LATERALITY, 2005, 10 (3), 193–205

Psychology Press
Taylor & Francis Group

Eye regeneration assay reveals an invariant functional left–right asymmetry in the early bilaterian, *Dugesia japonica*

Taisaku Nogi, Yan Emily Yuan, and Debra Sorocco
Forsyth Institute and Harvard School of Dental Medicine, Boston, MA, USA
Ricardo Perez-Tomas
Universitat de Barcelona, Spain
Michael Levin
Forsyth Institute and Harvard School of Dental Medicine, Boston, MA, USA

Consistent visceral asymmetry in vertebrates raises fascinating questions about the developmental mechanisms and evolutionary origin of fixed chirality of the left–right axis. One persistent controversy is whether consistently biased asymmetry is a later innovation imposed on a bilaterally symmetrical primitive body-plan, or whether asymmetry is a fundamental property predating the bilateria. The morphology of planaria suggests proximity to the origin of the bilateral body-plan, and they are commonly thought to be left–right symmetrical, as no consistent anatomical asymmetries have been described despite over a century of study of regeneration. Here, we show that *D. japonica* possess a consistent functional asymmetry in eye patterning defects caused by inhibition of H^+/K^+-ATPase activity (an ion flux mechanism recently shown to be an important early step in the asymmetry of several vertebrate embryos). Moreover, an endogenous transcript of the non-gastric H^+/K^+-ATPase subunit α is expressed in the head blastema shortly after amputation. Taken together, these data suggest that (1) left–right asymmetry is at least as old as planaria, (2) subtle functional asymmetries should be sought in other more primitive model systems that are believed to be symmetrical, and (3) symmetrical paired structures may in fact contain information about their position on the L or R side.

Address correspondence to: Michael Levin, Cytokine Biology Department, The Forsyth Institute, and Department of Oral and Developmental Biology, Harvard School of Dental Medicine, 140 The Fenway, Boston, MA 02115, USA. Emai: mlevin@forsyth.org

We thank Ralph Kent for advice on statistical analysis and Shing-Ming Cheng for technical assistance. This work was supported by the National Science Foundation (IBN #0347295), the National Institute of Health (R21-GM-068483-A1), and the American Cancer Society (RSG-02-046-01). This investigation was conducted in a Forsyth Institute facility renovated with support from Research Facilities Improvement Grant Number CO6RR11244 from the National Center for Research Resources, National Institutes of Health.

http://www.tandf.co.uk/journals/pp/1357650X.html DOI:10.1080/1357650054200001440

The consistent asymmetry of the heart, brain, and viscera in vertebrates highlights key aspects of the evolutionary origin of developmental patterning mechanisms (Burdine & Schier, 2000; Hamada, Meno, Watanabe, & Saijoh, 2002; Levin, 1999, 2005; Yost, 2001), as well as important issues in the medicine of birth defects in humans (Burn, 1991; Casey, 1998; Levin, Roberts, Holmes, & Tabin, 1996). One persistent question about the fixed chirality of the left–right axis concerns whether consistently biased asymmetry is a later innovation imposed on a bilaterally symmetrical primitive body-plan, or whether asymmetry is a fundamental property predating the bilateria (see Cooke, 2004a, 2004b; Palmer, 2004, for discussions of relevant issues). Planaria are a powerful model system for the study of patterning and regeneration (Newmark & Alvarado, 2002; Salo & Baguna, 2002). Their morphology suggests similarity to animals at the origin of the bilateral body-plan and they are commonly thought to be left–right symmetrical, as no consistent anatomical asymmetries have been described (Morgan, 1901; Oviedo, Newmark, & Sanchez Alvarado, 2003; Sakai, Agata, Orii, & Watanabe, 2000). In the context of a drug screen designed to identify ion transporters participating in regeneration (Levin, 2003; Nogi, Adams, & Levin, 2003), we discovered that inhibition of the H^+/K^+-ATPase ion pump (Sachs, Shin, Briving, Wallmark, & Hersey, 1995) induces defects in eye regeneration, which often affect only one eye. We used this assay to ask whether a consistently biased functional asymmetry existed in planaria, and whether H^+/K^+-ATPase-like transcripts may be expressed in the regenerating head. Here, we show that inhibition of H^+/K^+-ATPase activity, an ion flux mechanism recently found to be an important early step in the asymmetry of several vertebrate (Levin, Thorlin, Robinson, Nogi, & Mercola, 2002) and invertebrate (Ishii, Hibino, Nishino, Levin, & Amemiya, 2003; Shimeld, 2003) embryos, does indeed induce eye malformations more frequently on the right side. Moreover, we show the cloning and expression analysis of an endogenous transcript of the non-gastric H^+/K^+-ATPase subunit α which is transcribed in the head blastema shortly after amputation.

MATERIALS AND METHODS

For inhibition of the H^+/K^+-ATPase, mature *D. japonica* flatworms starved for 1 week were cut with a scalpel into head, tail, and trunk fragments on filter paper chilled on ice, with special care taken to perform cuts exactly perpendicular to the worm's long axis. The trunk fragments were then allowed to regenerate in Poland Spring water containing 70 μM of the potent and specific H^+/K^+-ATPase inhibitor SCH28080 (Vagin, Denevich, Munson, & Sachs, 2002). Exposure lasted for 2 days. Regenerating worms were monitored daily, and were scored for eye number and position 6 days after cutting. Worms were categorised as normal, cyclops (having a midline eye equidistant from the other two eyes), or

having an eye defect (duplicated or missing eye on one or both sides). Data were analysed using the χ^2 test.

