fischer. Proteins were

Fig. 1. Knockdown of Xwnt-2b results in reduced expression of tcf-4 and en-2. (A) Alignment of Xwnt-2b with the Xwnt-2b EGFP construct containing the 5’-UTR binding site for the Xwnt-2b morpholino (Wmo) but not for the control morpholino (Como). (B) Wnt-2b morpholino (Wmo), but not control morpholino (Como), represses the translation of exogenously expressed Xwnt-2b (Xwnt-2bEGFP). NOP lysates of a single embryonic equivalent of stage 13 (according to Nieuwkoop and Faber, 1967) stained with α-EGFP antibody. Counterstaining of the blot with α-Gemin was used as loading control. (C) Wnt-2b morpholino blocks the expression of XTcf-4 protein in the midbrain. Ten picomoles of the morpholino were injected together with 100 pg of myc-tagged EGFP mRNA into one blastomere of two-cell stage embryos. Transverse sections through the midbrain region of stage 30 embryos were stained with α-Tcf3/4 primary antibody and visualized with cy3-labeled secondary antibody. The EGFP signal indicates the injected side. (D) In situ hybridization of stage 30 embryos reveals that the Wnt-2b morpholino blocks the expression of tcf-4 and en-2, but not otx-2 and pax-6. Ten picomoles of the morpholino were injected together with 100 pg of myc-tagged EGFP mRNA into one blastomere of two-cell stage embryos. At late neurula stages, embryos were sorted by the fluorescence signal of EGFP. At stage 30, embryos were fixed and analyzed by whole-mount in situ hybridization. f: forebrain, m: midbrain, h: hindbrain.
visualized using ECL-Plus Western Blotting Detection System (Amersham).

**Results**

**Canonical Wnt signaling regulates tcf-4 expression in the midbrain**

The restricted and overlapping expression pattern of XTcf-4 and several Xwnts at the midbrain and the forebrain–midbrain boundary prompted us to investigate the function of Wnt/β-Catenin signaling in the patterning of the embryonic brain. Because zygotic expression of both genes starts at late neurula stages and is almost completely overlapping and restricted to the developing midbrain (König et al., 2000; Landesman and Sokol, 1997), we started our studies with the knockdown of Xwnt-2b. The efficiency of the antisense morpholino was tested by blocking the translation of a coinjected Wnt-2bEGFP construct containing the binding site for the morpholino (Fig. 1A). Indeed, the synthesis of Wnt-2bEGFP was...
repressed by coinjection of the Wnt-2b morpholino (Wmo), but not by the control morpholino (Como) (Fig. 1B). The spliceosome assembly factor Gemin was used as loading control.

Due to the colocalized expression of Xwnt-2b and XTcf-4, we speculated that one of the most prominent effects of Xwnt-2b knockdown would be altered tcf-4 expression. Therefore, we coinjected 10 pmol of the morpholino together with 100 pg of myc-tagged EGFP RNA into one blastomere of two-cell stage embryos. The coinjected EGFP-myc was used to trace the injected side, either by evaluating the EGFP signal or by immunological staining of the myc-epitope. Immunohistological studies using an αTcf3/4 antibody (Fig. 1C) and whole-mount in situ hybridizations (Fig. 1D) revealed that indeed, XTcf-4 expression was partially reduced at the injected side, both

