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Responses of hatchling *Xenopus* tadpoles to water currents: first function of lateral line receptors without cupulae

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SUMMARY

At later stages in larval life and also as adults, *Xenopus* can respond to water currents detected by their lateral-line sensory system. We have investigated when responses to water currents first appear and whether the first lateral line neuromasts operate in the same way as the adult organs. Just before and after hatching from their egg membranes we show that *Xenopus* embryos and tadpoles can respond to water currents by swimming into them. Local stimulation in immobilised animals where motor activity was recorded electrically suggested that the receptors detecting water currents were located between the eyes and the gills and were innervated by cranial nerves. In behaving tadpoles, responses to water currents were reduced following skin abrasion caudal to the eyes or treatment with neomycin, which is known to block hair cell function. We therefore used scanning electron microscopy to establish that rows of lateral line neuromasts with hair cells and kinocilia are present just caudal to the eyes at these stages of development. However, careful observations and manipulations of the kinocilia of neuromasts in living tadpoles failed to find any evidence that kinocilia were embedded in a jelly-like cupula. We conclude that, when they first start to function, these early neuromasts detect water movements which directly move their freely exposed hair cell kinocilia projecting out from the skin surface. Possible behavioural roles for the tadpoles responses to water are discussed.

Key words: lateral line, neuromast, *Xenopus laevis*.

INTRODUCTION

Aquatic fishes and amphibians have a lateral-line sensory system that enables them to detect water movements close to the surface of their body (Blaxter, 1987; Bleckmann, 1994; Coombs et al., 1989; Dijkgraaf, 1963; Montgomery et al., 1995) The sensory receptors responsible lie in the skin and the simplest type, found in amphibians, are free neuromasts. These are easily visible, for example around the eye in adult *Xenopus*, and form rows called stitches. Each neuromast contains a group of receptor hair cells with kino- and stereo-cilia embedded in a jelly-like cupula extending out from the skin surface (Gorner, 1963). It is well established that water currents can deflect the cupula of free neuromasts, move the kinocilia in its base and excite the hair cells, which then release transmitter to excite the cranial nerves which innervate them. Within each neuromast, the hair cells are arranged in different orientations in order to be able to detect cupula deflections in different directions.

The developmental origin of the lateral line neuromasts from neurogenic placodes lying underneath the skin and their innervation by cranial nerves has been studied in some detail in *Xenopus* (Schlosser and Northcutt, 2000; Winklbauer, 1989). There have also been studies using scanning electron microscopy and other methods to define the development of lateral line neuromasts and their receptive hair cells in the axolotl (Northcutt et al., 1994), the flounder (Otsuka, 2003) and the eel (Okamura et al., 2002). However, there have been few studies on the development of lateral-line neuromast function (Blaxter, 1987). In later larval stages both *Amblystoma* and *Xenopus* use their lateral line receptors to detect water currents and have been shown to turn towards a water current from a small pipette (Scharrer, 1932; Shelton, 1971) and to orient towards the source of water current in a flow chamber (Simmons et al., 2004). When does this capability first develop and do the newly formed neuromasts work in the same way when they first function?

After only 2 days of development and even before they hatch from the egg *Xenopus* embryos are capable of swimming when touched anywhere on the body (van Mier et al., 1989). However, responses to water currents do not appear to have been examined at these early stages of life. We have therefore investigated developing *Xenopus* embryos and larvae to see when they first respond to water currents, whether these responses depend on lateral line neuromasts, and how these newly formed neuromasts operate. We suggest that, unlike the mature neuromasts which have a jelly-like cupula to amplify water movements, the early responses of lateral-line hair cells may depend on the direct stimulation of hair cell kinocilia by water movements.

MATERIALS AND METHODS

Animals and observations on behaviour

Tadpoles developed from eggs laid following induced breeding of adult *Xenopus laevis* (Daudin), and were staged using normal tables (Nieuwkoop and Faber, 1956). Embryos were removed from their egg membranes before testing. Responses to water currents were studied initially in a 300 mm×300 mm tank containing dechlorinated tap water 20 mm deep at 17 to 21°C. A circular target was placed under the bottom of the tank and individual tadpoles placed over the centre of the target lying on their left side with their head facing in the ‘forwards’ direction (Fig. 1A). The target was divided into four equal 90° quadrants and the stimulating pipette was placed in the centre of one quadrant. This quadrant was called ‘towards’ and the other three were labelled as shown in Fig. 1A. When a tadpole swam, it was simple to see which quadrant they entered. This was used to judge their direction of swimming. A brief jet of water was

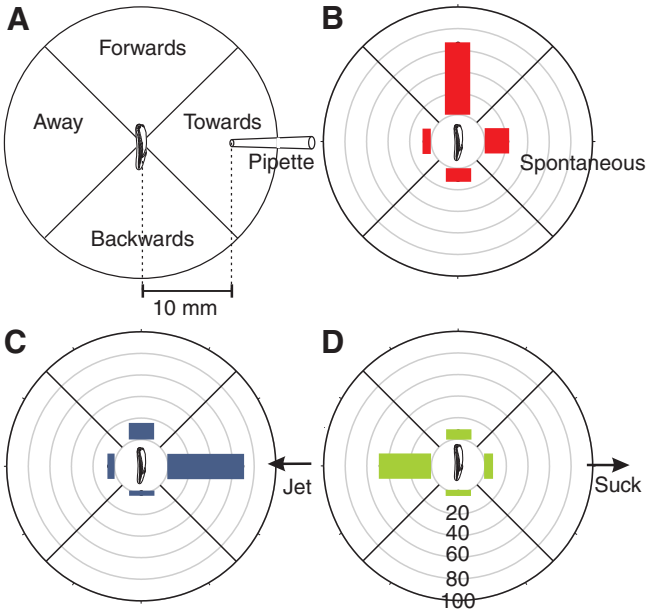

Fig. 1. Tadpole responses to water jets and suction. (A) Diagram of centre of test dish showing tadpole placed over crossed lines on target and position of pipette. (B–D) Polar plots showing the direction of swimming; rings are at 20% spacing. (B) Spontaneous swimming was mainly forward. (C) When a 10 ms water jet from a 50 µm diameter pipette was applied, tadpoles swam towards the pipette. (D) When suction was applied for 50 ms from a 5 mm diameter pipette, tadpoles swam away from the pipette.