Cloning of the *D. japonica* H^+/K^+-ATPase-like fragment was performed as follows. Planarian cDNA library constructed in λTriplEx2 was amplified by a nested-PCR using HKA191427FW1-ClaI
(5'-CCATCGATCGCCTAAAGAATTACCAGAAAT-3') and
HKA191427FW2-ClaI
(5'-CCATCGATTGTTAAATTTCTTAAAGAACTTACC-3') as the forward primers and λTriplEx 3' LD-Insert Screening primer
5'-ATACGACTCACTATAGGGCGAATTGGCC-3'; BD Biosciences Clontech) as the reverse primer. The product was digested by Cla I and Sac I and ligated to the Cla I and Sac I sites of pBluescript II KS+. The nucleotide sequence data will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB189739.

Whole-mount *in situ* hybridisation was performed essentially as described previously (Umesono, Watanabe, & Agata, 1999), with a few modifications. The worms were treated with 2% HCl solution for 5 min, and then fixed in Carnoy's fixative at 4°C for 2 hrs. To bleach the samples, fixed worms were incubated in methanol containing 5% H_2O_2 under light for 10 h at room temperature. Hybridisation was performed at 55°C in a solution containing 50% formamide, 5 × SSC, 0.1% Tween-20, 0.1 mg/ml heparin, 0.1 mg/ml yeast tRNA and 10% dextran sulphate with a DIG-labelled antisense RNA probe for 36 hrs. A mixture of BCIP/NBT was used for colour development of the alkaline-phosphatase-conjugated anti-DIG-antibody (Roche).

RESULTS

A variety of asymmetries (e.g., snail shell coiling and crab claw asymmetry) exists in invertebrates (Neville, 1976; Palmer, 1996), and it is not currently known whether the chirality of these forms derives from the same mechanisms that align the left–right axis in vertebrates (Boorman & Shimeld, 2002). Traditional phylogenetic trees based on morphology suggest planaria to be descendants of primitive bilaterians due to a triploblast, cephalised, and bilaterally symmetrical body-plan. Early acoelomate flatworms were believed to have been the first organisms to develop a third tissue layer, the mesoderm, and true bilateral symmetry, which differed from more primitive metazoa that are diploblast, and at most exhibit radial symmetry (Pechenik, 2000; Sarnat & Netsky, 1985). Recent studies based on 18s rRNA and *Hox* genes suggest a somewhat more advanced placement of planaria within the Lophotrochozoa (Adoutte, Balavoine, Lartillot, & de Rosa, 1999; Alvarado, 2003; Ruiz-Trillo, Ruitort, Littlewood, Herniou, & Baguna, 1999).

These flatworms are now once again becoming a popular system for addressing the mechanisms of morphogenesis during regeneration (Newmark &

Alvarado, 2002). As part of a reverse drug screen designed to probe the role of ion flux in establishing polarity control during regeneration (Dimmitt & Marsh, 1952; Marsh & Beams, 1957; Morgan, 1901; Nogi et al., 2003), we asked whether any consistently biased asymmetry existed in the regeneration of planaria exposed to various pharmacological reagents. We sought to determine whether LR asymmetry might exist among early bilateria, and to determine whether symmetrical paired organs might contain information bearing LR identity which was not apparent from morphology alone, or from intercalation experiments (Saito, Koinuma, Watanabe, & Agata, 2003). Such an asymmetry in symmetrically paired organs exists in mammalian embryos, and is revealed by the observation that several compounds specifically affect the left or right limb during development (Layton & Hallesy, 1965; Barr, 1973; Inouye and Murakami, 1978; Layton and Layton, 1979; Bochert, Platzek, Blankenburg, Wiessler, & Neubert, 1985).

A variety of blockers of ion transporters did not have specific effects on morphogenesis during regeneration (see Table 1). In contrast, omeprazole, lansoprazole, and Prodigiosin, reagents that specifically block the H^+/K^+-ATPase using different mechanisms (Matsuya et al., 2000; Sachs et al., 1995), induced eye defects during regeneration (see Table 2). Further experiments were performed with SCH28080, a potent and specific blocker of the H^+/K^+-ATPase (Vagin et al., 2002) which has the advantage that it does not need to be acid-activated prior to use. This reagent was chosen because of another important advantage. Because many species possess more than one H^+/K^+-ATPase gene, knock-down technologies targeting a single specific transcript (such as RNAi) can fail to reveal important phenotypes due to masking by compensation or redundancy effects. In contrast, pharmacological reagents simultaneously target

TABLE 1
Drugs targeting non-H^+/K^+-ATPase-mediated K^+ and H^+ flux that did not induce eye defects

Reagent	*Target/effect*	*Dosage*
Chromanol 293B	KCNK1 K^+ channel blocker	50 μM
Skelid (tiludronate)	V-ATPase inhibitor	250 μM
N-ethyl-maleimide	V-ATPase inhibitor	40 μM
SB-242784	V-ATPase inhibitor	3 μM
Triethylhexylammonium bromide	K_v K^+ channel blocker	700 μM
Phentolamine mesylate	K_{atp} K^+ channel blocker	10 μM
Diazoxide	K_{atp} K^+ channel opener	75 μM
Pinacidil	K_{atp} K^+ channel opener	30 μM
DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid)	I_{ks} activator	45 μM

Regenerating worm fragments were exposed to the reagents indicated during the first 2 days after cutting. Control worms (regenerating in vehicle alone) exhibited completely normal regeneration.

