RESEARCH ARTICLE

The vestibuloocular reflex of tadpoles (Xenopus laevis) after knock-down of the isthmus-related transcription factor XTcf-4

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SUMMARY

Development of the amphibian vestibular organ is regulated by molecular and neuronal mechanisms and by environmental input. The molecular component includes inductive signals derived from neural tissue of the hindbrain and from the surrounding mesoderm. The integrity of hindbrain patterning, on the other hand, depends on instructive signals from the isthmus organizer of the midbrain, including the transcription factor XTcf-4. If the development of the vestibular system depends on the integrity of the isthmus as the organizing centre, suppression of isthmus maintenance should modify vestibular morphology and function. We tested this hypothesis by downregulation of the transcription factor XTcf-4. If development of the vestibular system depends on the integrity of the isthmus as the organizing centre, suppression of isthmus maintenance should modify vestibular morphology and function. We tested this hypothesis by downregulation of the transcription factor XTcf-4. 10 pmol of XTcf-4-specific antisense morpholino oligonucleotide was injected in one blastomere of two-cell-stage embryos of Xenopus laevis. For reconstitution experiments, 500 pg mRNA of the repressing XTcf-4A isoform or the activating XTcf-4C isoform were co-injected. Overexpression experiments were included using the same isoforms. Otoconia formation and vestibular controlled behaviour such as the roll-induced vestibuloocular reflex (rVOR) and swimming were recorded two weeks later. In 50% of tadpoles, downregulation of XTcf-4 induced (1) a depression of otoconia formation accompanied by a reduction of the rVOR, (2) abnormal tail development and (3) loop swimming behaviour. (4) All effects were rescued by co-injection of XTcf-4C but not, or only partially, by XTcf-4A. (5) Overexpression of XTcf-4A caused similar morphological and rVOR modifications as XTcf-4 depletion, while overexpression of XTcf-4C had no effect. Because XTcf-4C has been described as an essential factor for isthmus development, we postulate that the isthmus is strongly involved in vestibular development.

Key words: axis formation, eye movement, postural control, vestibular development, Wnt-system.

Received 20 August 2012; Accepted 8 October 2012

INTRODUCTION

Development of the vestibular system, including the formation of sensory cells, the stimulus-transducing otoconia and otoliths, and the neuronal network, is regulated by molecular (Söllner et al., 2003; Söllner et al., 2004; Hughes et al., 2004; Blasiole et al., 2006; Kwak et al., 2006; Murayama et al., 2005; MacLennan et al., 2006; Petko et al., 2008; Zhao et al., 2007) and neuronal mechanisms (Edelmann et al., 2004), but environmental input is also needed (Wiederhold et al., 2003). Inner ear development, in particular, depends on inductive signals derived from the neural tissue of the hindbrain and from the surrounding mesoderm (Park and Saint-Jennet, 2008). The relevant morphogens belong to the Wnt and FGF growth factor families (Ladher et al., 2000). The integrity of hindbrain patterning depends on instructive signals from the isthmus organizer (Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001). The transcription factor XTcf-4, in particular its activating XTcf-4C isoform, expressed in the developing midbrain, is an essential factor for isthmus development (Kunz et al., 2004; Koenig et al., 2010).

These observations make a contribution of the isthmus to vestibular development likely. In quail and chick embryos, the isthmus is an organizer of tectum and cerebellum development (Nakamura et al., 2008) but its contribution to vestibular development has remained elusive. In the clawed toad (Xenopus laevis), depletion of zygotic XTcf-4 by morpholino injections has no effect on brain development until late neurula stages (Kunz et al., 2004). Furthermore, depletion of engrailed-2 results in a loss of isthmus-specific pax2 expression while otic expression of pax2 remains unchanged (Koenig et al., 2010). These observations make it unlikely that XTcf-4 morpholino-induced blockade of isthmus development causes defects in early otic development, in particular in placode induction.

We have, therefore, focused our interest on the question of whether impaired isthmus function modifies development of the inner ear in older stages; in particular, whether suppression of isthmus maintenance by XTcf-4 depletion results in malformations of the otoconia area and, consequently, of vestibular-induced behavior. To test this hypothesis, we selected the roll-induced vestibuloocular reflex (rVOR). Its development and adaptation to surgical lesions and modifications of the gravitational environment have been extensively studied in Xenopus (Horn et al., 1986a; Horn et al., 1986b; Rayer and Horn, 1986; Sebastian et al., 1996; Horn, 2006; Horn and Gabriel, 2011).