directed at the ventral side of the tadpole from a rigidly mounted 50 µm glass nozzle 10 mm from the tadpole and connected to a water bottle (water head 500 mm) by opening a solenoid valve for 10 ms. Suction stimuli were applied *via* a rigidly mounted 5 mm diameter glass tube 10 mm from the tadpole connected to a pipette bulb that could be rapidly released to draw in water.

Responses of animals at different developmental stages were also tested using similar methods, but were performed in a 90 mm diameter plastic Petri dish with water 7 mm deep. Smaller dishes were used in later experiments as it was easier to reach in to manoeuvre the animals prior to testing, and when they swam, they quickly hit the sides of the dish and stopped swimming in the segment they had entered. This made recording the direction of swimming very simple. In these tests suction was applied using a plastic Pasteur pipette. For lesion studies, tadpoles were anaesthetised in MS-222 in saline (see below) for 1 min, then transferred to a dish of saline and held down with crossed pins in a V-shaped groove in the Sylgard base of the dish. Under a dissecting microscope a tungsten needle was ground to a chisel shape about 100 µm wide. This was used to abraid the skin in a restricted region. The tadpole was then transferred to 50% saline for 1 h before testing. Control tadpoles received the same treatment but were not abraded. Tests on abraded and neomycin-treated animals were conducted in a double-blind fashion so that the person doing the tests did not know which were treated or control animals.

Electrophysiology

Tadpoles at stage 37–38, were used at 20±2°C in saline: 115 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 2.4 mmol l^{-1} $NaHCO_3$, 1 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Hepes; adjusted to pH 7.4 with 5 mol l^{-1} NaOH. Dissection and lesioning was carried out using finely etched tungsten needles and forceps. The methods for making recordings from the motor nerves have been described previously (e.g. Lambert et al., 2004). Briefly, tadpoles were anaesthetised with 0.1% MS-222 in saline for 20–30 s, then pinned onto a rotatable Sylgard block in a bath of saline. Tadpoles were slit along the dorsal fin and transferred to α-bungarotoxin (10 µmol l^{-1} in saline, Sigma, St Louis, MO, USA) for up to 20 min. After immobilisation, tadpoles were returned to the bath and re-pinned with their right side up. Some skin overlying the dorsal half of the body on the right side was removed to expose the myotomes. To record ventral root activity and see swimming responses, a suction electrode (60 µm tip diameter) was placed over an intermyotomal cleft. Display techniques were conventional, recording were viewed on an oscilloscope and permanent records made on a chart recorder.

Observations of living hair cells

Tadpoles were transferred to 50% physiological saline and decapitated just caudal to the gills. The head was then transferred to a 55 mm diameter plastic Petri dish containing 50% saline with a glass coverslip glued over a 10 mm diameter hole in its base. A 25 µm diameter tungsten pin was inserted rostrally so that it passed longitudinally through the isolated head and into the side of a small block of Sylgard fixed to the coverslip. The head was then rotated about its longitudinal axis so that dorsal was up and the head was secured with another tungsten pin. The dish was moved to the fixed stage of an Olympus BX50WI microscope. Observations were made with a ×40 water immersion lens using bright field or differential interference contrast (DIC) optics. Photomicrographs were taken using a Nikon Coolpix 990 camera. To stimulate the neuromasts, saline was ejected from the 3–4 µm diameter tip of a glass pipette mounted in a holder and connected to a flexible tube that allowed pressure to be applied using a 1 ml syringe. To manipulate the kinocilia, a fine glass probe or a fine tungsten needle with its end bent to point vertically was used. All probes were positioned and moved using a Huxley-type micromanipulator.

Scanning electron microscopy

Embryos and larvae were washed in 0.1 mol l^{-1} phosphate buffer; fixed for 2 h in 2% gluteraldehyde in phosphate buffer; washed in buffer and then dehydrated through a graded ethanol series before critical-point drying. The dried specimens were glued to aluminium stubs, sputter coated with gold, and examined using scanning electron microscopy (SEM) in a Philips 501B microscope.

All the procedures used have had University of Bristol ethical committee approval. Statistical tests were performed using Excel or Minitab and all means are given with their standard errors.

RESULTS

Basic swimming responses to water currents

When water currents from a small pipette are directed at hatchling tadpoles at stage 37/38 they often start to swim. We therefore investigated these responses systematically. Individual tadpoles were placed so they lay on their side with their head facing in the 'forwards' direction on the bottom of a dish of water at the centre of a target that could be used to judge their direction of swimming (Fig. 1A). Each tadpole was tested once. We checked that they were not attached to the bottom of the dish by cement from their cement gland since this inhibits responses (Lambert et al., 2004). We first evaluated spontaneous swimming in unstimulated tadpoles. Individuals were placed on the target, we waited until they swam, recorded the direction, and then tested another tadpole. Spontaneous swimming was mainly forwards (N=40: towards 8, away 2, forward 26, back 4; χ^2=15.24; d.f.=3, P=0.002). In response to a 10 ms water

jet from a 50 μm pipette they mainly swam towards the jet (N=50, towards 34, away 2, forward 6, back 0, no response 8; χ^2=29.39, d.f.=3, P<0.001, whereas they swam away from 50 ms of suction from a 5 mm pipette (N=50, towards 2, away 23, forward 3, back 0, no response 22; χ^2=22.82; d.f.=3, P<0.001). These tests indicate that hatchling tadpoles respond to water currents by swimming against the direction of flow.