TABLE 2
Drugs targeting the H^+/K^+-ATPase that specifically induced eye defects

Reagent	*% incidence of eye defects*	*Dosage*
Prodigiosin	35% ($N = 40$)	5 μM
Omeprazole/Lansoprazole	12% ($N = 98$)	75 μM

In all cases, posterior blastemas regenerated tails normally.

Regenerating worm fragments exposed to H^+/K^+-ATPase blockers exhibited defects in eye patterning.

all members of a given family, and are thus an ideal method to rapidly assay for an interesting phenotype and determine whether a particular transporter family is implicated. Moreover, pharmacological experiments have the advantage of allowing temporal control over the loss of function (many reagents are reversible, and can be washed out, unlike RNAi injections, reducing toxicity).

We first established a baseline dosage for SCH28080. Approximately 70 μM SCH28080 proved to be optimal; lower doses had a negligible effect on regeneration, whereas higher doses inhibited head regeneration and thus prevented scoring of eye asymmetry. This concentration (70 μM) is consistent with specific binding of SCH28080 to H^+/K^+-ATPase in vertebrates (Vagin et al., 2002) as well as with the dosage used to induce randomisation of left–right asymmetry in a number of species (Levin et al., 2002), and was thus used in all further experiments. Importantly, tail regeneration was not inhibited in SCH28080-treated worms, demonstrating that the effect was not due to general toxicity, nor to an induced inability to mount a regeneration response, but was rather specific to events involved in head regeneration patterning.

We next took advantage of the observed effects on eye regeneration to determine whether a consistent left–right asymmetry existed in planarians. A total of 1183 *D. japonica* flatworms were cut into head, tail, and trunk fragments in six separate experiments. Particular care was taken to ensure even cuts perpendicular to the worm's long axis. The trunk fragments were then allowed to regenerate in spring water containing SCH28080. The effects on head morphology are shown in Figure 1 and summarised in Table 3; the blocker inhibits head formation and results in defects in eye patterning. In 20% of the cases, only one eye was affected, and we compared the incidence of eye defects in the left and right eyes. The data are shown in Table 4: in 204 regenerates with only one eye affected, the right eye was 1.5 times more likely to be abnormal than the left. This difference is statistically significant ($p << .01$ by the χ^2 analysis, testing against the null hypothesis prediction of an equal number of defects in the left and right eye, if the worms were truly symmetrical in their response to the H^+/K^+-ATPase inhibitor). These drugs did not have teratological effects on