MATERIALS AND METHODS

Animals

The experiments were performed with tadpoles from the southern clawed toad, Xenopus laevis Daudin. Eggs from human choriongonadotropin (HCG)-treated females were fertilized by standard methods and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryos were reared at temperatures of 20–22°C for 2 weeks to elicit the rVOR (Horn et al., 1986a).
Knock-down of the transcription factor XTCf-4

Ten picomoles per liter XTCf-4-specific antisense morpholino oligonucleotide 5′-cgcattcaqggcgeatcgtc-3′ (XTCf-4 Mo) (cf. van Venrooy et al., 2008) was injected in the animal pole of one blastomere of *Xenopus* two-cell-stage embryos and co-injected with 200 pg GFP-mRNA to trace the injected side. mRNA was synthesized in vitro using the mRNA message machine kit (Ambion, Huntington, UK). As a control, the antisense morpholino 5′-atatattacagttactcctc-3′ (Control-Mo) was used. Details were described previously (Kunz et al., 2004). For rescue experiments, 500 pg mRNA of the repressing XTCf-4A isoform or the activating XTCf-4C isoform was co-injected. For overexpression experiments, 500 pg mRNA of the repressing XTCf-4A isoform or the activating XTCf-4C isoform was injected. In these experiments, preprolactin was used as the control. All constructs are as described earlier (Gratl et al., 2002). One day after injection, the groups were separated according to the side of injection and reared up until they were 2 weeks old.

Determination of appearance of the otoconia area in the utricle and sacculce

The appearance of the utricular and saccular otoconia areas was monitored when tadpoles were 2 weeks old. A qualitative description about presence, absence and partial development is possible because, with a lateral light source, the otoconia crystals appear bright through the transparent skin of the tadpoles. Transparency of the animal is still maintained after fixation in MEMFA (0.1 mol L−1 MOPS pH 7.2, 2 mmol L−1 EGTA, 1 mmol L−1 MgSO4, 3.7% formaldehyde) and dehydration to the level of 75% alcohol (Fig. 2). From each animal, pictures were taken from the dorsal view.

Recordings of the rVOR

The recording procedure has been described elsewhere (Horn et al., 1986a). Briefly, tadpoles were anesthetized by a low dose of MS222 (0.1 mg in 1000 ml spring water) to allow a mechanical immobilization within the observation chamber (Fig. 1). The chamber was attached to the stimulation machine, which was equipped with a camera to take frontal images from the tadpole. The animals were rolled around their longitudinal axis for 360 deg in 15 deg steps. This stimulation induced a downward (depression) or upward (elevation) movement of both eyes, which was directed opposite to the roll stimulus. Each step was maintained for 8 s. A picture recorded 7 s after each step was used to determine the eye posture for the respective roll of the animal. At that time, the eye posture was maintained constantly for approximately 5 s (Sebastian et al., 1996).

The eye posture was defined by the angle between the margin of the eye cup and the dorsoventral body axis. Plotting the eye position against the body position angle revealed the sine-like rVOR characteristic curve (Fig. 1). This curve was used to calculate the rVOR amplitude, which is the maximal peak-to-peak movement of the eyes during a 360 deg lateral roll. Previous studies in *Xenopus* laevis, young fish (*Oreochromis mossambicus*) and salamander larvae (*Pleurodeles waltl*) have shown that the rVOR amplitude is a sensitive indicator to describe (1) modifications of the rVOR during development (Horn et al., 1986a; Sebastian and Horn, 1999; Gabriel et al., 2012), (2) the susceptibility to changes within the vestibuloocular system induced by vestibular lesions (Horn et al., 1986b), (3) the extent of vestibular plasticity after such lesions (Rayer and Horn, 1986) and (4) the impact of altered gravitational conditions on vestibular function (Horn 2006; Sebastian et al., 2001; Horn and Gabriel, 2011; Gabriel et al., 2012). The rVOR amplitude was determined by the angular difference between the mean of eye angles recorded for roll angles between 60 deg to 120 deg and 240 deg to 300 deg, respectively (cf. Fig. 1, open circles). Immediately before the rVOR recording, we documented tail morphology. Previous studies of the relationship between tail shape and vestibuloocular reflex after spaceflights revealed that the rVOR in tadpoles with abnormal tails differs from those with normal tails in an age-related manner (Horn, 2006; Horn and Gabriel, 2011).