Fig. 3. Knockdown of XTcf-4 results in malformed isthmus. (A) Alignment of XTcf-4 (XTcf-4myc) with the XTcf-4UTRmyc construct containing the 5'-UTR binding site for the XTcf-4 morpholino (Tmo) but not for the control morpholino (Como). (B) XTcf-4 morpholino (Tmo) but not control morpholino (Como) suppresses the translation of exogenous XTcf-4UTRmyc. NOP lysates equivalent to one embryo were separated on a 7.5% SDS PAGE, transferred onto nitrocellulose, and stained with the α-myc antibody 9E10. Counterstaining of the blot with α-Gemin was used as loading control. (C) XTcf-4 morpholino (Tmo), but not control morpholino (Como), blocks the expression of XTcf-4 protein in the midbrain. Ten picomoles of the morpholino were injected together with 100 pg of myc-tagged EGFP mRNA into one blastomere of two-cell stage embryos. Transversal sections through the midbrain region stained with αTcf3/4 antibody reveals that following knockdown XTcf-4 protein is strongly reduced in the midbrain. (D) XTcf-4 protein reappears at the injected side during late tadpole stages. Ten picomoles of the morpholino were injected together with 100 pg of myc-tagged EGFP mRNA into one blastomere of two-cell stage embryos. Explanted brains of stage 50 embryos were incubated with αTcf3/4 and visualized with a Cy3-coupled goat anti-mouse. The injected side is traced by EGFP. Note the reduction of the mesencephalic part of the isthmus at the injected side (arrow). f: forebrain, m: midbrain, h: hindbrain.
at RNA and protein level. To our surprise, knockdown of Xwnt-2b did not reduce the expression of other coexpressed marker genes, including the paired-box gene *pax-6* or the orthodentrical homologue *otx-2* (Fig. 1D). Thus, Xwnt-2b is not the major regulator of the anterior–posterior patterning of the CNS. Interestingly, in about 50% of the injected embryos (*n = 16*), we observed a partial reduction in expression of the direct Wnt/β-Catenin target gene *engrailed-2 (en-2)* (Fig. 1D), whereas other isthmus marker genes including *fgf-8* and *wnt-1* were not affected (data not shown).

Based on the reduced *tcf-4* expression, we hypothesized that an autoregulatory loop of Xwnt-2b/β-Catenin/XTcf-4 is necessary for maintaining *tcf-4* expression. If so, *tcf-4* expression should be restored by the activation of the canonical Wnt pathway. For this approach, we coinjected 100 pg cDNA encoding Xwnt-2b, Xwnt-8, Xwnt-5A, or Xwnt-11 under the control of the CMV promoter with the Xwnt-2b antisense morpholino. We found that Xwnt-2b and Xwnt-8 restored the morpholino-induced phenotype almost completely (Fig. 2). Ninety-four to ninety-seven percent of the coinjected embryos showed no reduction in *tcf-4* expression, compared to 37% of embryos injected with morpholino alone. Interestingly, injection of the noncanonical Xwnts including Xwnt-5A and Xwnt-11 did not restore the morpholino phenotype (Fig. 2). This indicates that *tcf-4* expression is activated by the canonical Wnt pathway. Because several Xwnts are coexpressed in the *tcf-4* expression field, we postulate that Xwnt-2b, Xwnt-3A, Xwnt-1, and Xwnt-8b regulate their downstream transcription factor *tcf-4* in a partially redundant manner.

**XTcf-4 is essential for midbrain development**

After we have identified *tcf-4* as a target of Wnt/β-Catenin signaling, we focused on the role of this transcription factor in brain development. For this approach, we knocked down XTcf-4 translation by antisense morpholino injection (Tmo) (Fig. 3A). The immunoblot demonstrates that translation of exogenous XTcf-4UTRmyc was efficiently blocked by the antisense morpholino but not by the control morpholino (Como) (Fig. 3B). In immunohistochemical studies, we analyzed the effect of the antisense morpholino on endogenous XTcf-4 protein (Fig. 3C) and found that it was absent at the injected side (traced by EGFP) of stage 30 embryos. Transverse sections illustrate that in some cases, traces of XTcf-4 protein remained expressed in the dorsal part of the midbrain. In stage 50, the XTcf-4 signal reappeared at the injected side, as shown by α-Tcf-4 staining of explanted brains (Fig. 3D). However, the posterior part of the midbrain adjacent to the isthmus was malformed, suggesting that XTcf-4 is essential for proper structural organization of the isthmus. The control morpholino (Como) had no effect on XTcf-4 expression or brain morphology (Figs. 3C, D).