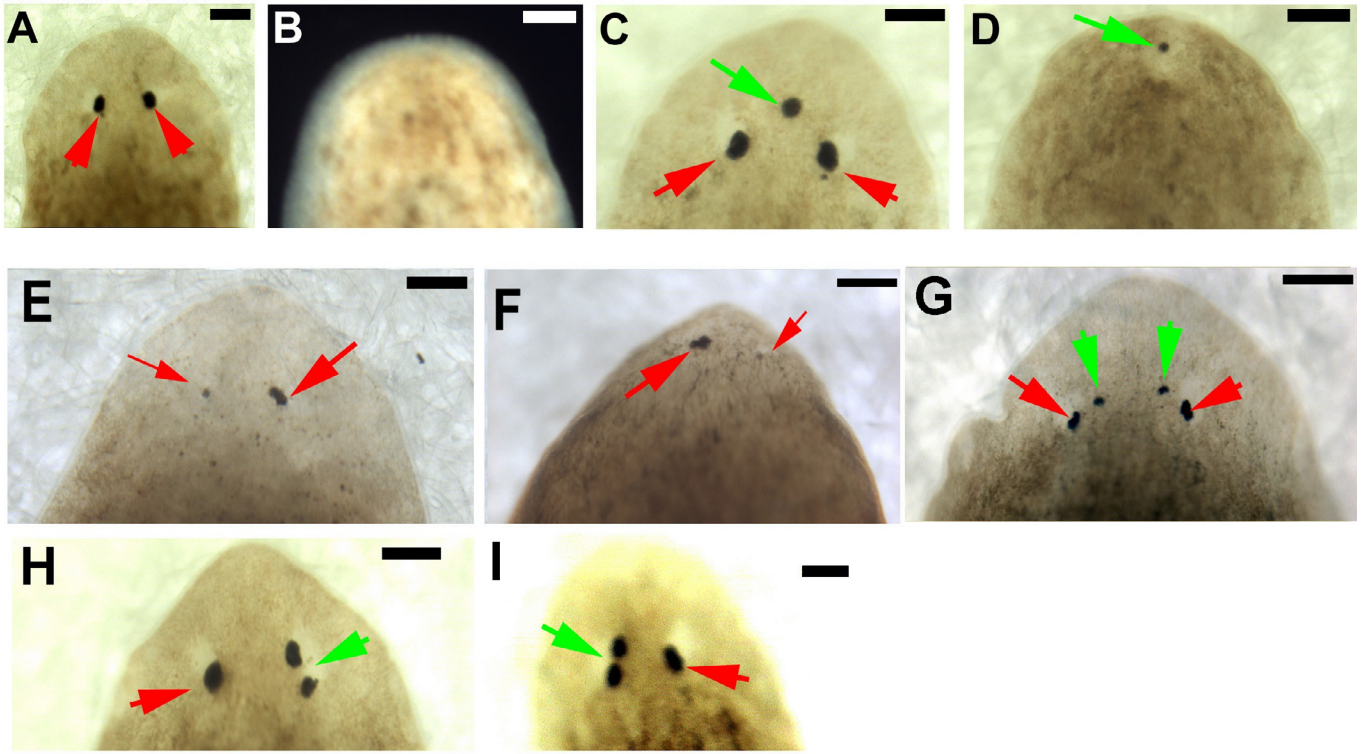

Figure 1. Head abnormalities caused by regeneration in SCH28080. (A) Worms exposed to vehicle alone develop wild-type heads with two eyes. In contrast, worms exposed to the H,K-ATPase inhibitor SCH28080 frequently exhibit defects in eye regeneration, including: (B) missing eyes, (C) ectopic eye in the midline, (D) single eye in midline location, (E,H) defect in left eye, (F,I) defect in right eye, and (G) defects in both eyes. Dysmorphic eyes occurred significantly more frequently on the right side, $p = .003$. Light grey (red online) arrows indicate normal eyes, Dark grey (green online) arrows indicate abnormal eyes. Scale bar is 0.25 mm. To see a colour version of the figures, please view the online version of the journal.

TABLE 3
Distribution of head phenotypes caused by regeneration in SCH28080

Phenotype:	*SCH28080*	*Controls*
Normal	57% (672)	90% (221)
One eye absent or duplicated	20% (231)	4% (10)
Three eyes	3% (38)	4% (9)
No head	16% (191)	1% (3)
Cyclops	4% (51)	1% (2)
Total:	1183	245

TABLE 4
Comparison and statistical analysis of effects in the left and right eye

	SCH28080-exposed	*Null hypothesis*
Right eye abnormal:	60% (123)	50% (102)
Left eye abnormal:	40% (81)	50% (102)
Total:	204	204

Regenerating worm fragments exposed to the H^+/K^+-ATPase blocker SCH28080 exhibited abnormalities more often in the right eye, at a significance of $p = .003$ using the χ^2 test with 1 degree of freedom.

intact worms; consistent with their current biomedical use in human patients (Thitiphuree & Talley, 2000)—this suggests that at levels used to specifically inhibit the H^+/K^+-ATPase in a number of vertebrate and protozoan systems (Meade & Stringer, 1995; Jiang, Meadows, Anderson, & Mukkada, 2002), general toxicity is not induced by these compounds.

We then sought to identify and characterise the expression of potential endogenous targets of the SCH28080 in planaria. A single H^+/K^+-ATPase-like EST (accession number BP191427) was found by BLAST analysis in the database of ESTs produced from head fragments (Mineta, Nakazawa, Cebria, Ikeo, Agata, & Gojobori, 2003). Using PCR based on this fragment sequence, we isolated a longer partial clone (990 bp, predicted to encode 330 amino acids) from a *D. japonica* library (submitted as accession number AB189739) that bore 67.1% homology to the *Xenopus laevis* non-gastric H^+/K^+-ATPase subunit α at the amino acid level. *In situ* hybridisation with a probe antisense to this clone revealed punctate expression throughout the body of intact worms, with low intensity staining at the midline (Figure 2A); especially strong expression was observed in the peripheral region of the head (light grey arrows in Figure 2B).