In *Xenopus*, effective swimming (powerful enough to move the animal through the water) first appears at about embryonic stage 31/32 (van Mier et al., 1989). No responses to water currents were seen prior to stage 31/32. We therefore examined the development of swimming responses to water currents from nearly a day before hatching, at stage 31, to stage 41, which is about 1 day after hatching. For each stage of development we tested 10 animals once and recorded the number swimming towards a water jet or away from suction; swimming in other directions and not responding (as above). Swimming responses to both stimuli were seen from the first stage tested but the proportion of tested animals responding to stimulation with swimming increased with age up to the time of hatching (stage 37/38; Fig. 2; regression analysis of proportion swimming against age: for water jet R^2=0.75, P=0.026 and for suction: R^2=0.94, P=0.001). From stages 31 to 41 swimming was predominantly towards a water jet (81%) or away from suction (74%).

Localising the receptors

Since embryos and hatched tadpoles showed responses to water currents, we then investigated the location of the receptors, expecting that the post-orbital lateral-line neuromasts might be involved (Winklbauer, 1989). All observations were made on stage 37/38 tadpoles as these are most convenient for motor nerve recording. In preliminary observations we used tadpoles immobilised in α-bungarotoxin and made recordings from the motor nerves to the trunk muscles (Fig. 3A). To establish that recording was successful we touched the skin with a fine hair and checked that fictive swimming activity was evoked with motor bursts at intervals within the normal swimming range (40–100 ms; Fig. 3B) (Kahn et al., 1982). We then tested water jet stimulation with a narrow, 10 ms jet of water from a 20 μm pipette 20 mm from the ventral side of the tadpole. This stimulation was tested over the whole body surface but was only found to be effective in evoking fictive swimming if directed at the head (Fig. 3B).

Lesions were then used to find what cranial pathways were necessary for such fictive swimming responses. After all lesions, 5 min were allowed for recovery, the recording electrodes were put in place, and the trunk skin was stroked with a fine hair to check that this stimulus evoked fictive swimming. We found that water jet stimulation still evoked swimming after removal of the midbrain and forebrain (N=8/8) and removal of the otic capsules on both sides (N=8/8). Since lateral line neuromasts are innervated by cranial nerves, the next operation was to cut vertically along each side of the hindbrain to sever all hindbrain sensory cranial nerves (V to XI). After this lesion, swimming responses to water jets were dramatically reduced (N=1/9).

To follow up observations on immobilised tadpoles we tested the effects of cranial nerve lesions on the behaviour of tadpoles. In 30 tadpoles at stage 37/38 short vertical cuts (~0.1 mm long) were made rostral and caudal to the otic capsule on both sides. When each tadpole was tested once to water jet stimulation, lesioned tadpoles

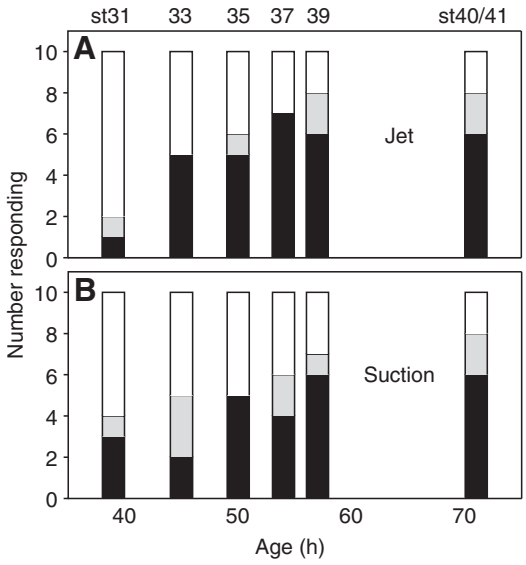

Fig. 2. Responses to water currents as a function of age and developmental stage. (A) Numbers swimming into a water jet (black bars); (B) numbers swimming away from suction (black bars). Grey bars show numbers swimming in other directions and white bars numbers not responding. Stage (st) numbers are given at the top.

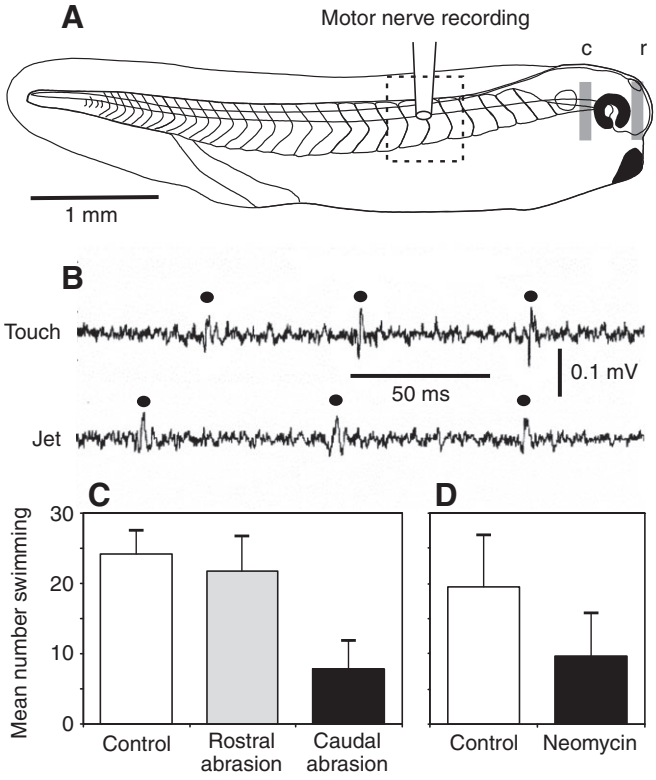

Fig. 3. Responses to water jets in immobilised tadpoles and effects of skin abrasions and neomycin on behaviour. (A) Diagram of the stage 37/38 tadpole from the side to show placement of a suction electrode on muscle in an area where skin has been removed (dashed square) to record motor nerve activity. The skin was abraded in the grey shaded areas just rostral (r) and caudal (c) to the eye. (B) Example recordings of motor nerve bursts (at dots) during fictive swimming evoked by touch to the skin and a water jet stimulus to the head region. (C,D) Behavioural tests on swimming responses. (C) Bar chart showing effects of local skin abrasion on mean number of swimming responses to a water jet. (D) Bar chart showing reduction in mean number of swimming responses after neomycin treatment. Error bars indicate s.e.m.

