# Formation of the head organizer in hydra involves the canonical Wnt pathway

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## Summary

**Stabilization of β-catenin by inhibiting the activity of glycogen synthase kinase-3β has been shown to initiate axis formation or axial patterning processes in many bilaterians. In hydra, the head organizer is located in the hypostome, the apical portion of the head. Treatment of hydra with alsterpaullone, a specific inhibitor of glycogen synthase kinase-3β, results in the body column acquiring characteristics of the head organizer, as measured by transplantation experiments, and by the expression of genes associated with the head organizer. Hence, the role of the canonical Wnt pathway for the initiation of axis formation was established early in metazoan evolution.**

Key words: Hydra, Head organizer, Canonical Wnt pathway



## Introduction

Molecular mechanisms underlying processes of axis formation and axial patterning often have common features among bilaterians. For example, Hox genes and the genes of the canonical Wnt pathway are involved in axis formation and axial patterning in both deuterostomes and protostomes (e.g. McGinnis and Krumlauf, 1992; Cox and Pfeifer, 1998). How early in metazoan evolution did these genes, and their roles in axis formation/axial patterning, appear?

Among the diploblasts, these processes have been extensively investigated in the cnidarian hydra. The hydra polyp consists of a single axis with radial symmetry. The regions along the axis are the head, body column and foot. Because of the tissue dynamics of an adult hydra, the processes governing axial patterning are continuously active (e.g. Bode, 2003). Similarly, as bud formation, a form of asexual reproduction, also occurs continuously in hydra, the processes governing the initiation of axis formation are also constantly active. Axial patterning, especially of the head, is well understood in hydra at the tissue level. The head organizer (Browne, 1909; Technau et al., 2000; Broun and Bode, 2002) located in the hypostome, the upper portion of the head in a hydra, produces a signal and transmits it to the body column. The signal sets up a morphogenetic gradient that decreases down the body column. This gradient, referred to as a positional value gradient (Wolpert, 1971), a source density gradient (Gierer and Meinhardt, 1972) or the head activation gradient (MacWilliams, 1983b), confers head formation capacity on tissue of the body column. The head organizer also produces a second signal, head inhibition, which is transmitted to and graded down the body column. Head inhibition prevents body column tissue from forming heads (MacWilliams, 1983a).

The molecular basis of the head organizer and these gradients is not well understood. Homologs of a number of genes that affect axial patterning in bilaterians have been isolated from hydra, and appear to play similar roles. For example, the expression patterns of *Cnox-3*, a homolog of the Hox gene *labial/Hox-1*, and of the parahox gene *Cnox-2*, a homolog of *Gsx*, in several experimental situations suggest that these two genes are involved in the control of head formation (Shenk et al., 1993a; Shenk et al., 1993b; Bode, 2001).

With respect to the head organizer, genes of the canonical Wnt pathway have been identified in hydra, and their expression patterns suggest that this pathway plays a role in this structure (Hobmayer et al., 2000). *HyWnt* is expressed exclusively in the hypostome, where the head organizer is located. The gene is also expressed very early in the apical tip of the developing head during head regeneration and bud formation as the head organizer is developing. *HyTcf* has a similar, although slightly more extended, range of expression, and *Hyβ-Cat* is expressed strongly during early stages of head formation.

In addition, there are data implicating glycogen synthase kinase-3β in the activity of the head organizer. Treatment with LiCl, which inhibits GSK-3β (e.g. Phiel and Klein, 2001), results in the formation of ectopic tentacles along the body column (Hassel et al., 1993). Exposure to diacylglycerol, which activates protein kinase-C (PKC) (Nishizuka, 1992), which in turn blocks GSK-3β activity (Goode et al., 1992), causes the formation of individual ectopic tentacles or complete heads along the body column (Mueller, 1989). However, both reagents have other effects. For example, $Li^+$ blocks, and diacylglyercol catalyzes, the traverse of the phosphoinositol pathway (Hallcher and Sherman, 1980).

To gain more direct evidence for the role of the canonical Wnt pathway in the formation of the head organizer, we made use of alsterpaullone, which specifically blocks the activity of GSK-3β (Leost et al., 2000; Bain et al., 2003). Treatment with

this inhibitor blocked GSK-3β activity throughout the animal as well as elevating the level of β-catenin in the nuclei of body column cells. The treatment also conferred characteristics of the head organizer on the body column as well as inducing the expression of genes of the Wnt pathway in the body column. These results provide direct evidence for the role of the canonical Wnt pathway in the formation and maintenance of the head organizer in hydra.

## Materials and methods

### Animals and culture conditions

Experiments were carried out with 1-day starved animals of the Zurich L2 strain of *Hydra vulgaris*. Animals were fed three times per week, and maintained in hydra medium as described previously (Smith et al., 1999).

### Tissue manipulations

#### Treatment with alsterpaullone

Hydra were exposed to 5 μmol/l alsterpaullone (A.G. Scientific, Inc.) in 0.025% DMSO in hydra medium for 1 or 2 days. Thereafter, they were rinsed several times and cultured in hydra medium for 2–5 days. Treatment with 0.025% DMSO had no effect on hydra. To determine if treatment with alsterpaullone affected cell cycle traverse, 1 μmol/l BrdU was injected after 2 days of treatment and the labeling index was measured, as described by Bode et al. (Bode et al., 1990).

#### Treatment with aminopurvalanol

Hydra were exposed to several concentrations of aminopurvalanol for 2 days, and then returned to hydra medium without inhibitor. The highest concentration of the reagent used, 20 μmol/l, resulted in a final concentration of 0.2% DMSO, which had no effect on hydra.

#### Transplantation experiments

To assay the inductive capacity of body column tissue treated with alsterpaullone, one-eighth of the body column of a donor animal was isolated, cut into four equal-sized pieces, and one of these quarters grafted into the body column of a host animal, as described previously (Broun and Bode, 2002). Host animals were injected with India ink (Campbell, 1973) before transplantation to distinguish transplanted from host tissue.