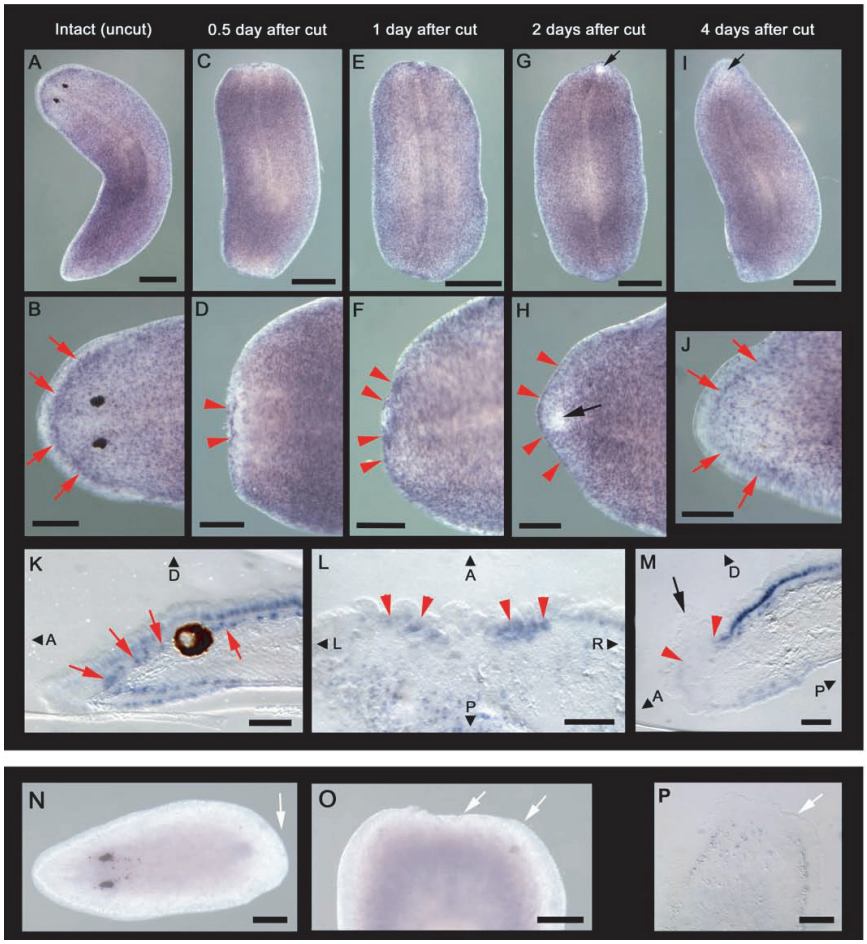

Figure 2. Endogenous expression of H^+/K^+-ATPase-like transcript. Expression of H^+/K^+-ATPase α subunit in the intact worms and regenerating trunk fragments revealed by whole-mount *in situ* hybridisation. (A, C, E, G, I) Whole images of specimens. Anterior end is at the top of the figure. (B, D, F, H, J) Close-up views of the head region in worms shown in panels A, C, E, G and I, respectively. Anterior end is to the left. (A, B) Intact worm. The light grey arrows indicate the strong signal at the peripheral region in the head. (C, D) 0.5 days after cutting. The light grey arrowheads indicate the expression at the wound closure. (E, F) 1 day after cutting. The expression is detected around the wound closure, indicated by the light grey arrowheads. (G, H) 2 days after cutting. The light grey arrowheads indicate the expression at the edge of anterior blastema. Note that expression is absent from a small spot in the head region, indicated by the black arrow. (I, J) 4 days after cutting. The black arrow indicates the weak level of the expression in the middle of the head, and the light grey arrows indicate the strong signal at the peripheral region in the head. (K–M) Plastic sections of whole-mount *in situ* hybridisation specimens. Orientation is indicated by the arrowheads and initial letters: A, anterior; P, posterior; D, dorsal; L, left; R, right. (K) Sagittal section of the head fragment

(Continued on facing page)

Then, 12 to 24 hours after cutting, the transcript was expressed in the border of the dorsal and ventral epithelium in the wound (Figures 2C and 2E, and light grey arrowheads in Figures 2D and 2F). Two days after cutting, the expression was absent from a small spot in the anterior blastema (black arrows in Figures 2G, 2H, and 2M). Four days after cutting, the expression was weaker around the regenerating eyes (black arrows in Figure 2I), but positive cells were scattered around the eyes in the head region (Figure 2J), and a high expression region appeared at the peripheral region in the anterior blastema (light grey arrows in Figure 2J). Sectioning revealed the tissue distribution of the transcript. Staining was observed within the epithelium and in a thin layer in the mesenchyme underneath the muscle tissue (Figure 2K). In the head region, the mesenchymal layer expressing the H^+/K^+-ATPase subunit α subunit surrounds the eye and lines the inside of the head region (light grey arrows in Figure 2K). Horizontal sections demonstrate the specific expression of the transcript within the epithelium in the head blastema (Figure 2L,M). Hybridisation with a sense probe revealed no signal within the blastema (Figure 2N–P).

DISCUSSION

While most planaria do not appear to possess consistent anatomical asymmetries—although the genus *heteroplana* is a rare exception (Wiley, 1897), exhibiting partial atrophy of the left side—they have been shown to be able to distinguish left from right in behavioural tasks (Corning, 1964), demonstrating that some mechanism must exist allowing the planarian nervous system to distinguish left from right. Our fundamental finding is that the left and right sides of the worm exhibit consistently different sensitivities to the SCH28080; this is a functional difference between cells on the left and right sides of the head. This effect is specific to SCH28080—differential sensitivity was not observed using a number of V-ATPase and ion channel inhibitors (Table 1). Thus, we are currently pursuing the hypothesis that an endogenous asymmetry in H^+/K^+-ATPase function may function in head or eye regeneration. Since H^+/K^+-ATPase activity has already been shown to be important for the asymmetry of a number of species (Ishii et al., 2003; Levin et al., 2002; Shimeld, 2003), it is

at 2 days after cutting, close-up of the anterior region. The signal is detected in a thin mesenchymal layer underneath the muscle tissue, indicated by light grey arrows. Note the mesenchymal layer expressing H^+/K^+-ATPase α subunit is surrounding the eye. Anterior is to the left and dorsal to the top. (L) Horizontal section of the regenerating trunk fragment at 0.5 days after cutting, close-up of the anterior region. The light grey arrowheads indicate the expression at the wound closure. Anterior is to the top. (M) Sagittal section of the regenerating trunk fragment at 2 days after cutting, close-up of the anterior region. The expression is absent from the region between the light grey arrowheads, indicated by the black arrow. Anterior to the left and dorsal to the top. Sense probe negative controls show the expected lack of specific stain throughout the animal (N) and in the anterior blastema (O). Sectioning confirms lack of specific signal in sense probe controls (P). Scale bars: (A, C, E, G, I) 500 μm, (N) 300 μm, (B, D, F, H, J, O, P) 200 μm, and (K–M) 100 μm.

likely that these data support a shared ancestral mechanism for the bilateria, rather than a derived characteristic of planaria.