The knockdown of XTcf-4 did not cause any early developmental effects as failures in gastrulation or spina bifida (data not shown). But the structure of the neural tissue in the midbrain region revealed severe defects. Immunological staining for the pan-neural marker NCAM shows that the dorsal part of the midbrain is reduced or even missing at the injected side (Fig. 4A). The missing neural tissue does not seem to be replaced by non-neural tissue; instead, the dorsal midbrain is less thick (Fig. 4A, brightfield). Transversal sections in the midbrain region of Tmo-injected embryos stained for the early pan-neural marker gene *nrp-1* revealed that the roof plate was well formed, but again the dorsal neural tissue was reduced (Fig. 4B). As the remaining tissue does express *nrp-1* but not NCAM, we conclude that it consists of unspecified neural tissue. To demonstrate that the lack of neural tissue is caused by reduced cell proliferation, we stained transverse sections of stage 25 embryos with the proliferation marker α-phosphoH3 (Fig. 4C). This immunostaining reveals that in the dorsal half of the brain, the number of dividing cells is dramatically decreased (53.4 ± 4.0% PhosphoH3-positive nuclei relative to the uninjected side) whereas proliferation of the ventral cells in the brain was normal (93.4 ± 6.5% PhosphoH3-positive nuclei relative to the uninjected side) (Fig. 4D). Thus, blocking XTcf-4 synthesis results in a loss of cell proliferation in the tissue that normally expresses XTcf-4. Consistent with this lack of neural tissue, we observed a downregulation of all marker genes coexpressed with *tcf-4* or adjacent to the *tcf-4* expression field. These include *wnt-2b, pax-6, otx-2, en-2, and fgf-8* (Fig. 5) as well as *tcf-4*, *wnt-1*, and *wnt-3A* (not shown). More detailed analyses of transverse sections revealed that the ventral staining for *lim* remained more or less unchanged, while the dorsolateral *lim* staining disappeared following Tcf-4 knockdown (not shown). Forebrain markers like *bf-1* (Fig. 5), *anf-1*, or *arx-2* (not shown) and hindbrain markers like *krox-20* (Fig. 5) were not affected by the antisense morpholino. Consistent with the observation that the antisense morpholino did not affect early embryogenesis, we did not see any reductions of neural markers before the onset of *tcf-4* expression. *Pax-6* expression, although reduced at stage 28, was not altered by the morpholino at stage 13 (Fig. 5).

**XTcf-4 isoforms differ in regulating midbrain development**

The data presented so far provide evidence that the Wnt-regulated *tcf-4* expression is essential for proper development of the midbrain. Recently, we found that isoforms of XTcf-4, most likely derived by alternative splicing, are differentially expressed in embryogenesis (Gradl et al., 2002). XTcf-4C, an isoform that acts as activator is expressed from stage 16 onward, followed by the repressor XTcf-4A from stage 18 onward. To dissect the role of the individual isoforms, we tested their rescue properties by coinjecting cDNA of XTcf-4A and XTcf-4C together with...
the antisense morpholino. The ability to rescue the antisense morpholino phenotype was evaluated by the reappearance of NCAM. For this purpose, the reduction degree in NCAM staining was scored according to the following categories: strongly reduced (2), reduced (1), and not reduced (0) (Fig. 6A). The evaluation revealed that both isoforms can rescue
the morpholino-induced phenotype but XTcf-4A was more potent than XTcf-4C (Fig. 6B). Coinjection of XTcf-4C decreased the frequency of category 2 phenotype from 76.6% to 16%. Eighteen percent of the sections showed no reduction (category 0). This rescue effect was even more pronounced in the XTcf-4A coinjected embryos. Only 14.9% of the transverse sections revealed a strong reduction of NCAM (category 2) and in 42.6% NCAM staining was not reduced (Fig. 6B). To quantify the rescue effects of the different XTcf-4 isoforms, we measured the thickness of the dorsal midbrain in the injected side in relation to the noninjected side, and in Tmo-injected embryos, a reduction to 47% (±2%) was found (Fig. 6C). Again, both XTcf-4 isoforms were able to restore the midbrain. Consistent with the observed rescue of NCAM expression, we observed a statistically significant difference (P < 0.005 in Student’s t test) between XTcf-4A and XTcf-4C in restoring the thickness of the midbrain at the injected side.