Experiments to measure the capacity of body column tissue to produce either head inhibition or the signal for setting up the head activation gradient were carried out as described by Wilby and Webster (Wilby and Webster, 1970a; Wilby and Webster, 1970b). Donor animals were labeled with India ink, and then treated with alsterpaullone for 2 days. Thereafter, one-eighth of the length of the body column of the treated animal was isolated and grafted onto the basal end of a bisected host animal, as described by Rubin and Bode (Rubin and Bode, 1982).

### Isolation of GSK-3β

GSK-3β was isolated from hydra by affinity chromatography using axin sepharose beads, as described by Primot et al. (Primot et al., 2000). Two adult hydra were homogenized in 100 μl of a buffer, which was a combination of the homogenization buffer and the lysis buffer used for cell culture (Primot et al., 2000). This combination buffer consisted of the components of the lysis buffer plus 60 mmol/l β-glycerophosphate, 50 mmol/l Na vanadate, 500 mmol/l NaF, 10 μg/ml leupeptin, 10 μg/ml aprotenin, 10 μg/ml SBTI and 100 μmol/l benzamidine. The lysate was centrifuged at 14,000 ***g*** in a microfuge for 5 minutes at 4°C. Subsequently, the supernatant was mixed by rotation for 30 minutes at 4°C with 5 μl axin-sepharose beads in 25 μl bead buffer (beads were stored as a 20% suspension in a bead buffer) plus another 25 μl of bead buffer. Then the beads were washed 3× with 100 μl of bead buffer, 2× with 100 μl kinase buffer and, finally, resuspended in 50 μl kinase buffer as described by Ryves et al. (Ryves et al., 1998).

### GSK-3β kinase assay

GSK-3β kinase activity was measured using $^{32}$P-ATP as described by Ryves et al. (Ryves et al., 1998) using the GSM [RRRPASVPPSPSLSRHSSHQRR] peptide, in which the underlined serine is phosphorylated. GSM(nP), which is the same peptide without the phosphorylated serine, served as a control. Three microliters of either hydra lysate or GSK-3β purified on axin-sepharose beads was used in a 12 ml reaction mixture. To assay the inhibitory effect of alsterpaullone on GSK-3β, the kinase assay was carried out on axin-sepharose-isolated GSK-3β in the presence of different concentrations of alsterpaullone.

### Immunocytochemistry on whole mounts

Staining with the TS-19 monoclonal antibody was carried out as described by Bode et al. (Bode et al., 1988). For the anti-β-catenin antibody the following procedure was used. Animals were relaxed in 2% urethane for 2 minutes, and then fixed in Lavdowsky's fixative (50% ethanol, 10% formalin, 4% acetic acid, 40% water) for 10-15 minutes. Then the animals were washed 3× 5 minutes in PBS, and once for 5 minutes by rotation with PBST. Next, the animals were incubated with rotation in 4% goat serum in PBST for 60 minutes at 4°C. After this, they were incubated overnight with a 1:100 dilution of an affinity-purified guinea pig anti-sea urchin β-catenin polyclonal antibody (Miller and McClay, 1997). After washing 4-5 times for 5 minutes with 4% serum, samples were incubated for 60 minutes at room temperature with a 1:100 dilution of a FITC-conjugated donkey anti-guinea pig polyclonal antibody (Jackson ImmunoResearch Laboratories). Subsequently, the sample was incubated in a 1:100 dilution of propidium iodide for 20-30 minutes at room temperature. Finally, the animals were washed 2× 10 minutes in PBST, and then 2× 10 minutes in PBS. They were mounted using Vectashield mounting medium (Vector Laboratories, Inc.) and stored at –80°C. Samples were examined with confocal microscopy.

### In situ hybridization

In situ hybridization analysis was carried out on whole mounts of hydra as described previously (Grens et al., 1996; Martinez et al., 1997). The antisense RNA probe for *HyBra1* was a fragment of the gene containing the 5′ end of the ORF including the T-box domain. For the *HyWnt and HyTcf* genes, the antisense RNA probes contained the entire open reading frame. Probes were used at concentrations 0.025 ng/μl for *Hybra1*, 0.1 ng/μl for *HyWnt* and 0.01 ng/μl for *HyTcf*.

## Results

### Treatment with alsterpaullone induces formation of ectopic tentacles on the body column of hydra

To determine the effects of blocking GSK-3β on axial patterning, hydra were treated with different concentrations of alsterpaullone. Treatment with 5 μmol/l alsterpaullone for 2 days followed by a return to hydra medium resulted in the formation of ectopic tentacles 3-4 days later along the upper 80% of the body column (Fig. 1A,B). The fact that the ectopic structures were tentacles was confirmed by staining control (Fig. 1C) and alsterpaullone-treated animals (Fig. 1D) with TS-19, an antibody that specifically binds to an antigen on the surface of ectodermal epithelial cells of the tentacles (Bode et al., 1988). Occasionally, complete ectopic heads were observed by 9-10 days along the body column (data not shown). Treatment for longer periods of time, or with higher concentrations of the reagent, led to disintegration of the