The family of H^+/K^+-ATPase proteins has not yet been exhaustively characterised in planarians, but these transporters have been studied pharmacologically and biochemically in a number of invertebrates where they are known to be effectively blocked by the reagents used in our study (Jiang, Anderson, Winget, & Mukkada, 1994; Jiang et al., 2002; Meade, Hudson, Stringer, & Stringer, 1989; Meade, Shaw, Lemaster, Gallagher, & Stringer, 1987). Aside from the molecular details of the eye development pathway disrupted by SCH28080, these data demonstrate that the left and right sides of the flatworm possess a consistently biased inherent difference underlying the phenotypes we observed. Our cloning and expression data indicate that the *D. japonica* genome does indeed contain a homologue of the H^+/K^+-ATPase which is likely to be targeted by the SCH28080 loss-of-function reagent. The transcript we characterised is expressed within the early blastema during regeneration (Figure 2D,L), consistent with its being the mediator of SCH28080 effects on eye regeneration. Its lack of expression in eye tissue *per se* suggests that its role in eye development is not cell autonomous, and is consistent with signalling from cells that express this putative ion antiporter to cells that actually participate in eye formation during head regeneration. Interestingly, the expression of the gene we characterised is not itself asymmetric. While additional, as yet uncharacterised, members of this family may exhibit asymmetries at the level of transcription, the H^+/K^+-ATPase is subject to complex functional regulation. It is possible that, as in vertebrates (Fujita et al., 2002; Levin, 2003; Levin et al., 2002), the activity of additional K^+ transporters contributes to a net-asymmetric change in membrane voltage.

Functional and behavioural asymmetries exist in the visual system of many fishes and toads (Bisazza, Rogers, & Vallortigara, 1998; Cantalupo, Bisazza, & Vallortigara, 1996; Halpern, Liang, & Gamse, 2003; Lippolis, Bisazza, Rogers, & Vallortigara, 2002; Malashichev, 2002; Okada, Takagi, Seikai, Tanaka, & Tagawa, 2001; Rogers, 2002; Wassersug & Yamashita, 2002), although consistent asymmetry in the eyes themselves have not been described. Interestingly, exposure to a vertical static electric field has previously been reported to produce right-sided eye defects in mice (Marino, Berger, Mitchell, Duhacek, & Becker, 1974), suggesting that while anatomically symmetrical, eyes may in fact possess a consistently oriented asymmetry conserved across phyla. Our data suggest that further examples of fixed laterality may await discovery in species that morphologically appear to be symmetrical—such as *Drosophila*, where recently a subtle but consistent asymmetry was detected in wing size (Klingenberg, McIntyre, & Zaklan, 1998). While much remains to be learned about the molecular basis of the phenotypes we observed, these observations suggest that asymmetry of the left–right axis is an ancient feature of animal morphology, and that the ability to orient the left–right axis with respect to the

anterior–posterior and dorsal–ventral axes may exist in lineages that do not utilise that information to control morphogenesis of anatomical structures.

Manuscript received 10 February 2005
Revised manuscript received 23 February 2005

REFERENCES

Adoutte, A., Balavoine, G., Lartillot, N., & de Rosa, R. (1999). Animal evolution the end of the intermediate taxa? *Trends in Genetics*, *15*, 104–108.

Alvarado, A. S. (2003). The freshwater planarian *Schmidtea mediterranea*: Embryogenesis, stem cells and regeneration. *Current Opinion in Genetics & Development*, *13*, 438–444.

Barr, M. (1973). The teratogenicity of cadmium chloride in two stocks of Wistar rats. *Teratology*, *7*, 237–242.

Bisazza, A., Rogers, L. J., & Vallortigara, G. (1998). The origins of cerebral asymmetry: A review of evidence of behavioural and brain lateralization in fishes, reptiles and amphibians. *Neuroscience & Biobehavioral Reviews*, *22*, 411–426.

Bochert, G., Platzek, T., Blankenburg, G., Wiessler, M., & Neubert, D. (1985). Embryotoxicity induced by alkylating agents: Left-sided preponderance of paw malformations induced by acetoxymethyl-methylnitrosamine in mice. *Archives of Toxicology*, *56*, 139–155.

Boorman, C. J., & Shimeld, S. M. (2002). The evolution of left–right asymmetry in chordates. *Bioessays*, *24*, 1004–1011.

Burdine, R., & Schier, A. (2000). Conserved and divergent mechanisms in left–right axis formation. *Genes & Development*, *14*, 763–776.

Burn, J. (1991). Disturbance of morphological laterality in humans. *CIBA Foundation Symposium*, *162*, 282–296.