Fig. 5. XTcf-4 regulates the expression of midbrain and isthmus marker genes. Ten picomoles of XTcf-4 morpholino were coinjected with 100 pg EGFP-RNA. At late neurula stages, embryos were sorted by the fluorescence signal of the coinjected EGFP. At stage 30, embryos were fixed and stained by in situ hybridization for the expression of the indicated genes. The midbrain marker genes wnt-2b, otx-2, and pax-6 and the isthmus marker genes en-2 and fgf-8 were markedly reduced at the XTcf-4 morpholino (Tmo)-injected side (marked with an asterisk), while the forebrain marker bf-1 and the rhombomere 3 and 5 marker krox-20 were not reduced. The control morpholino (Como) did not reduce the expression of any of the marker genes analyzed. In early neurula (stage 13), the XTcf-4 morpholino did not alter the expression of pax-6. f: forebrain, m: midbrain, h: hindbrain.
Surprisingly, we found that the individual isoforms restored different parts of the neural tissue. Expression of the midbrain marker genes *pax-6* and *wnt-2b* was much better restored by XTcf-4A than by XTcf-4C (Figs. 7A, B). Expression of the isthmus marker genes *en-2*, *fgf-8*, and *wnt-1*, however, was better restored by XTcf-4C than by XTcf-4A (Figs. 7A, B). Thus, the endogenous function of XTcf-4 in brain development can be subdivided in two

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**Fig. 6.** XTcf-4A and XTcf-4C differ in restoring NCAM expression. (A) The upper panel shows the evaluation of NCAM reduction in transverse sections through the midbrain region of stage 30 embryos according to the categories 0: no reduction; 1: partial reduction of dorsal neural tissue; 2: total reduction of dorsal neural tissue. The lower panel shows examples of embryos coinjected with 10 pmol Tcf-4 morpholino (Tmo) and 100 pg XTcf-4A cDNA or 10 pmol Tcf-4 morpholino and 100 pg XTcf-4C cDNA together with 100 pg EGFP mRNA to mark the injected side. Transverse sections through the midbrain region of stage 30 embryos were incubated with α-NCAM antibody and visualized with a Cy3-coupled secondary antibody. The bars in the brightfield image on the right indicate the area used for measuring the relative thickness of the dorsal midbrain. (B) Quantification of NCAM reduction in the midbrain at the injected side. Given is the percentage of sections showing the different categories of NCAM reduction. The number of embryos and the number of sections are given. (C) Quantification of the thickness of the dorsal midbrain. Lateral extension of the midbrain was measured using Openlab software (Improvision). Relative thickness of the dorsal midbrain at the injected side is given in relation to the uninjected side. The asterisks indicate that XTcf-4A restores midbrain thickness significantly better (*P* < 0.005 in Student’s *t* test) than XTcf-4C.
differentially regulated regions: (1) the regulation of dorsal neural tissue development in the midbrain and (2) the control of the expression of isthmus organizer genes. The first mentioned appears to be controlled by the repressing isoform XTcf-4A, the latter by the activating isoform XTcf-4C.

Discussion

The knockdown and rescue experiments in our study demonstrate that an autoregulatory Wnt/β-Catenin/Tcf-4 loop is essential for cell proliferation in the dorsal midbrain and for midbrain and isthmus specification.
Because XTcf-4 is not obviously coexpressed with other Lef/Tcf family members (König et al., 2000; Molema et al., 1998; Roel et al., 2003), we assume that activation of the Wnt pathway via Xwnt-2b in the midbrain is transduced in the nucleus by XTcf-4. Thus, XTcf-4 seems to maintain its own expression. A similar autoregulatory loop has been reported for the expression of hLef-1 in cancer cells (Hovanes et al., 2001), but an autoregulatory Wnt/Tcf-loop important for embryogenesis has not been reported so far. As possible regulators of tcf-4 expression, the canonical Wnt signaling molecules Xwnt-1, Xwnt-2b, and Xwnt-3A overlap in the tcf-4 expression field (König et al., 2000). This redundancy might explain that the suppression of tcf-4 by the Xwnt-2b morpholino was not complete and that subsequently other midbrain markers including pax-6 and otx-2 were not reduced. Surprisingly, we found that the isthmus marker en-2, although not exactly coexpressed, was reduced in the Wnt-2b knockdown embryos. The en-2 reduction could rely on a paracrine effect of Xwnt-2b, directly regulating en-2 expression in the isthmus, or it could be due to secondary effects caused by reduced tcf-4 expression in the midbrain. We showed that XTcf-4 is essential for cell proliferation in the midbrain and that loss of XTcf-4 results in a decrease of dorsal neural tissue. Most likely, these midbrain defects subsequently result in malformations of the posterior midbrain and the isthmus. Currently, we try to identify the molecules mediating the Xwnt-2b/XTcf-4 signal from the anterior midbrain to the isthmus. Interestingly, expression of other isthmus markers like wnt-1 and fgf-8 was not changed after the Wnt-2b knockdown (not shown). In a selective subtractive hybridization screen, McGrew et al. (1999) identified en-2 as a direct target gene of Wnt/β-Catenin signaling. Apparently, different midbrain and isthmus marker genes differ in their sensitivity to Xwnt-2b/XTcf-4 signal. We attempt to speculate that the most sensitive marker genes tcf-4 and en-2 are direct Wnt/β-Catenin targets.