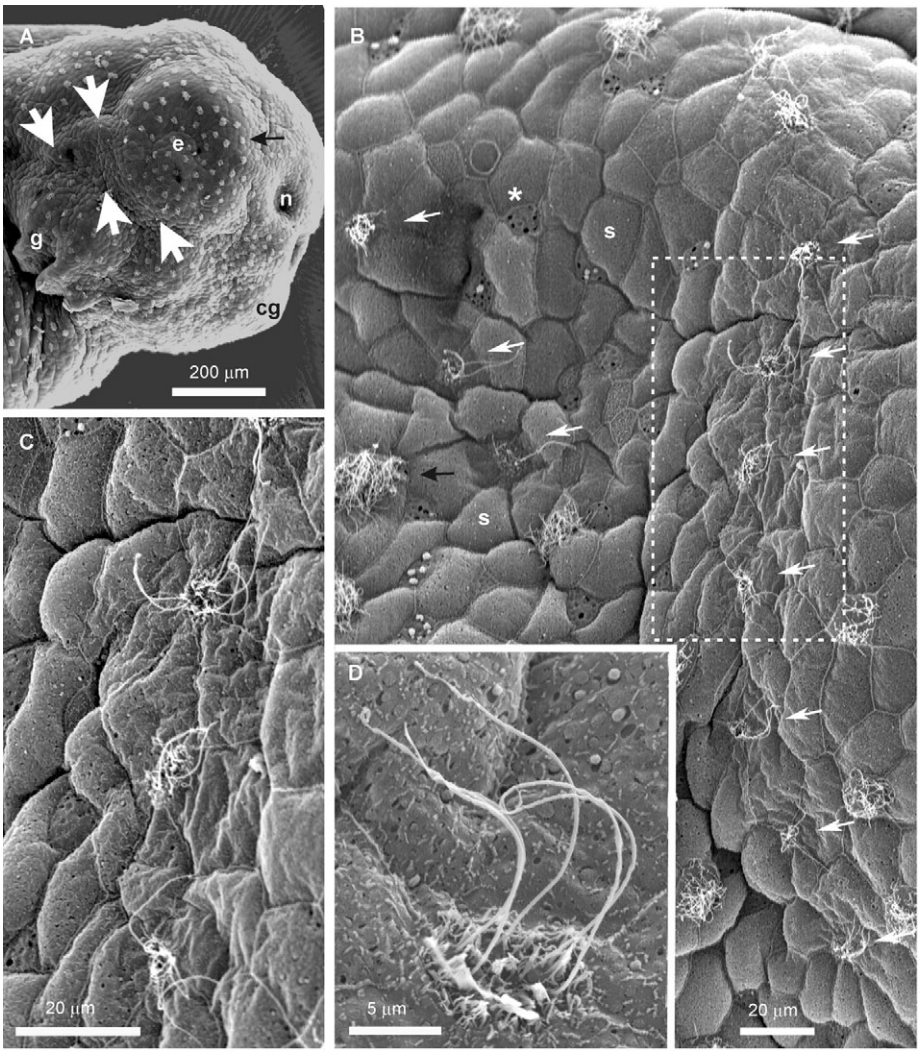

Fig. 4. Scanning EM views of the side of the head of a stage 37/38 tadpole to show lateral-line neuromasts. (A) Whole head with many ciliated cells (black arrow indicates an example), eye (e), nasal opening (n), cement gland (cg) and gills (g). Two rows of neuromasts lie between the large white arrows just caudal to the eye. (B) At higher magnification two rows of neuromasts (white arrows) lying among skin cells (s) can be distinguished by their long kinocilia. Ciliated cells (black arrow indicates an example) and mucus cells (asterisk) are also present. The dashed rectangle shows area enlarged in C. (C) Area in B, with three neuromasts, each with many long kinocilia. (D) A neuromast with five long curved kinocilia emerging from bundles of stereocilia at their bases.

swam (11/30) significantly less than controls (26/30; contingency table analysis, d.f.=1; $P<0.005$).

The evidence so far implicated lateral-line receptors in mediating responses to water currents. Since lateral-line primordia develop just behind the eye (Winklbauer, 1989), we used a narrow jet (40 μm) to give a very local stimulus to tadpoles lying on their side on the bottom of a dish (N=70 individuals, five repeats each = 350 trials). Current just caudal to the eye resulted in significantly more swims (153) than current rostral to the eye (83; Mann-Whitney W=5740.0 N=70, P=0.008). To locate the receptors we then abraded the skin just rostral or caudal to the eyes with a small, chisel-shaped needle (Fig. 3A, see grey shaded areas) and then tested responses to the water jet from a 1 mm pipette 15 mm from the tadpole. For each treatment five tadpoles were each tested 40 times and the number of swimming responses out of 40 trials was determined. In the control group, without abrasions, the number of swimming responses (24.2±3.35; mean ± s.e.m.) was similar to tadpoles with abrasions rostral to both eyes (21.8±4.97; P=0.65) but the number of swimming responses was significantly reduced after abrasions caudal to both eyes (7.8±4.15; Fig. 3C) when compared with both the operated and unoperated controls ($P<0.001$ in each case; ANOVA with Tukey's pairwise comparisons, F=6.48, d.f.=6).