Cantalupo, C., Bisazza, A., & Vallortigara, G. (1996). Lateralization of displays during aggressive and courtship behaviour in the Siamese fighting fish (*Betta splendens*). *Physiology & Behavior*, *60*, 249–252.

Casey, B. (1998). Two rights make a wrong: Human left–right malformations. *Human Molecular Genetics*, *7*, 1565–1571.

Cooke, J. (2004a). Developmental mechanism and evolutionary origin of vertebrate left/right asymmetries. *Biological Reviews of the Cambridge Philosophical Society*, *79*, 377–407.

Cooke, J. (2004b). The evolutionary origins and significance of vertebrate left–right organisation. *Bioessays*, *26*, 413–421.

Corning, W. C. (1964). Evidence of right–left discrimination in planarians. *The Journal of Psychology*, *58*, 131–139.

Dimmitt, J., & Marsh, G. (1952). Electrical control of morphogenesis in regenerating *Dugesia tigrina*. 2. Potential gradient vs current density as control factors. *Journal of Cellular and Comparative Physiology*, *40*, 11–23.

Fujita, A., Horio, Y., Higashi, K., Mouri, T., Hata, F., Takeguchi, N. et al. (2002). Specific localization of an inwardly rectifying K+ channel, Kir4.1, at the apical membrane of rat gastric parietal cells; its possible involvement in K+ recycling for the H+-K+ pump. *Journal of Physiology*, *540*, 85–92.

Halpern, M. E., Liang, J. O., & Gamse, J. T. (2003). Leaning to the left: Laterality in the zebrafish forebrain. *Trends in the Neurosciences*, *26*, 308–313.

Hamada, H., Meno, C., Watanabe, D., & Saijoh, Y. (2002). Establishment of vertebrate left–right asymmetry. *Nature Reviews Genetics*, *3*, 103–113.

Inouye, M., & Murakami, U. (1978). Teratogenic effect of N-methyl-N′-nitro-N-nitrosoguanidine in mice. *Teratology*, *18*, 263–268.

Ishii, Y., Hibino, T., Nishino, A., Levin, M., & Amemiya, S. (2003). An inhibitor for H+/K+-ATPase disrupted the left–right asymmetry in the echinoid embryo. *74th Annual Meeting of the Zoological Society of Japan*. Hakodate, Japan.

Jiang, S., Anderson, S. A., Winget, G. D., & Mukkada, A. J. (1994). Plasma membrane K+/H(+)-ATPase from *Leishmania donovani*. *Journal of Cellular Physiology*, *159*, 60–66.

Jiang, S., Meadows, J., Anderson, S. A., & Mukkada, A. J. (2002). Antileishmanial activity of the antiulcer agent omeprazole. *Antimicrobial Agents & Chemotherapy*, *46*, 2569–2574.

Klingenberg, C. P., McIntyre, G. S., & Zaklan, S. D. (1998). Left–right asymmetry of fly wings and the evolution of body axes. [Erratum appears in *Proc R Soc Lond B Biol Sci*, 1998, Dec *22*;265(1413):2455.] *Proceedings of the Royal Society of London—Series B: Biological Sciences*, *265*, 1255–1259.

Layton, W., & Hallesy, D. (1965). Forelimb deformity in rats: Association with acetazolamide. *Science*, *149*, 79.

Layton, W., & Layton, M. (1979). Cadmium induced limb defects in mice. *Teratology*, *19*, 229–236.

Levin, M. (1999). Twinning and embryonic left–right asymmetry. *Laterality*, *4*, 197–208.

Levin, M. (2003). Electric embryos: Endogenous ion fluxes and voltage gradients in left–right asymmetry. *Developmental Biology*, *259*, 482.

Levin, M. (2005). Left–right asymmetry in embryonic development: A comprehensive review. *Mechanisms of Development*, *122*, 3–25.

Levin, M., Roberts, D., Holmes, L., & Tabin, C. (1996). Laterality defects in conjoined twins. *Nature*, *384*, 321.

Levin, M., Thorlin, T., Robinson, K. R., Nogi, T., & Mercola, M. (2002). Asymmetries in H+/K+-ATPase and cell membrane potentials comprise a very early step in left–right patterning. *Cell*, *111*, 77–89.

Lippolis, G., Bisazza, A., Rogers, L. J., & Vallortigara, G. (2002). Lateralisation of predator avoidance responses in three species of toads. *Laterality*, *7*, 163–183.

Malashichev, Y. B. (2002). Asymmetries in amphibians: A review of morphology and behaviour. *Laterality*, *7*, 197–217.

Marino, A. A., Berger, T. J., Mitchell, J. T., Duhacek, B. A., & Becker, R. O. (1974). Electric field effects in selected biologic systems. *Annals of the New York Academy of Sciences*, *238*, 436–444.

Marsh, G., & Beams, H. (1957). Electrical control of morphogenesis in regenerating *Dugesia tigrina*. *Journal of Cellular and Comparative Physiology*, *39*, 191–211.

Matsuya, H., Okamoto, M., Ochi, T., Nishikawa, A., Shimizu, S., Kataoka, T. et al. (2000). Reversible and potent uncoupling of hog gastric (H(+)+K(+))-ATPase by prodigiosins. *Biochemical Pharmacology*, *60*, 1855–1863.