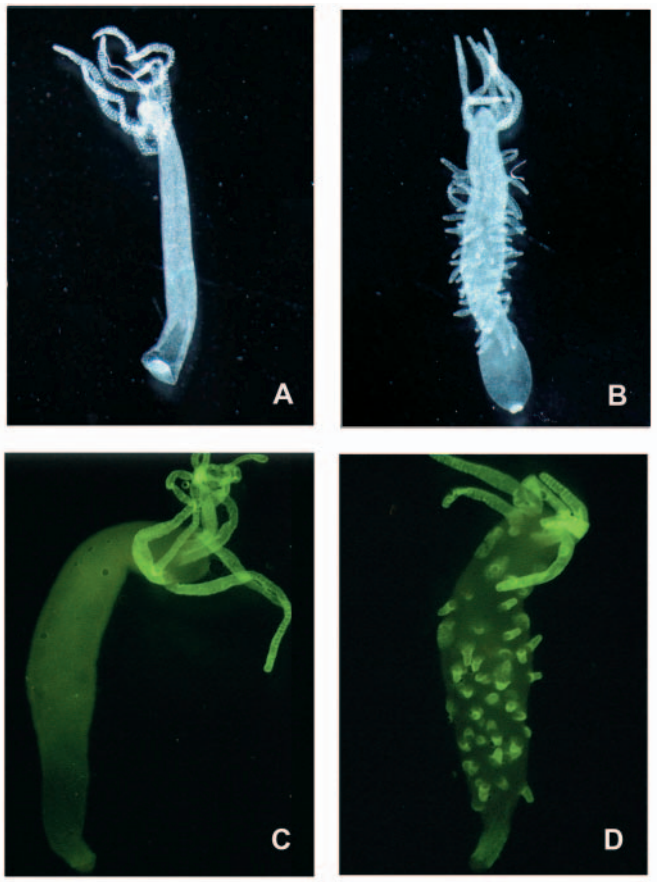



**Fig. 1.** Effect of a 2-day treatment with 5 µmol/l alsterpaullone on the formation of ectopic tentacles in adult hydra. Control (A) and alsterpaullone-treated (B) animals. (C,D) Control and alsterpaullone-treated animals stained with the TS-19 antibody.

animals. Treatment with lower concentrations had no visible effects.

### Alsterpaullone specifically blocks GSK-3β activity

Alsterpaullone is known to be specific in its activity. It strongly blocks the activity of GSK-3α and GSK-3β, and to a lesser extent inhibits three cyclin-dependent kinases, CDKS 1, 2 and 5 (Leost et al., 2000). To determine if alsterpaullone blocked the activity of hydra GSK-3β, the enzyme was isolated and its activity assayed. GSK-3β was isolated from a hydra lysate using a method based on the affinity of GSK-3β for axin attached to sepharose beads (Primot et al., 2000). The activity of the affinity-purified GSK-3β was assayed by the phosphorylation of GSM, a 22-aa peptide that is specifically phosphorylated by GSK-3β (Ryves et al., 1998). The much higher level of label incorporated into samples exposed to the purified GSK-3β compared with the negligible level seen with axin-sepharose beads not exposed to hydra lysate indicated the presence of GSK-3β on the lysate-treated beads (Fig. 2A). Exposure of the lysate-treated beads to GSM(nP), a modified version of GSM only poorly phosphorylated by GSK-3β (Ryves et al., 1988), provided additional evidence for the presence of GSK-3β on the beads (Fig. 2A).

When alsterpaullone was added directly to the GSK-3β-axin-sepharose beads in concentrations of 1 µmol/l and 10 µmol/l, the enzyme activity was reduced to around 1-2% of the control level (Fig. 2B). Similar results have been reported for GSK-3β isolated from an insect cell line (Leost et al., 2000). In addition, when hydra lysates were directly treated with 5 µmol/l alsterpaullone, the activity of GSK-3β was reduced to 30-40% of the control level (Expt 1, 1788 versus 4830 CPM; Expt 2, 417 versus 1254 CPM). Hence, this inhibitor blocks the activity of GSK-3β in hydra.

Alsterpaullone also blocks three cyclin-dependent kinases, CDK 1, 2 and 5 (Leost et al., 2000). The fact that the observed effects of alsterpaullone on the formation of head structures was not due to inhibition of these enzymes was shown as follows. Aminopurvalanol is a specific inhibitor of CDKs (Leost et al., 2000) but has no effect on GSK-3β (Bain et al., 2003). Treatment of hydra with several concentrations of aminopurvalanol (1, 2 or 5 µmol/l) for 2 days did not induce the formation of ectopic head structures, nor did it have any other effects on the morphology of the animal. Treatment with 10 or 20 µmol/l aminopurvalanol resulted in the disintegration of hydra within 1 day, indicating that the inhibitor penetrated the tissues of the animal (data not shown).

Another assay involved the roles of CDK1 and CDK2 in cell cycle traverse. If alsterpaullone was blocking these enzymes

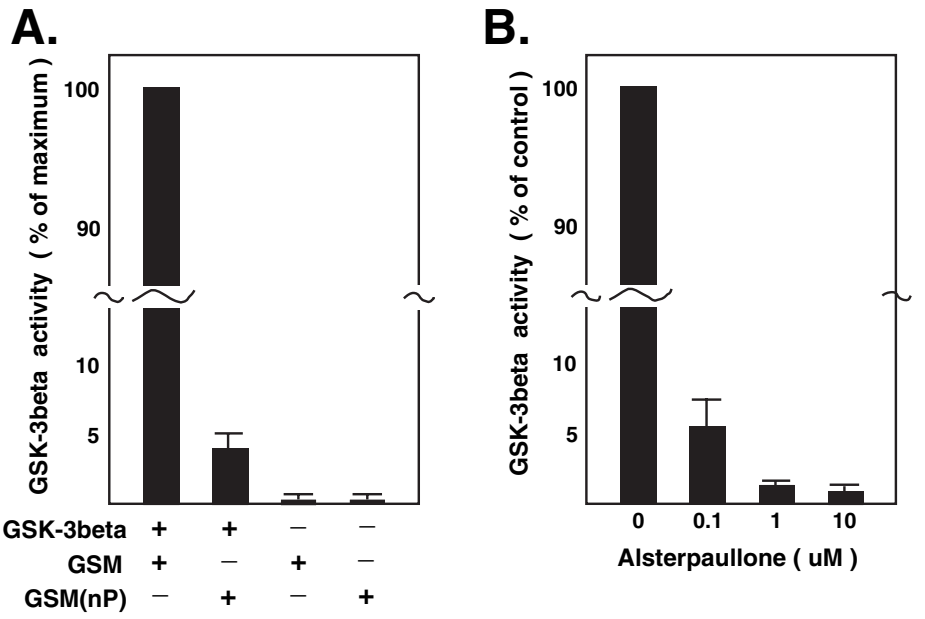



**Fig. 2.** Effect of alsterpaullone on hydra GSK-3β activity. (A) Activity of purified GSK-3β isolated from a hydra lysate using GSM or GSM[nP) as a substrate. Results expressed as percentage of the activity in the lysate using GSM. (B) Effect of alsterpaullone on isolated GSK-3β activity. Data are presented as the mean value of two independent experiments.