The antibiotic neomycin is known to produce loss of function in sensory hair cells in the mammallian auditory system (Gale et al., 2001) and in the amphibian (mudpuppy) lateral line system (Shiozawa and Yanagisawa, 1979). We therefore looked at the effects of a 30 min wash in 10 μmol l^{-1} neomycin sulphate (pH 7.4). After 60 min in normal tap water, we tested the responses of ten treated and ten control tadpoles to the water jet. As in the previous abrasion tests each tadpole was tested 40 times. We found a significant reduction in the number of swimming responses to the water jet in neomycin-sulphate-treated tadpoles (Fig. 3D; 9.6±6.2 treated and 19.6±7.3 control tadpoles; t-test: t=3.31, P=0.004, d.f.=18).

Features and development of lateral-line neuromasts

The evidence from electrical recording and behavioural tests suggested that the receptors involved in detection of water currents could be lateral-line neuromasts lying just caudal to the eye and innervated by cranial nerves. To investigate when and where lateral line neuromasts first appear and how they then develop from stage 29/30 to 42 we used scanning electron microscopy to examine the outer surface of the skin in gluteraldehyde-fixed and critical-point dried specimens (at least four at each stage). The head skin has three types of cells: normal skin cells with a fairly smooth surface (longest dimension often >20 μm), ciliated cells which have very large numbers of short cilia and are distributed over the whole body surface (longest dimension ~ 10 μm), and mucus cells with a small

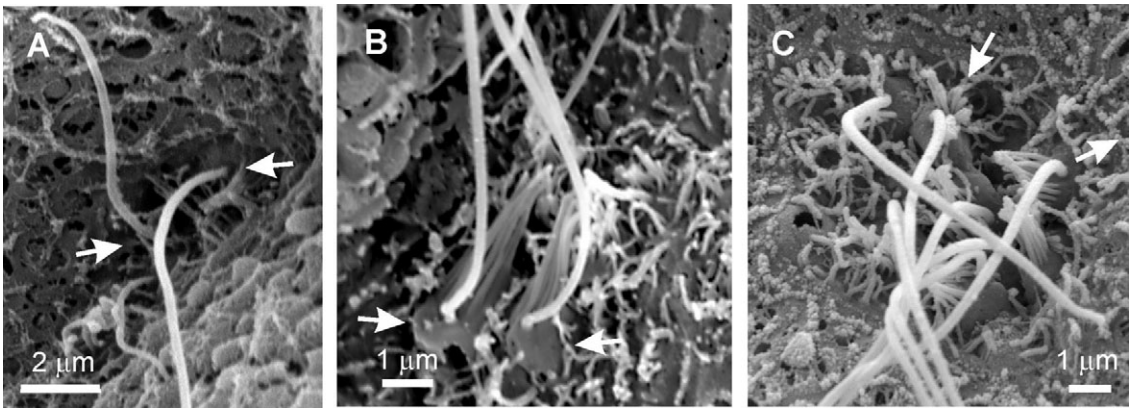

Fig. 5. High magnification images of individual neuromasts to show kinocilia and stereocilia. (A) Neuromast of stage 37/38 tadpole with two kinocilia (marked by arrows) just emerging from between skin cells. (B) Neuromast of stage 42 tadpole with three kinocilia, in which the apical ends of two hair cells (arrows) are clearly visible with the kinoclia emerging next to the bases of a group of stereocilia. (C) Neuromast of stage 37/38 tadpole with seven kinocilia. Stereocilia can be seen in two cases, and the orientation of the kinocilia and stereocilia (indicated by arrows) is nearly opposite.

exposed surface (longest dimension <10 μm) usually with clear holes (Fig. 4). Neuromasts were found at stage 37/38 just caudal to the eye (Fig. 4B–D) and were easily distinguished from ciliated skin cells as their exposed surface was smaller (longest dimension <10 μm) and they had fewer, longer cilia (two to eight).

The features of the neuromasts were examined in higher magnification SEM pictures of 55 neuromasts in ten animals. The simplest neuromasts had only two kinocilia and often lay in a slight depression between skin cells (Fig. 5A) so their outer surface and stereo cilia were difficult to see. Such neuromasts may be at an early stage of differentiation and in the process of erupting to the surface of the skin. In most neuromasts the external faces of supporting cells and the groups of stereo cilia with emerging kinocilia were visible (Fig. 5B,C). Neuromasts were between 4.4 and 11.6 μm across (means: widths 6.85±1.37 μm, lengths 8.21±1.67 μm). In a few cases the exposed ends of hairs cells were clear enough to see the bases of kinocilia situated close to a bundle of stereocilia (Fig. 5B) and sometimes, the different orientations of the stereo and kinocilia could be resolved (Fig. 5C). When the kinocilia had collapsed close to the skin surface, their lengths could be measured (mean 21.77±2.59 μm, N=13). Surprisingly, none of the neuromasts examined showed any evidence of a jelly cupula around the kinocilia.

Neuromasts were not found at stage 29/30 but by stage 32 a short row of three to six had formed just caudal to the eye (Fig. 6A). By stage 37/38 the number of neuromasts in this first row (infra orbital) had increased and a second row had appeared just dorsal to the gills (Fig. 6B). At stage 42 the number of neuromasts was similar (Fig. 6C).