PCR and western blot

RT-PCR

We tested the possibility whether XTCf-4 is expressed in different parts of the body of an embryo. RT-PCR was used to determine XTCf-4 expression in head, trunk and tail regions of *Xenopus* embryos. Complete RNA was extracted from pooled pieces of five dissected embryos using TRIZOL reagent (Life Technologies, Darmstadt, Germany) followed by a DNase digest. One microgram of RNA was reverse transcribed using MMLV reverse transcriptase (Life Technologies). cDNA according to 25 ng RNA was amplified with the following primers: Histone4 forward: 5′-CGGGATAAACATTCCGGATATCG-3′; Histone4 back, 5′-ATCCATGGCAGGTATGTTTCT-3′, 28 cycles; XTCf-4 forward, 5′-CCGCTCATACCTACTACAGCAAC-3′; XTCf-4 back, 5′-CAGCATGAACGCGTTAGGG-3′, 34 cycles.

Western blot

To prove that our XTCf-4 rescue constructs are not suppressed by the morpholino, we analysed their expression in the presence and absence of the morpholino. RIPA (radioimmunoprecipitation assay) lysate according to one half of a stage 14 embryo was loaded on 7.5% SDS PAGE, transferred onto nitrocellulose, and incubated with the 9E10 anti-myc antibody. Secondary antibody was a goat anti-mouse antibody coupled with horseradish peroxidase. Visualization was performed using ECL substrate (Amersham Pharmacia, Freiburg, Germany).

Statistics

Unilateral injection into one cell of the two-cell-stage embryos required analysis of the responses of both eyes separately. Either a parametric one-way analysis of variance or the Kruskal–Wallis one-way analysis of variance on ranks was used. For the rescue test, the
control morpholino group, the XTcf-4 morpholino group, and the rescue groups for additional injection of XTcf-4A and XTcf-4C were included in the one-way analysis of variance. For the gain of function test, the groups developing either from preprolactin-, XTcf-4A- or XTcf-4C-injected embryos were included in the one-way analysis of variance. If the normality test passed, subsequent pairwise multiple-comparison procedures were performed by means of the Holm–Sidak method; if the normality test failed, the Dunn’s test was used for all pairwise multiple-comparison procedures. In the figures, we always present mean values and standard errors of means (s.e.m.). The untreated group was not included in any statistical test; it served only as an indicator of the rVOR development under standard conditions concerning the shape of the rVOR characteristic curve and rVOR amplitude. The level of significance is at least $P<0.05$. Ratios as recorded for the number of affected or not affected animals in the rescue and overexpression approaches, the relationship between modifications in the utricular and saccular otoconia area or the relationships between the utricular otoconia area and the body shape were statistically tested by means of the $\chi^{2}$-test from Brandt and Snedecor (Sachs, 1997) or the 3x3-table $\chi^{2}$-test (Weber, 1967).

RESULTS

Morphological modifications

Morphological modifications were studied in 317 tadpoles. Forty-two tadpoles were untreated, 139 had received an injection in the right cell of the two-cell embryo and 136 in the left cell of the two-cell embryo. The most obvious effects were a reduced formation of the otoconia area in the utricle and saccule and the occurrence of an upward bended tail (synonymous tail lordosis) (Fig. 2).

Table 1. Correlation between the extent of modification of the otoconia area in the sacculus and utriculus

<table>
<thead>
<tr>
<th>Utriculus</th>
<th>Symmetric</th>
<th>I&lt; C</th>
<th>I&lt;; C+</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symmetric</td>
<td>31</td>
<td>2</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>I&lt;; C</td>
<td>5</td>
<td>11</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>I&lt;; C+</td>
<td>0</td>
<td>3</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>N</td>
<td>36</td>
<td>16</td>
<td>18</td>
<td>Total N=70</td>
</tr>
</tbody>
</table>

I, ipsilateral eye; C, contralateral eye.