**Table 1. Effect of alsterpaullone on cell cycle traverse**

| Treatment | Length of treatment (days) | Fraction of cells labeled (%) | |
|---|---|---|---|
| | | Epithelial cells | Interstitial cells |
| Control | 1 | 21.2±2.0 | 56.2±0.6 |
| Alsterpaullone | 1 | 27.1±4.5 | 70.0±5.9 |
| Control | 2 | 18.9±0.6 | 39.1±13.0 |
| Alsterpaullone | 2 | 35.0±1.1 | 54.9±12.4 |

one would expect a reduction in the fraction of cells in S-phase, as the two enzymes are involved in the G1/S and G2/M transitions (Murray, 2004). Animals were treated with 5 μmol/l alsterpaullone for 2 days and then injected with 1 μmol/l BrdU. The labeling index of the epithelial cells and the interstitial cells did not decrease (Table 1), indicating that neither of the two enzymes was inhibited by alsterpaullone. Instead, the labeling index increased, which is consistent with another effect of blocking GSK-3β activity. CDK4, which is essentially not affected by alsterpaullone (Leost et al., 2000), also affects cell cycle progression. Phosphorylation of CDK4 by GSK-3β leads to its degradation and, hence, slows down cell cycle traverse (Ewen et al., 1993). Blocking GSK-3β with alsterpaullone increases the level of CDK4 activity, thus, enhancing cell cycle traverse, which could account for the observed increase in the labeling index.

In sum, alsterpaullone blocks the activity of the hydra GSK-3β, but has no inhibitory effect on the CDKs.

## Inhibition of GSK-3β increases the level of β-catenin in the nucleus and cell membranes of hydra

In the canonical Wnt pathway, GSK-3β phosphorylates β-catenin, targeting it for degradation (Yost et al., 1996). Blocking the activity of GSK-3β results in the accumulation of β-catenin in cell membranes, where the protein has a role in cell-cell adhesion (Nagafiuchi and Takeichi, 1989). Accumulation of β-catenin also occurs in the nuclei of cells, where, along with Tcf, it is involved in transcription of genes downstream of the Wnt pathway (Behrens et al., 1996). To determine if blocking GSK-3β activity by treatment with alsterpaullone had similar effects on β-catenin in hydra, we made use of an affinity-purified guinea pig anti-sea urchin β-catenin polyclonal antibody (Miller and McClay, 1997). This antibody is known to bind to the β-catenin of tunicates and amphioxus as well as sea urchins (D. McClay, personal communication). Three different circumstances were analyzed.

Hydra treated with 5 μmol/l alsterpaullone for 24 hours were subjected to immunocytochemistry with anti-β-catenin antibody and examined with confocal microscopy. The treatment raised the level of β-catenin associated with the cell membranes (Fig. 3B) compared with controls (Fig. 3A).

*HyWnt*, the hydra *Wnt* homolog, is expressed exclusively in the apical tip of the hypostome of an adult hydra (Hobmayer et al., 2000). If GSK-3β is a negative regulator of β-catenin downstream of *Wnt* in hydra as it is in other systems, one would expect higher levels of nuclear β-catenin in the hypostome than elsewhere in the adult. This was confirmed by examining the head, body column and foot regions of an adult. As shown in Fig. 4A,B, many of the nuclei in the apical tip of the hypostome were strongly labeled with the antibody. The smaller nuclei are the nuclei of neurons, while the larger nuclei are those of

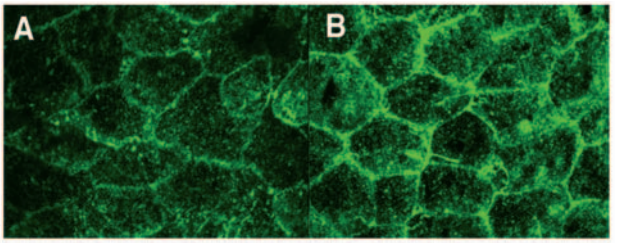



**Fig. 3.** Effect of alsterpaullone on the accumulation of β-catenin in cell membranes in the body column, as measured with an anti-β-catenin antibody. (A) Control. (B) Animal treated with 5 μmol/l alsterpaullone for 24 hours.

epithelial cells. This was observed nowhere else in the adult, for example in the body column (Fig. 4C,D).

Finally, *Hyβ-Cat* and *HyGSK3,* the hydra homologs of *β-catenin* and *GSK-3β* are expressed fairly uniformly throughout a hydra, although *Hyβ-Cat* is expressed at higher levels in the hypostome (Hobmayer et al., 2000). Hence, blocking GSK-3β would be expected to result in the accumulation of β-catenin in the nuclei of body column cells. Treatment of hydra with increasing levels of alsterpaullone (0.3, 1.25 or 3.0 μmol/l) for 24 hours led to an increased level of the accumulation of β-catenin in the nuclei of several cell types of the body column (Fig. 5) compared with such nuclei in control animals (Fig. 4C,D).

Thus, blocking the activity of GSK-3β raised the levels of β-catenin in the cell membranes and in cell nuclei in hydra as has been observed in bilaterians (Yost et al., 1996; Pai et al., 1997; Logan et al., 1999).

## Treatment with alsterpaullone confers characteristics of the hypostome on the body column

The increased level of nuclear β-catenin in the body column of animals treated with alsterpaullone suggests the body column may have taken on characteristics of the hypostome. To examine this possibility, the expression patterns of three genes expressed exclusively or predominantly in the hypostome were observed.

*HyBra1*, a hydra *brachyury* homolog, is expressed in the hypostome of an adult hydra (Fig. 6A) (Technau and Bode, 1999). Treatment with 5 μmol/l alsterpaullone for 24 hours expanded the expression domain of the gene throughout most of the body column (Fig. 6B).