Observations on living hair cells

The SEM images showed that early neuromasts were present from stage 32 when embryos first respond to water currents. However, the images suggested that the kinocilia projecting from lateral line hair cells were not embedded in a jelly cupula. Since the cupulae may have been dissolved or destroyed during preparation for SEM, we made direct observations of living head skin in 11 tadpoles at stage 37/38 in 50% physiological saline using a ×40 water immersion lens. When viewed from the dorsal side using bright-field or DIC optics, two or three groups of straight, static, kinocilia about 20 μm long were seen at different dorsoventral positions projecting from the skin just caudal to the eyes (Figs 7 and 8). There were from three to 11 kinocilia in each group. The groups of kinocilia lay in the positions where neuromasts had been found in the SEM investigation (see above). In two cases a jet of 50% saline was directed at the group of kinocilia from a glass pipette with 5 μm diameter tip opening. When the current flowed, the kinocilia remained straight along most of their length, but pivoted around their bases to become deflected by the current (Fig. 7).

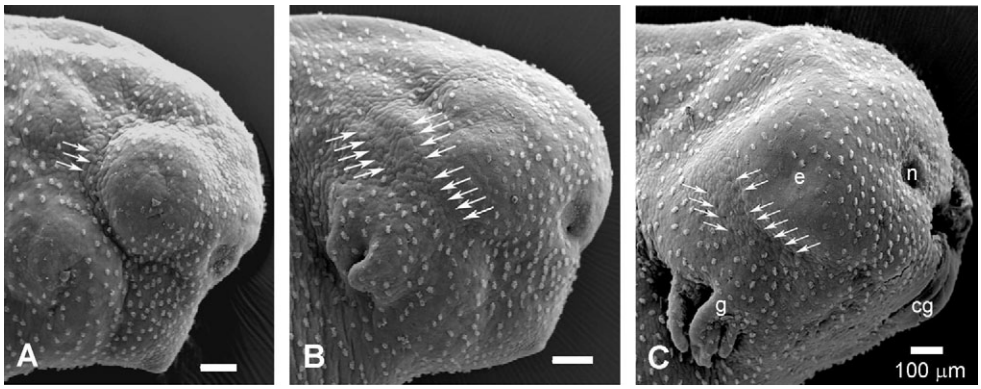

Fig. 6. Low magnification images of heads, in lateral view from the right, to show rows of neuromasts (each marked by white arrow) at stages (A) 32, (B) 37/38 and (C) 42. Labels in C are as in Fig. 4. Scale bars, 100 μm.

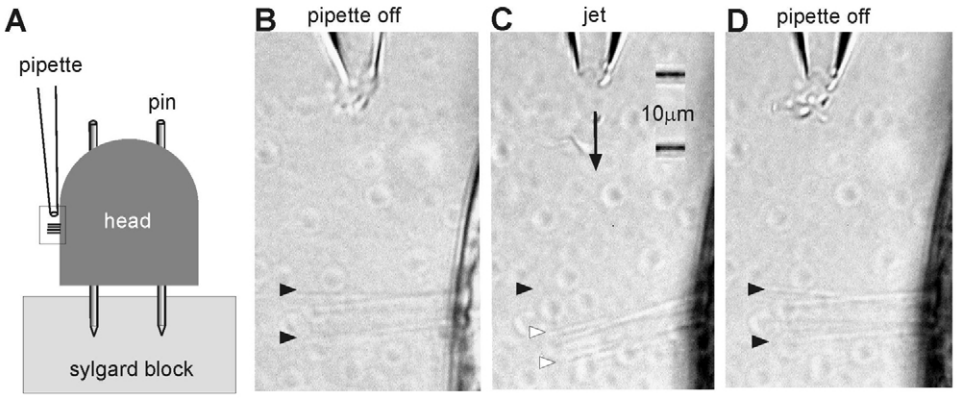

Fig. 7. Effects of water current on living kinocilia at stage 37/38. (A) Schematic diagram of the tadpole head pinned dorsal side up into a Sylgard block. Kinocilia were viewed on left side and stimulated by a water jet from a glass pipette. (B–D) Images of the area in the rectangle in A with a group of kinocilia emerging from the left side of the head (dark area bottom right). (B) With no current; black arrowheads mark positions of the outside kinocilia in the group at rest. (C) During head to tail water current (in direction of the arrow) from pipette at top; kinocilia are deflected caudally (to positions marked by open arrowheads). (D) After current stops kinocilia return to their resting position.

Surprisingly, no cupulae could be seen in the 30 neuromasts examined, using either bright-field or DIC optics. To test if cupulae were present but invisible, fine glass or tungsten probes were manipulated into or around the kinocilia. The preparation and rationale of these two tests is illustrated in Fig. 8A,B which shows a diagram of a typical excised fish neuromast where the kinocilia are embedded in a jelly-like cupula (Van Trump and McHenry, 2008). In the first test, probe 1 is moved in towards the kinocilia and it should contact the cupula first. By moving the cupula, the kinocilia inside should all move with the cupula and to the same extent. In the second test, probe 2 is moved over the ends of the kinocilia. If a cupula is present extending beyond the ends of the kinocilia this should move and take the kinocilia with it.

The first test was performed on 11 tadpole neuromasts. When a probe was moved in towards the sides of the kinocilia, there was no indication that they moved before being contacted by the probe (Fig. 8C,D). Furthermore, when the first kinocilium was moved by the probe, the others in the same neuromast did not move (Fig. 8E,F). During these tests it was noticed that, after contacting the kinocilia, the probe could often move them as it was moved away and without apparent contact. The simplest explanation of this is that the kinocilia are covered in mucus which becomes attached to the probe and can then pull on the kinocilia.

In the second test on nine neuromasts, a probe was moved backwards and forwards past groups of kinocilia less than 20 μm from their tips. No movements of the kinocilia were seen (Fig. 8G–L). This would not be expected if there was a jelly-like cupula extending beyond the kinocilia.