Otoconia area in the utricle and saccule

XTcf-4 depletion affected otoconia formation within the utricular area in 52.9% ($N=37$) of animals, mostly on the injected side. The remaining 47.1% ($N=33$) of XTcf-4 morphants revealed symmetry of the otoconia area. Injection of the control morpholino revealed no effect in the 34 tadpoles used in this experiment. Untreated tadpoles ($N=42$) revealed always symmetrical shapes of the otoconia area. The appearance of modifications within the saccular area was highly correlated to those in the utricular area (Table 1). In 59 out of 70 XTcf-4 tadpoles, the modifications correspond to those in the utricle; in the remaining 11 animals, modifications were only weak and could be classified in the adjacent category to that of the utricle ($\chi^{2}$-test; $P<0.0001$). In the control-morpholino group and the untreated group, the similarity was 100%.

Right- and left-side symmetry of the otoconia was restored in all tadpoles after co-injection of the activating XTcf-4C isofom (20 out of 20 tadpoles) but in only eight out of 30 tadpoles by repressing the XTcf-4A isofom. The $\chi^{2}$-table $\chi^{2}$-test from Brandt and Snedecor, however, revealed that even this reduction in the number of animals with ipsilateral-reduced or absent otoconia area by co-injection of XTcf-4A was significant ($P<0.05$) (Fig. 3A).

Overexpression of the repressing XTcf-4A isofom caused asymmetry between the left and right otoconia area in seven tadpoles (25%), two animals (7.2%) revealed bilateral absence of otoconia, while the remaining 19 tadpoles (67.8%) developed normal otoconia areas. This appearance of abnormal presentation of the otoconia area on the ipsilateral side was significant ($P<0.05$). Overexpression of the activating XTcf-4C isofom, instead, revealed otoconia area symmetry in all ($N=50$) treated tadpoles. Control-injection with preprolactin caused no modification of the otoconia formation ($N=40$ tadpoles) (Fig. 3B).

Body shape

Tadpoles with lordotic tails were not observed in the untreated ($N=42$) and preprolactin-treated ($N=40$) controls and were observed in only one out of 34 animals in the control-morpholino group. Knock-down of XTcf-4 induced tail lordosis in 34 out of 71 embryos (15 of which had a strong lordosis). The appearance of tail lordosis was strongly correlated with modifications in the utricular area (Table 2). Partial rescue was observed by co-injection with XTcf-4A mRNA. This rescue, however, was not significant ($P>0.05$).
Instead, co-injected XTcf-4C mRNA rescued normal tail formation almost completely. Only one out of 21 treated animals revealed a weak lordosis (Fig. 3C).

Overexpression by XTcf-4 mRNA4A caused tail lordosis in four out of 28 animals. This change in the appearance of tail lordosis was significant compared with preprolactin-treated tadpoles (P<0.05; χ2-table χ2-test from Brandt and Snedecor). In contrast, overexpression by XTcf-4C mRNA had no impact on the body shape; only one out of 50 animals developed a weak tail lordosis by this treatment (Fig. 3D).

Behavioral modifications

Roll-induced vestibuloocular reflex

The roll-induced vestibuloocular reflex (rVOR) was studied in the same 317 tadpoles. The analysis of the rVOR was done in relation to the otoconia formation in the utricle because signals from both vertically oriented macula organs (saccule and lagena) do not contribute to eye responses induced by roll stimulation (Horn et al., 1986b; Hess and Precht, 1984; Riley and Moorman, 2000; Rohregger and Dieringer, 2002; Mo et al., 2010).

The characteristic sine-like shape of the rVOR curve was not affected by the knock-down of XTcf-4. However, the response of both eyes was significantly reduced in tadpoles with reduced or absent crystalline structures in the utricular otoconia area of the treated side compared with those that were untreated or injected with the control morpholino. We observed no significant changes in XTcf-4 morphant tadpoles that had developed a symmetric otoconia area on both the treated side and non-treated side (Fig. 4). Interestingly, the depression of the response of the ipsilateral eye was higher than that for the contralateral eye in those animals that developed a reduced otoconia area. This difference in the rVOR amplitude was significant (P<0.05) (Fig. 5A). Those embryos that developed symmetrically shaped otoconia areas did not show such differences. Thus, impaired rVOR in XTcf-4 morphants correlates with reduced otoconia area size.