The two other genes examined, *HyWnt* and *HyTcf*, the hydra homologs of *Wnt* and *Tcf*, encode proteins that are part of the canonical Wnt pathway. The change in the expression pattern of *HyTcf* in response to alsterpaullone was similar to that of *HyBra1*. Normally *HyTcf* is expressed in the head region of an adult (Fig. 6C), with the most intense level of expression in the apex of the hypostome (Hobmayer et al., 2000). After treatment with alsterpaullone for 1 day, *HyTcf* was expressed throughout the body column (Fig. 6D). *HyWnt* is expressed exclusively in the apical tip of the hypostome (Hobmayer et al., 2000). After 24 hours of treatment the *HyWnt* expression domain expanded from the apical tip (Fig. 6E) to cover a larger area of the hypostome (data not shown). After 48 hours of treatment, spots of *HyWnt* expression similar in size to the spot in the apical tip of the hypostome appeared in the upper two-

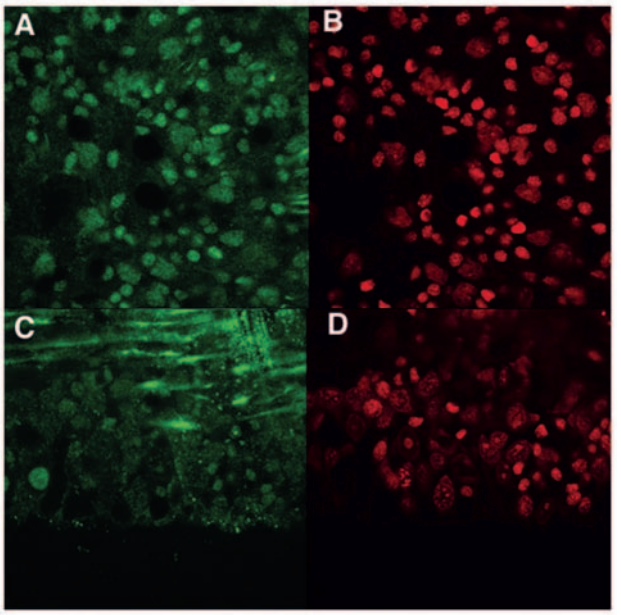



**Fig. 4.** A higher level of β-catenin in nuclei of the hypostome (A,B) compared with nuclei of the body column (C,D). (A,C) Staining with the anti-β-catenin antibody; (B,D) staining with propidium iodide. The intense horizontal lines of stain are in the membranes of processes extending from the epithelial cells along the basement membrane.

thirds of the body column in a relatively regularly spaced pattern (Fig. 6F).

Thus, treatment with alsterpaullone, which inhibited GSK-3β activity, induced ectopic expression of the head-specific genes, *Hybra1*, *HyWnt* and *HyTcf*, and subsequently the formation of tentacles and ectopic heads along the body column.

## Alsterpaullone promotes formation of the head organizer in the body column

As the expression of *HyWnt* is associated with the head organizer, the expression of the gene in the body column suggests the body columns of alsterpaullone-treated animals may have acquired characteristics of the head organizer. As described in the Introduction, the head organizer is defined by three properties: (1) its ability to induce a second axis when transplanted to the body column of a host animal; (2) the production and transmission of a signal that sets up the head activation gradient; and (3) the production and transmission of a head inhibition signal that prevents head formation from occurring in the body column. These three properties were examined to determine if treatment with alsterpaullone had conferred head organizer characteristics on tissue of the body column.

### Capacity for induction of a second axis

When a hypostome is transplanted into a body column, it invariably induces body column tissue to form a second axis consisting of a head and body column (Browne, 1909; Broun and Bode, 2002). As a similar-sized piece of tissue (1/32nd) taken from any part of the body column does not form a second axis upon transplantation (Yao, 1945; Broun and Bode, 2002), this inductive capacity is restricted to the hypostome.

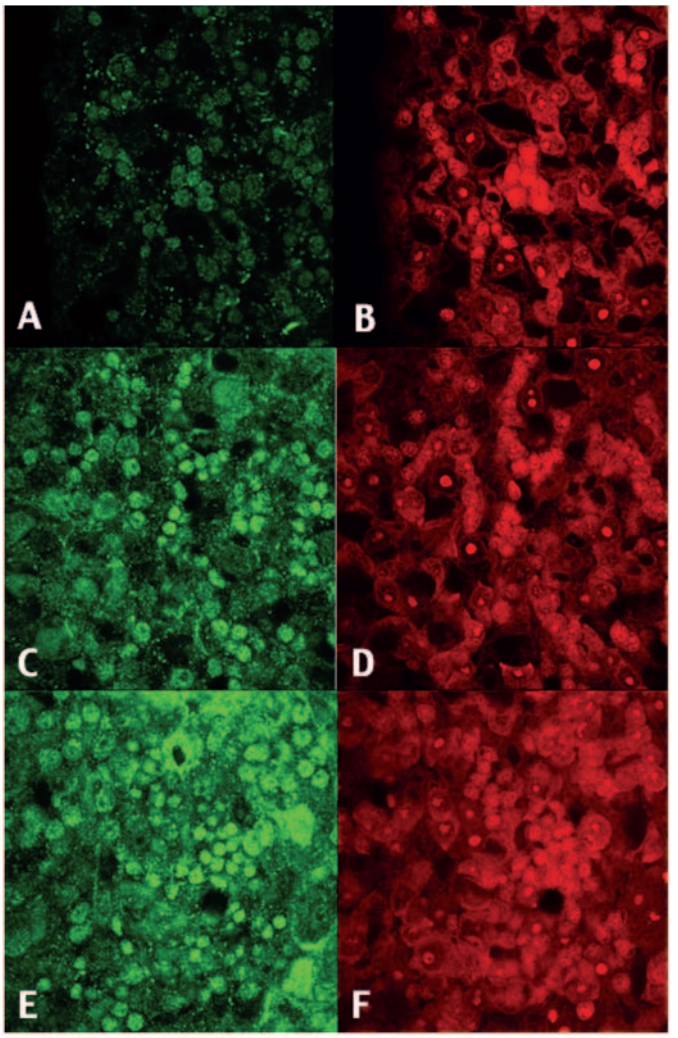



**Fig. 5.** Increasing accumulation of β-catenin in the nuclei of body column cells as a function of the concentration of alsterpaullone. (A,B) 0.3 mmol/l; (C,D). 1.25 mmol/l; (E,F) 3.0 mmol/l. (A,C,E) Staining with the anti-β-catenin antibody; (B,D,F) staining with propidium iodide.