Similar observations were made on four neuromasts from two older tadpoles at stages 41 and 44. As expected from the SEM observations, older neuromasts had more kinocilia but probe manipulations showed that kinocilia could still be moved independently and moving the probe over the ends of the kinocilia did not displace them. These observations suggest that cupulae are not present at these later stages.

DISCUSSION

Behaviour and role of responses to water currents

We have shown that as soon as the kinocilia of lateral line hair cells appear just caudal to the eye in developing *Xenopus* embryos at stage 32 (Nieuwkoop and Faber, 1956), embryos are able to respond to water currents even though there may be as few as three or four neuromasts on each side of the head. Over the next 10 h or so of further development, more neuromasts appear and a higher proportion of tested animals respond to water currents. When they detect a water current the embryos and young tadpoles start to swim and this swimming is directed into the water current. This type of directed turning towards a water current had been shown in much older *Ambystoma* and *Xenopus* larvae (Scharrer, 1932; Shelton, 1971). In *Ambystoma* this response was interpreted as a feeding response allowing the larvae to detect small prey that produced local water movements. Since the embryo and young larval *Xenopus* tadpoles do not yet have a mouth, and do not feed, a role in prey detection seems unlikely at these early stages.

What are the other possible roles of the young tadpole's directional swimming response to water currents? Rheotaxis is a behavioural orientation to water currents that is common in fish. Swimming into water currents may help them to maintain their position in a stream and avoid being swept away by currents. In fish (Montgomery et al., 1997) and larval *Xenopus* (Simmons et al., 2004) this behaviour has been shown to depend in part on the superficial, free neuromasts of the lateral-line system. Unfortunately, in the *Xenopus* study although 18 tadpoles were tested at stages 37 to 45, there is no information on how many were tested at each different stage. Furthermore, the animals were not viewed from above so orientation angles of swimming into the water current in the horizontal plane were not accurately measured. This makes it difficult to assess the behaviour of the younger tadpoles in the sample. Rheotaxis is therefore well established in *Xenopus* tadpoles but may be more important in later free swimming stages [after stage 43 but see Shelton (Shelton, 1971)] than at the earlier stages we have examined (up to stage 40) when tadpoles are very inactive. They spend most of their time (99%) hanging from a strand of mucus secreted by the cement gland on their head (Jamieson and Roberts, 2000). Another role was suggested by the tadpoles responses to suction (Fig. 1D). In many fish (Wainwright et al., 2007) and aquatic amphibians (Lauder and Shaffer, 1986) suction feeding is the main method to catch prey. An ability to initiate swimming and then swim against a water current could help young tadpoles avoid being sucked up by fish or older amphibian larvae or adults.