After co-injection of the activating XTcf-4C isoform, the tadpoles revealed rVOR amplitudes for the ipsi- and contralateral eyes similar to both control-morpholino and XTcf-4 morpholino-treated tadpoles with bilateral otoconia symmetry. Slight differences between these groups were not significant, i.e. the size of the amplitude rVOR was completely rescued for both eyes. Co-injection of the repressing XTcf-4A isoform did not restore the rVOR amplitude. In the tadpoles of this group with still-reduced otoconia areas on the treated side, the rVOR amplitude was still depressed compared with the animals treated with the control morpholino or with symmetrical otoconia area after XTcf-4A co-injection. However, on the ipsilateral side, the rVOR amplitude increased significantly, indicating a partial functional rescue (P<0.05) compared with that of non-rescued animals (Fig. 5A; Table 3).

Overexpression of the activating XTcf-4C and the repressing XTcf-4A isoform had no effect on the sine-like shape of the rVOR characteristic. However, overexpression of XTcf-4C caused a significant depression of the rVOR amplitude of the ipsilateral eye while the rVOR amplitude of the contralateral eye remained at the level of the control embryos. Overexpression for XTcf-4A caused a similar splitting of the response as was observed after XTcf-4 morpholino treatment: in seven tadpoles with asymmetrical otoconia areas, the rVOR amplitude was significantly (P<0.05) depressed for both eyes. In the 19 tadpoles with normally developed utricular otoconia areas in the left and right vestibular organ, the rVOR amplitude remained at the level of the preprolactin control. The slight

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**Table 2. Correlation between the extent of modification of the otoconia area in the utricle and the shape of the body**

<table>
<thead>
<tr>
<th>Body shape</th>
<th>Symmetric</th>
<th>I–C</th>
<th>C+</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straight</td>
<td>29</td>
<td>5</td>
<td>4</td>
<td>38</td>
</tr>
<tr>
<td>Weak lordosis</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Strong lordosis</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>N</td>
<td>33</td>
<td>17</td>
<td>20</td>
<td>Total N=70</td>
</tr>
</tbody>
</table>

I, ipsilateral eye; C, contralateral eye.
differences recorded for the left and right eyes were not significant (Fig. 5B; Table 4). Throughout all of these experiments it turned out that (1) whenever the size of the ipsilateral otoconia area was reduced, the rVOR amplitudes of both eyes were reduced as well (XTcf-4 morpholino and XTcf-4 morpholino + XTcf4A) while (2) whenever the otoconia areas were symmetric (XTcf-4 morpholino + XTcf4C, control morpholino, preprolactin), the rVOR characteristics remained indistinguishable from control tadpoles.

Swimming

XTcf-4 knock-down tadpoles mainly rested; spontaneous swimming was rare. Induced swimming was mainly characterized by looping in circles backwards with diameters smaller than the body length (Fig. 6B) and rarely by spinning around the longitudinal axis or by irregular zig-zag movements. Thus, it resembled movements recorded from Xenopus tadpoles immediately after a spaceflight (Hor, 2006; Horn and Gabriel, 2011) rather than those recorded from aquatic animals with unilateral utricular lesions [cf. Schaefer and Meyer, 1974; for Xenopus tadpoles (Rayer et al., 1983)].

We determined the ratio of abnormally swimming tadpoles after XTcf-4 knock-down in a group different from that used for the morphological investigations and for rVOR recordings; but all parameters of treatment as well as the time of observation were the same. Swimming was induced by both pushing the animal slightly and by shaking the aquarium weakly. Tadpoles were counted as circlers if abnormal swimming could be induced in both conditions. The test showed that untreated controls (N=79) never swam in circles after stimulation. Control-morpholino-injected tadpoles behaved