Meade, J. C., Hudson, K. M., Stringer, S. L., & Stringer, J. R. (1989). A tandem pair of *Leishmania donovani* cation transporting ATPase genes encode isoforms that are differentially expressed. *Molecular & Biochemical Parasitology*, *33*, 81–91.

Meade, J. C., Shaw, J., Lemaster, S., Gallagher, G., & Stringer, J. R. (1987). Structure and expression of a tandem gene pair in *Leishmania donovani* that encodes a protein structurally homologous to eucaryotic cation-transporting ATPases. *Molecular & Cellular Biology*, *7*, 3937–3946.

Meade, J. C., & Stringer, J. R. (1995). Cloning and characterization of an ATPase gene from *Pneumocystis carinii* which closely resembles fungal H+ ATPases. *Journal of Eukaryotic Microbiology*, *42*, 298–307.

Mineta, K., Nakazawa, M., Cebria, F., Ikeo, K., Agata, K., & Gojobori, T. (2003). Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. *Proceedings of the National Academy of Science USA*, *100*, 7666–7671.

Morgan, T. (1901). *Regeneration*. New York: Macmillan.

Neville, A. (1976). *Animal asymmetry*. London: Edward Arnold.

Newmark, P. A., & Alvarado, A. S. (2002). Not your father's planarian: A classic model enters the era of functional genomics. *Nature Reviews Genetics*, *3*, 210–219.

Nogi, T., Adams, D. S., & Levin, M. (2003). Electric controls of regeneration in planaria. *Developmental Biology*, *259*, 586.

Okada, N., Takagi, Y., Seikai, T., Tanaka, M., & Tagawa, M. (2001). Asymmetrical development of bones and soft tissues during eye migration of metamorphosing Japanese flounder, *Paralichthys olivaceus. Cell Tissue Research*, *304*, 59–66.

Oviedo, N. J., Newmark, P. A., & Sanchez Alvarado, A. (2003). Allometric scaling and proportion regulation in the freshwater planarian *Schmidtea mediterranea. Developmental Dynamics*, *226*, 326–333.

Palmer, A. R. (1996). From symmetry to asymmetry: Phylogenetic patterns of asymmetry variation in animals and their evolutionary significance. *Proceedings of the National Academy of Science USA*, *93*, 14279–14286.

Palmer, A. R. (2004). Symmetry breaking and the evolution of development. *Science*, *306*, 828–833.

Pechenik, J. A. (2000). *Biology of the invertebrates* (4th ed.). Boston, MA: McGraw Hill.

Rogers, L. J. (2002). Lateralised brain function in anurans: Comparison to lateralisation in other vertebrates. *Laterality*, *7*, 219–239.

Ruiz-Trillo, I., Riutort, M., Littlewood, T. J., Herniou, E. A., & Baguna, J. (1999). Acoel flatworms: Earliest extant bilaterian metazoans, not members of platyhelminthes. *Science*, *283*, 1919–1922.

Sachs, G., Shin, J. M., Briving, C., Wallmark, B., & Hersey, S. (1995). The pharmacology of the gastric acid pump: The H+,K+ ATPase. *Annual Review of Pharmacology & Toxicology*, *35*, 277–305.

Saito, Y., Koinuma, S., Watanabe, K., & Agata, K. (2003). Mediolateral intercalation in planarians revealed by grafting experiments. *Developmental Dynamics*, *226*, 334–340.

Sakai, F., Agata, K., Orii, H., & Watanabe, K. (2000). Organization and regeneration ability of spontaneous supernumerary eyes in planarians. *Zoological Science (Tokyo)*, *17*, 281–412.

Salo, E., & Baguna, J. (2002). Regeneration in planarians and other worms: New findings, new tools, and new perspectives. *Journal of Experimental Zoology*, *292*, 528–539.

Sarnat, H. B., & Netsky, M. G. (1985). The brain of the planarian as the ancestor of the human brain. *Canadian Journal of Neurological Sciences*, *12*, 296–302.

Shimeld, S. M. (2003). Regulation of left right asymmetry in Ciona. In P. Lemaire (Ed.), *International Urochordate Meeting* France: Carry le Rouet.

Thitiphuree, S., & Talley, N. J. (2000). Esomeprazole, a new proton pump inhibitor: Pharmacological characteristics and clinical efficacy. *International Journal of Clinical Practice*, *54*, 537–541.

Umesono, Y., Watanabe, K., & Agata, K. (1999). Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes. *Development, Genes, and Evolution*, *209*, 31–39.

Vagin, O., Denevich, S., Munson, K., & Sachs, G. (2002). SCH28080, a K+-competitive inhibitor of the gastric H,K-ATPase, binds near the M5-6 luminal loop, preventing K+ access to the ion binding domain. *Biochemistry*, *41*, 12755–12762.

Wassersug, R. J., & Yamashita, M. (2002). Assessing and interpreting lateralised behaviours in anuran larvae. *Laterality*, *7*, 241–260.

Wiley, A. (1897). On Heteroplana, a new genus of planarians. *Quart. Journ. Micr. Sci. N.S.*, *xl*, 203–205.

Yost, H. J. (2001). Establishment of left–right asymmetry. *International Review of Cytology*, *203*, 357–381.