Blocking the translation of Xwnt-2b and XTcf-4 with antisense morpholinos did not result in any early developmental defects. Thus, maternally provided Xwnt-2b and XTcf-4 (Houston et al., 2002; Landesman et al., 2002) seem to play a minor role in early embryogenesis. Down-regulation of XTcf-4 by the corresponding antisense morpholino caused severe defects in the midbrain and a loss of dorsal neural tissue in tadpole embryos. Although in tadpole stages (stage 50), XTcf-4 protein reappears, indicating that the tcf-4-expressing tissue can regenerate even in the absence of XTcf-4 protein; the posterior mesencephalon remains malformed. Unlike the tcf-3 mutant headless in zebrafish (Kim et al., 2000), the effect of the Tcf-4 knockdown in Xenopus was restricted to the midbrain and the adjacent isthmus. We did not observe an altered anterior–posterior patterning of the brain; instead, the XTcf-4-expressing tissue at the dorsal half of the midbrain was missing on the Tcf-4-depleted side. Thus, XTcf-4 is essential for midbrain development. Recently, it has been shown that in the spinal cord of chick embryos, an active Wnt/β-Catenin signal is necessary for proliferation of neuronal precursors and that dominant-negative Tcf-4 blocks cyclinD1 expression and cell proliferation (Megas and McMahon, 2002). Consistently, we found that the number of proliferating cells in the dorsal half of the brain was dramatically decreased after Tcf-4 knockdown. Thus, the Xwnt-2b/XTcf-4 target XTcf-4 regulates brain development by controlling cell proliferation of the dorsal midbrain.

Although brain-specific expression of tcf-4 is conserved in vertebrates (Cho and Dressler, 1998; Young et al., 2002), the knockout mice did not reveal dramatic brain malformations. Instead, Tcf4−/− mice have a defect in the development of the small intestine (Korinek et al., 1998). The lack of brain defects in these mice could be due to a partial redundancy of this transcription factor with other family members as it has been reported for Tcf-1 and Lef-1 in a double knockout (Galceran et al., 1999). Lef-1−/− mice, however, showed a severe malformation of the brain (Galceran et al., 2000). Compared to our XTcf-4 morpholino embryos, the brain of Tcf4−/− mice was affected more anteriorly, exhibiting a disturbed development of the hippocampus. Thus, the different Lef/Tcf family members appear to play the development of different areas in the CNS.

The knockdown of XTcf-4 in this study gives an explanation for the striking accumulation of nuclear β-Catenin in the midbrain (Schohl and Fagotto, 2002): A self-maintaining Wnt/β-Catenin signal is transduced by XTcf-4 and controls cell proliferation in the dorsal midbrain. Furthermore, this autoregulatory Wnt/β-Catenin/XTcf-4 loop is essential for the expression of midbrain marker genes, including wnt-2b, tcf-4, otx-2, and pax-6 as well as the isthmus specific genes wnt-1, fgf-8, and the direct Wnt/β-Catenin target en-2.

Surprisingly, alternatively expressed isoforms of XTcf-4 differ in their endogenous function in brain development. The isoform XTcf-4A, which is characterized as antagonist of Wnt/β-Catenin signaling (Gradi et al., 2002; Pukrop et al., 2001), is essential for the maintenance of the dorsal midbrain and the expression of midbrain-specific marker genes, but not for the expression of isthmus-specific marker genes. XTcf-4C, an agonist of Wnt/β-Catenin signaling, however, reactivated en-2, fgf-8, and wnt-1 expression after the XTcf-4 knockdown. This indicates that a fine balance of Wnt/β-Catenin signaling including both Wnt agonists—such as Xwnt-1, Xwnt-2b, Xwnt-3A, and Xwnt-8b—and Wnt antagonists—such as Xaxin and XTcf-4A—specify the development of the dorsal neural tissue in the midbrain.

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References