#### Table 3. Tests for significance in the reconstitution test (cf. Fig. 5A)

<table>
<thead>
<tr>
<th>Control Mo</th>
<th>XTcf-4 Mo</th>
<th>XTcf-4 Mo &amp; XTcf-4A</th>
<th>XTcf-4 Mo &amp; XTcf-4C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sym</td>
<td>Sym</td>
<td>Sym</td>
<td>Sym</td>
</tr>
<tr>
<td>I&lt;C</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Cells in the upper and lower part of the matrix (separated by gray shading) indicate significant differences (X) between treatment groups for the roll-induced vestibuloocular reflex (rVOR) amplitudes recorded for the eye ipsilateral (I) and contralateral (C), respectively, to the treated side. Levels of significance: P<0.05. Sym, symmetric otoclonia area; I<C, ipsilateral otoclonia area is smaller than the contralateral otoclonia area, or even absent; OA, otoclonia area. Statistics for otoclonia area: one-way analysis of variance because normality test passed followed by Holm–Sidak test for all pairwise multiple comparisons.
similarly; only two out of 47 tadpoles swam abnormally. In contrast, 80 out of 87 (92%) XTcf-4 morpholino-treated tadpoles were circling. This change in behavior was significant (P<0.001; χ²-test) (Fig. 6A).

Normal swimming was restored by co-injection of either the XTcf-4C or XTcf-4A isoform, but not in all animals. The ratio of circlers after XTcf-4C as well as after XTcf-4A co-injection always differed significantly from treatment with either the control morpholino or XTcf-4 morpholino (P<0.001; χ²-test). Consistent with the rescue of otoconia symmetry, the activating XTcf-4C was more effective than the XTcf-4A isoform. Among the 62 XTcf-4C co-injected tadpoles, only 20 (32.3%) were circling whereas 27 (65.9%) of the 41 XTcf-4A co-injected tadpoles were still circling. This difference was significant (P=0.002; χ²-test). Thus, target gene activation by XTcf-4 seems to be essential for normal swimming.

**PCR and western blot**

In contrast to in situ hybridization, which showed XTcf-4 expression exclusively in the midbrain (König et al., 2000; Kunz et al., 2004), RT-PCR revealed additional XTcf-4 expression in the trunk and tail (Fig. 7A). Obviously, in the trunk and tail, XTcf-4 expression is not restricted to a distinct organ or tissue, because we never observed a specific signal in RNA in situ hybridization. The western blot shows that translation of the rescue-constructs is not suppressed by the morpholino (Fig. 7B, upper part).

**DISCUSSION**

The function of the vestibular system is closely related to the orientation of the body axes (Magnus, 1924). Vestibular reflexes compensate for passive displacements of the center of gravity and bring the body back to its symmetrical position. In addition, gravity receptors of the vestibular system control movements of the eyes (vestibuloocular reflexes) (Cohen, 1974). In aquatic animals, modification of the vestibular input either by vestibular lesions or by a transient deprivation (exposure to weightlessness during spaceflights) leads to profound depression of the reflexes (Schaefer and Meyer, 1974) [for amphibians (cf. Agosti et al., 1986; Rohregger and Dieringer, 2002; Horn et al., 1986b; Horn, 2006)]. The underlying vestibular networks that connect the peripheral sensory structures with the neuronal and muscular elements are formed during development under the influence of environmental, in particular gravitational, conditions (Moorman et al., 1999; Moorman et al., 2002; Sebastian et al., 2001; Horn and Gabriel, 2011) and genetic factors. Genetics factors were mainly studied for the development of otoliths in zebrafish and include contributions of otoc1 (Petko et al., 2008), zOMP-1 (Murayama et al., 2005), pax5 (Kwak et al., 2006) and starmaker (Söllner et al., 2003; Söllner et al., 2004). Knock-down techniques also revealed malformations of otoliths without affecting the sensory epithelium or other structures within the vestibulum (Hughes et al., 2004; Petko et al., 2008). Our studies extend the analysis of molecular mechanisms involved in vestibular development. They demonstrate that *Xenopus* XTcf-4 morphants showed significant modifications of the sensory structures in their vestibular organs and body shape (Figs 2, 3) and deficits in the performance of vestibular-related behaviors such as vestibuloocular reflexes (Figs 4, 5) and swimming (Fig. 6). They also show that these behavioral modifications are related to the depression of otoconia formation, which occurred in a predictable manner. The cell of the two-cell embryo that received the XTcf-4 morpholino...
We could also show that the swimming modification in these morphants correlated with an abnormal tail development (Figs 2, 6). Furthermore, reconstitution studies indicated that the isthmus is strongly involved in the development of the vestibular system because the behavioural (Figs 4–6) and morphological effects (Fig. 3) were rescued by co-injection of the activating XTcf-4C isoform. This XTcf-4 isoform is an essential factor for isthmus development (König et al., 2000; Koenig et al., 2010). The repressing XTcf-4A isoform instead restored the XTcf-4 morpholino effects only partially. The following discussion focuses on (1) neurobiological aspects of these observations that are related to vestibular-related behavioral responses and (2) developmental aspects that are related to the contribution of the isthmus to vestibular development.