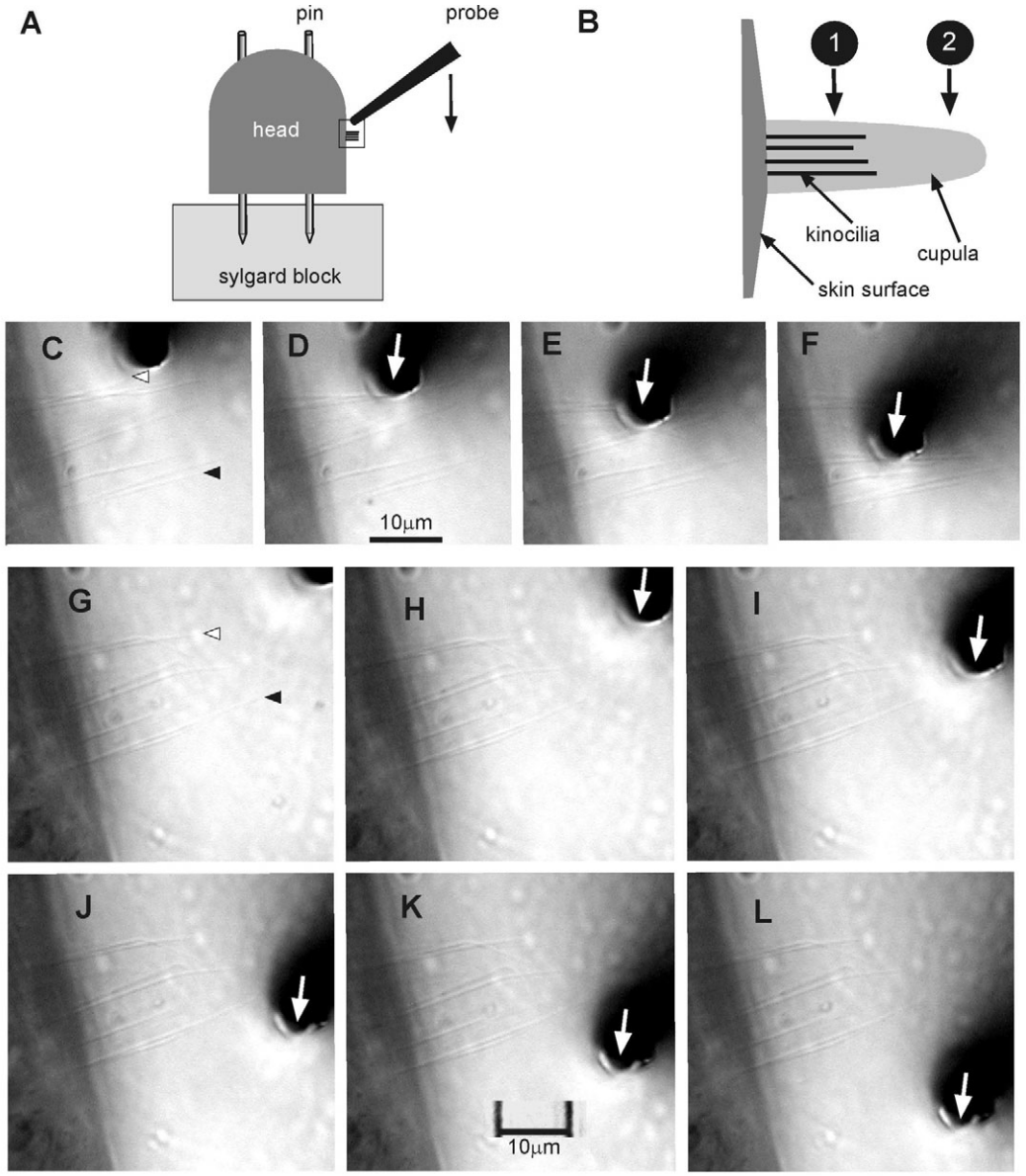

Fig. 8. Observations on living kinocilia. (A) Schematic diagram of the tadpole head from above. Kinocilia were viewed on the right side and a tungsten probe (moving in the direction of the arrow) was used to manipulate the kinocilia. The small square shows the area of images below. (B) Diagram of an excised neuromast with jelly cupula (light grey) surrounding four kinocilia. If probe 1 is moved into the cupula, the kinocilia should all move together before they appear to be contacted directly. If probe 2 is moved beyond the ends of the kinocilia it should also move the cupula and all kinocilia should move with it. (C–F) A vertical tungsten probe is moved caudally (as indicated by white arrows) into a group of kinocilia protruding from the right side of the head. (D) More rostral kinocilia move when directly contacted, but more caudal kinocilia remain in place. (E,F) Kinocilia only move when directly contacted by the probe. (G–L) Moving the same probe past the ends of the kinocilia does not move them. In all images top is rostral, bottom is caudal and dark shadow on the left is the right side of the head from which kinocilia emerge. Black and white arrowheads in C and G mark the outermost kinocilia.

Development of neuromasts and operation without cupulae

Lateral line kinocilia and neuromasts were seen well before hatching in SEM images of stage 32 embryos. About 5 h earlier at stage 29/30, close examination of the skin caudal to the eye showed some small depressions where neuromasts might be about to erupt but no sensory kinocilia. In a few hours a functional lateral-line sensory system develops that allows the embryos to respond to water currents by swimming. This implies that within this period the hair cells become capable of detecting kinocilium deflection and releasing transmitter, and that the sensory neurons that innervate them are excited and carry an impulse discharge into the hindbrain to synaptically activate the circuits that drive swimming (Li et al., 2006). These conclusions do not rule out a role for saccular hair cells in detecting movements of the whole body resulting from strong water currents.

As they develop, the neuromasts form lines or rows. The first to form by stage 32 is the infra-orbital line just caudal to the eye and this then extends ventrally. The second aortic line forms by stage 37/38, slightly more caudally and dorsal to the gills. The pattern of extension of neuromast lines is very much as expected from the development of the underlying neurogenic placodes in *Xenopus* (Schlosser and Northcutt, 2000) and is similar to that found in the axolotl (Northcutt et al., 1994). Many features of the earliest

neuromasts of axolotl are similar in SEM images to our findings in *Xenopus*. However, in both the axolotl and teleosts evidence of cupulae have been detected (including by SEM) at very early stages of neuromast development (Blaxter, 1984; Otsuka, 2003). By contrast, we found no evidence for the presence of a gelatinous cupula in *Xenopus* neuromasts from embryonic stage 32 to the tadpole, 1.5 days after hatching, at stage 44.

Do the early neuromasts in newly hatched *Xenopus* tadpoles work without a gelatinous cupula? Since the preparative methods for SEM examination might have destroyed cupulae, we made observations in living tadpoles. Neuromast kinocilia could be seen using a ×40 water immersion lens but there were no signs of cupulae. Rather than trying to see cupulae, for example by coating their surface with reflective polystyrene microspheres (Van Trump and McHenry, 2008), we chose to use mechanical tests to seek evidence for their existence by moving probes into and around groups of neuromast kinocilia. In the developmental stages that we examined, there was no indication of any invisible structure surrounding kinocilia or linking them together. We looked at the oldest tadpoles permitted by British Home Office regulations (stage 44) and still found no evidence for cupulae. However, direct observations also showed that living kinocilia could be deflected by local water currents. Taken together, our evidence indicates that young *Xenopus* tadpoles detect water currents which directly move the kinocilia protruding some 20 μm from the surface of neuromasts forming two rows just caudal to the eyes. The young *Xenopus* tadpole, therefore, provides an opportunity to investigate the properties of naked kinocilia.

What is the significance of neuromasts without cupulae? If early neuromasts operate without cupulae at early stages of development then one can guess that they will have reduced sensitivity as there is good evidence that sensitivity relates to cupula length (McHenry et al., 2008; Van Trump and McHenry, 2008). Cells in the tadpole skin secrete mucus and this is driven caudally over the body surface by the numerous ciliated skin cells. Our observations that probes which have touched kinocilia can move them when pulled away, suggests that the kinocilia are coated with mucus. If the kinocilia can move independently, then they could give independent directional information. We looked at the orientation of kinocilia and stereocilia but in many cases clear measurement of orientation angle was not possible because a tangle of kinocilia lay over the surface of the neuromast, which was not viewed orthogonally. Our impression was that there was a range of orientations rather than the strictly opposed orientation found in mature neuromasts (Bleckmann, 1994; Coombs et al., 1989; Montgomery et al., 1995). However, we rarely saw a neuromast with only a single kinocilium which may support the idea that hairs cells develop in pairs (Rouse and Pickles, 1991).

Finally, the lateral-line neuromasts in the young *Xenopus* tadpole appear to be the simplest so far described. Even if Haeckel's idea that ontogeny recapitulates phylogeny (Haeckel, 1992) is no longer accepted, the young *Xenopus* tadpole appears to give us an insight into the simplest possible vertebrate sensory system to detect water movements over the body surface.

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