To determine if treatment with alsterpaullone confers this inductive capacity on the tissue of the body column, the following experiment was carried out. Animals were treated with 5 μmol/l alsterpaullone for 48 hours and then transferred to hydra medium. Periodically after start of treatment, a quarter of the 3-region (=1/32nd of the body column) of a treated animal was transplanted to the 3-region of an untreated host labeled with India ink (Fig. 7A). Because the host head can inhibit the formation of a second axis (MacWilliams, 1983a), the host animals were decapitated just before transplantation to make the induction assay more sensitive.

None of the control transplants, that is, a quarter of the 3-region of a control animal, formed a second axis (Fig. 7C). By contrast, the same piece of tissue from an animal treated for 1 day with alsterpaullone formed a second axis in ~40% of the transplants, while a 2-day treatment resulted in all of the transplants forming a second axis (Fig. 7C). The fact that the second axis was due to induction is shown in Fig. 7B, in which a quarter of a 3-region of an

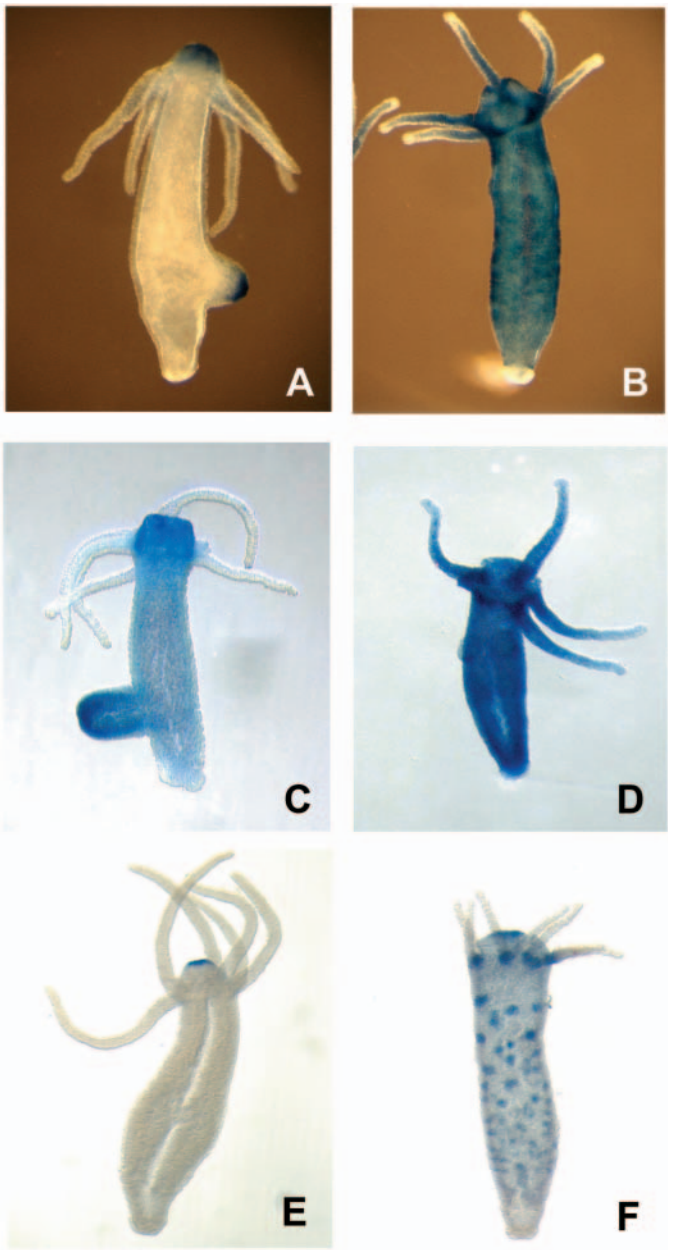



**Fig. 6.** Changes in the expression patterns of *HyBra1* (A,B), *HyTcf* (C,D) and *HyWnt* (E,F) after treatment with alsterpaullone, as visualized using in situ hybridization. (A,C,E) Control animals; (B,D,F) animals treated with alsterpaullone for 24 hours (B,D) or 48 hours (F).

alsterpaullone-treated animal was transplanted into a host animal stained with India ink. The hypostome of the second axis is derived from the unstained transplant, while the rest of the second axis (tentacles and body column) is made up of host tissue.

### Capacity to produce an inhibitor of head formation

Wilby and Webster (Wilby and Webster, 1970a) described an experiment that directly demonstrates that a head, and hence the head organizer, produces and transmits head inhibition along the body column. When the 2-4 region of the body column (see Fig. 8A) is isolated, a head regenerated in most of the samples at the 2-end (Fig. 8B, sample 2-4). If a head was grafted to the 4-end of an excised H-1-2-3-4 piece of a host, and 6 hours later the host H-1 region was removed (Fig. 8A, 2-4/H), a different result was obtained. Only a small fraction of the samples regenerated a head at the 2-end (Fig. 8B), indicating that the grafted head produced and transmitted head inhibition up the body column, which inhibited head regeneration at the original apical end.

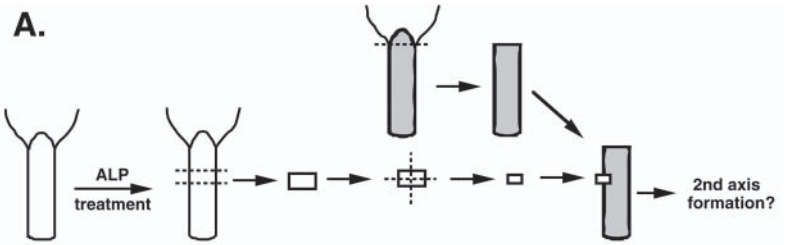
If instead of a head, a 4-region of a normal animal was grafted to the basal end of the H-1-2-3-4 region (2-4/4) the result was similar to the one in which the 2-4 region is allowed to regenerate. Most of the samples regenerated a head at the 2-end (Fig. 8B, 2-4/H), indicating that the grafted 4-region has little or no capacity to inhibit head regeneration. By contrast, when a 4-region from a donor treated with 5 μmol/l alsterpaullone for 2 days was grafted to the basal end (2-4/ALP-4), and the same experiment was carried out, none of the samples regenerated a head (Fig. 8B, 2-4/ALP-4). This indicates that the alsterpaullone-treated 4 region has the same capacity as a head to produce and transmit head inhibition along the body column to prevent head regeneration at the apical end.