**Neurobiological aspects**
In general, muscles responsible for eye movements induced by angular acceleration, linear acceleration or roll and tilt of the body (gravity related) receive their information from both vestibular organs (Precht, 1976). Thus, complete unilateral vestibular lesions as well as selective elimination of one utricular organ affect both eyes and reduce the rVOR of both eyes (Horn et al., 1986b). About 50% of XTcf-4 morphants have strongly depressed or even absent formation of otoconia on the injected side (Fig. 3), which leads to a depression of the rVOR of both eyes (Fig. 4, upper plots). We can exclude the possibility that morpholino treatment influences the induction of the otic placode because zygotic XTcf-4 is not transcribed before stage 16 (König et al., 2000) and morpholino-injected embryos show normal development until late neurula stages (Kunz et al., 2004). This result, revealing the depression of the rVOR

**Fig. 6.** (A) Frequency of circling swimming in untreated tadpoles, after knock-down of XTcf-4 by XTcf-4 morpholino injection in one cell of the two-cell stage embryo and after injection of a control morpholino. N, number of circling or normal swimming animals for the respective treatment group. Statistics: $\chi^2$-test. (B) The pictures show normal swimming of a Control-Mo treated animal (top), and circling swimming recorded from XTcf-4 Mo treated tadpoles with a lordotic tail (lower left) and with a normal tail (lower right). The frames were taken from video-recordings; time between two positions is 80ms. For the lordotic animal, only the margin of the head is represented in moving steps 2 to 8 because the tail always remained lordotic throughout the movement, while in the not lordotic tadpole the tail posture always changes between the bended and stretched posture. The circles in the eye indicate its position during the movement.

**Fig. 7.** Supplemental experiments to show specificities of the approach. (A) RT-PCR of dissected embryos revealed that XTcf-4 is not exclusively expressed in the head (h), but also in the trunk (b) and tail (c) regions. The upper panel illustrates the section planes for a stage 25 embryo. cDNA (representing 25 ng of total RNA) was amplified with primers specific for XTcf-4 and the housekeeping gene histone 4. –RT shows the amplification of the housekeeping gene in samples without reverse transcriptase. (B) Western blot showing expression of the rescue constructs XTcf-4A and XTcf-4C in the presence (+) and absence (–) of the XTcf-4 morpholino. The western blot in the upper part shows that translation of the rescue-constructs is not suppressed by the morpholino. The lower part shows a Coomassie stain to demonstrate equal loading.
of both eyes, indicates that, despite absence of utricular otoconia, and therefore utricular input from one side, bilateral vestibulococular pathways had developed in the XTcf-4 morphants.

However, the rVOR amplitude of XTcf-4 morphants was significantly more strongly suppressed for the ipsilateral eye compared with the contralateral eye response (Fig. 5). In general, a unilateral surgical lesion of the utricular organ depresses the response of the ipsi- and contralateral eye equally. Thus, the fine tuning between ipsi- and contralateral projection was obviously modified by the XTcf-4 knock-down. The behavioral study does not give any indication where this modification in the developing neuronal network happened; cerebellar locations cannot be excluded as they are involved in vestibular plasticity (Precht, 1979).

Abnormal swimming of XTcf-4 morphants (Fig. 6) might rather be related to the abnormal body shape than to the lack of input from the labyrinth of the injected side. Based on observations in animals with unilateral vestibular lesions (Schaefer and Meyer, 1974; Rayer et al., 1983), swimming movements should be expected in XTcf-4 morphants. Instead, looping swimming was dominant in these morphants, which resembled tadpole swimming recorded after spaceflights (Horn and Gabriel, 2011).

According to [3H]thymidine autoradiographic studies, first cells of the nucleus isthmi are formed at stage 20 and continue to be formed until stage 62. The majority of isthmus cells were generated between stages 45 and 55 (Tay and Straznicky, 1980). The early part of this period, up to stage 47, covers those stages to which embryos could develop in the experiments dealt with here. It also includes the period during which the tail is susceptible to gravity deprivation (Horn and Gabriel, 2011). Taking into consideration that the activating XTcf-4C isoform is an essential factor for isthmus development (König et al., 2000; Koenig et al., 2010) and restores tail development (Fig. 3A,C), the results of swimming in XTcf-4 morphants make it likely that isthmus influences contribute to tail development. The route of the isthmus influence on tail development, however, remains unclear. It might be indirect via the vestibular system. In general, tadpoles develop curved tails after destruction of the labyrinth of one body side (Rayer et al., 1983), probably due to the absence of excitatory effects on the developing tail muscles. It is very likely that absence of the ipsilateral otoconia area in XTcf-4 morphants has similar effects.

However, nine out of 38 morphant tadpoles had straight tails, although they developed otoconia asymmetries, and two out of 33 lordotic morphants displayed symmetric otoconia areas (Table 2). This observation contradicts an exclusively vestibulum-mediated effect of the isthmus. It rather points to an additional direct XTcf-4 effect on tail development as this transcription factor is also expressed in the tail region of the embryo (Fig. 7A).

The contribution of the isthmus to vestibular development: comparative aspects

The importance of the isthmus in the control of vestibular development is demonstrated by the results of the reconstitution experiments (Figs 3–5). Kunz et al. have shown that this region depends on the presence of the activating XTcf-4C isoform (Kunz et al., 2004). Consistently, the malformation of labyrinths on the treated side (Fig. 2) caused by the XTcf-4 specific morpholino was rescued by co-injection of the activating XTcf-4C isoform and not by the repressing XTcf-4A isoform. Interestingly, otic placode and otic vesicle formation do not require an intact isthmus because, following en-2 depletion, isthmus-located pax2 was downregulated while otic staining for pax2 remained unchanged at least until the tail bud stages (Koenig et al., 2010). Thus, we provide evidence for a novel function of the isthmus in inner ear development, which is essential for proper otoith formation and subsequently for vestibulococular reflexes and swimming behavior. For this isthmus function, activating (XTcf-4C) but not repressing (XTcf-4A) Tcf family members seem to be required. Thus, we assume that active Wnt/β-catenin signaling in the isthmus regulates otoith formation in the otic vesicles.

In mammals, noradrenergic mechanisms contribute to the regulation of postural control because of the close coupling between the locus coeruleus (LC) and vestibular pathways (Pompeiano, 2006). Pharmacological activation or blockade of the LC, which is the basic noradrenergic nucleus of the brain, modifies vestibulospinal and vestibulocular responses (Pompeiano et al., 1991). Vice versa, labyrinthine stimulation modifies the firing rate of LC neurons, indicating a functional coupling between the LC and the vestibular network (Pompeiano, 2006). In amphibians, a similar functional connection between the isthmus region, the amphibian homolog of the LC (McLean et al., 2000) and the vestibular network has not yet been described. However, noradrenergic modulation of spinal motor networks is known in Xenopus (Fischer et al., 2001). Tyrosine hydroxylase immunoreactive neurones in the isthmus region provide the catecholaminergic innervation of the spinal cord. They were shown first at stage 41; their spinal projections became visible at stage 43 (Sánchez-Comacho et al., 2002). Thus, the comparative approach supports the postulation of an isthmus influence on vestibular development.

In conclusion, this study shows that the isthmus can be defined as a center for the regulation of vestibular development. Its contribution is, at least, related to the formation of macular structures of the utricle within the vestibular system, which is responsible for the control of eye movements and swimming.

ACKNOWLEDGEMENTS

All experiments comply with the ‘Principles of Animal Care’, publication No. 86-23, revised 1985 of the National Institutes of Health, and with the ‘Deutsches Tierschutzgesetz’, BGBi from 17 February 1993. Permission for the experiments was given by the Regierungspräsidium of Tübingen (Germany), no. 796ULM, and by the Regierungspräsidium Karlsruhe (Germany), AZ 35-9185.81/G-161/03.

FUNDING

N.A.E.-Y. was supported by a grant from the Egyptian Government.

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