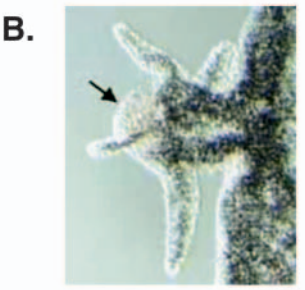
### Capacity to produce and transmit a signal that sets up the head activation gradient

The head activation gradient is maximal at the apical end of the body column and declines along its length (e.g. MacWilliams, 1983b). This gradient confers regeneration polarity on the body column. That is, when a piece of the body column is isolated, a head invariably regenerates at the apical end. Wilby and Webster (Wilby and Webster, 1970b) demonstrated that grafting a head to the basal end of a piece of the body column will invert this regeneration polarity. When the grafted head is removed, a head will regenerate at the basal end due to this inversion. This experiment provided direct evidence that the head produces a signal that sets up the head activation gradient.

A similar experiment was carried out using the four conditions described in the previous experiment. An isolated 2-4 region regenerated a head at the apical or 2-end (Fig. 9B). When a head was grafted to the 4-end (2-4/H), the host head removed after 6 hours, and then the remaining graft left intact for 6 days before removing the grafted head (Fig. 9A), a different result was obtained. Half the grafts regenerated a head at the basal end, while very few regenerated a head at the apical end (Fig. 9B, 2-4/H), which indicates that the head activation gradient had been inverted in many of the animals. Variations of this graft were carried out using a 4-region from an alsterpaullone-treated animal (2-4/ALP-4), and a control 4-region (2-4/4). Invariably, a head regenerated at the apical end when a control 4-region was used (Fig. 9B, 2-4/H). By contrast, the alsterpaullone-treated 4-region behaved like a head, in that in a similar number of samples the head activation gradient was inverted (Fig. 9B, 2-4/ALP-4). Hence, treatment with alsterpaullone confers the characteristic of the head organizer on the body column to send out a signal setting up the head activation gradient.

Thus, these three experiments indicate that alsterpaullone treatment confers characteristics of the head organizer on the body column.




C. Effect of Alsterpaullone on the Head Formation Capacity of Body Column Tissue

| Treatment [xd ALP + yd HM] | Number of Grafts | Number of 2nd Axes |
|---|---|---|
| Control | 30 | 0 |
| 1d ALP | 29 | 12 |
| 2d ALP | 24 | 24 |

**Fig. 7.** Acquisition by the body column of the capacity to induce a second axis after treatment with alsterpaullone. (A) Diagram of the experiment. (B) An example of a transplant inducing a second axis. The arrow indicates the unlabeled donor tissue as a part of the induced axis (host tissue labeled with India ink). (C). Results of the grafting procedure.

## Discussion

The canonical Wnt pathway is involved in patterning events in a number of bilaterians. In *Xenopus* and zebrafish, β-catenin is involved in organizer formation, which subsequently sets up the dorsoventral axis (Guger and Gumbiner, 1995; Schneider et al., 1996), while in mice β-catenin affects the formation of the anteroposterior axis (Haegel et al., 1995). In sea urchins β-catenin regulates the patterning of the anteroventral axis so that the vegetal end forms endoderm (Logan et al., 1999). Similarly, in the urochordate, *Ciona*, the Wnt pathway controls endoderm formation (Imai et al., 2000). The Wnt pathway is also active in axial patterning in protostomes. In *Drosophila* it is involved in determining the anteroposterior polarity of segments (Nusslein-Volhard and Wieschaus, 1980), and later affects the development of the dorsoventral axis in the wing and leg imaginal discs (Struhl and Basler, 1993). Recently β-catenin has been shown to be involved the process of gastrulation and germ layer specification in a cnidarian, *Nematostella vectensis* (Wikramanayake et al., 2003).

A crucial step in the canonical Wnt pathway is the stabilization and subsequent accumulation of β-catenin in the nucleus due to the inhibition of GSK-3β activity. By blocking the activity of GSK-3β, the formation of an ectopic organizer or ectopic axial structures can be induced in a number of bilaterians (Dominguez et al., 1995; Diaz-Benjumea and Cohen, 1994; Sumoy et al., 1999). We show here that a similar phenomenon occurs in hydra.

### The Wnt pathway is involved in the head organizer in hydra

Three sets of results indicate that the Wnt pathway plays a central role in the formation and maintenance of the head organizer in hydra. One is the set of expression patterns of three genes, *HyWnt, HyTcf* and *Hyβ-Cat*, of the canonical Wnt pathway. They are expressed in the mature hypostome or in a developing hypostome (Hobmayer et al., 2000), suggesting that this pathway plays a role in the head organizer.

#### Transplantation experiments indicate that blocking GSK leads to HO formation

The second set involves the transplantation experiments, which demonstrate that blocking GSK-3β activity throughout the animal leads to head organizer formation in the body column. When hydra were treated with alsterpaullone, it clearly blocked the activity of GSK-3β but had no inhibitory effect on the CDKs. Blocking GSK-3β activity resulted in tissue of the body column, acquiring the three known characteristics of a head organizer.

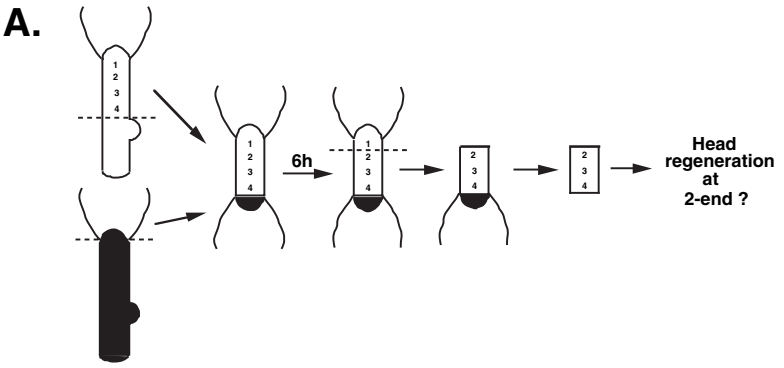
The fundamental property of the head organizer in hydra is its ability to induce a second axis when the hypostome is transplanted to the body column (Broun and Bode, 2002). A piece of normal body column tissue similar in size to that of a hypostome does not have this ability (Yao, 1945; Broun and Bode, 2002). By contrast, a hypostome-sized piece of alsterpaullone-treated body column tissue does have this inductive capacity, and has acquired a level similar to that found in a normal head.

The other two properties involve the two signals produced by the head organizer and transmitted down the body column, which set up the head activation and head inhibition gradients. Normal body column tissue does not generate either of these two signals. However, the tissue of an alsterpaullone-treated body column produces both signals. A piece of such a body column can invert the head activation gradient when grafted to the basal end of a body column, indicating that it is producing the signal for setting up this gradient. And, at the same time, this piece of tissue produces head inhibition and transmits it up the body column. The produced and transmitted level of head inhibition is sufficiently high to prevent head regeneration from taking place at a considerable distance (one-third to half the body length) from the source of this head inhibition, the alsterpaullone-treated piece of body column.

Thus, blocking the activity of GSK-3β conferred all three characteristics that define a head organizer onto tissue of the body column.

#### Blocking GSK-3β results in head genes expressed in the body column

As inhibition of GSK-3β confers head organizer characteristics on the body column, one would expect genes expressed in the hypostome or associated with the head organizer, such as genes of the canonical Wnt pathway, to be expressed in the body




**B.** **Capacity for inhibiting head regeneration**

| Graft Type | Graft Number | Regeneration at 2-end Head | Tentacle | None |
|---|---|---|---|---|
| 2-4 | 18 | 14 | 0 | 4 |
| 2-4/4 | 22 | 14 | 1 | 7 |
| 2-4/H | 24 | 5 | 1 | 18 |
| 2-4/ALP-4 | 18 | 0 | 0 | 18 |

**Fig. 8.** Acquisition by the body column of the capacity to produce head inhibition after treatment with alsterpaullone. (A) Diagram of the experiment. (B) Results of two experiments using the grafting procedure.

column. Normally, *HyBra1*, the hydra homolog of *Brachyury,* and *HyWnt* are expressed only in the head, while *HyTcf* is expressed much more strongly in the head than in the body column (Technau and Bode, 1999; Hobmayer et al., 2000). In addition, staining with an anti-β-catenin antibody indicates that the nuclei of the hypostome contain high levels of the β-catenin protein, while levels in the body column are much lower.

In animals treated with alsterpaullone, *HyBra1* and *HyTcf* were expressed strongly throughout the body column 1 day after initiation of the block in GSK-3β activity. In addition, the level of β-catenin was elevated in the nuclei of the body column after 1 day. *Brachyury* and *Tcf* are direct transcription targets of the β-catenin/TCF complex in vertebrates (Yamaguchi et al., 1999; Hovanes et al., 2001). Thus, it is quite likely that inhibition of GSK-3β resulted in the stabilization of β-catenin and its translocation into the nucleus, which in turn led to the transcription of *HyBra1* and *HyTcf* throughout the body column.

The timing of the change that occurred in *HyWnt* expression was slightly different. Two days after start of treatment, *HyWnt* expression expanded from the apex of the hypostome throughout much of the body column. The discovery of a TCF-binding site in the promoter of the *Drosophila Wg* gene (Heslip et al., 1997; Lessing and Nusse, 1998) indicates that the Wnt gene may be a direct target of the Wnt pathway. Thus, the elevated level of *HyTcf* coupled with the elevated level of nuclear β-catenin throughout the body column after 1 day of treatment most likely led to the transcription of *HyWnt* in the body column by the second day.

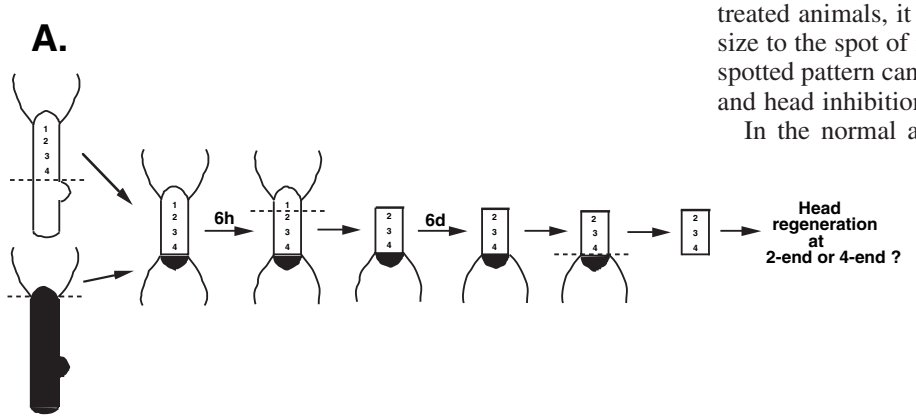
In sum, the latter two sets of data provide more direct evidence for the involvement of the Wnt pathway in the formation of the head organizer. Blocking the activity of GSK-3β results in the body column acquiring the characteristics of the head organizer as well as expressing genes associated with the Wnt pathway.

## The pattern of *HyWnt* expression following GSK-3β inhibition reflects the patterning processes governing head formation

Another result is consistent with the Wnt pathway playing a role in the head organizer. Instead of *HyWnt* being uniformly expressed throughout the body column of alsterpaullone-treated animals, it was expressed in spots that were similar in size to the spot of its expression found in the hypostome. This spotted pattern can be explained in terms of the head organizer and head inhibition.

In the normal animal, the organizer in the head produces head inhibition, which prevents the formation of new head organizers nearby. Only further down the body column where the head inhibition level falls below a threshold value, will a new head organizer arise. This results in the formation of the axis of a new bud, hydra's asexual form of reproduction. *HyWnt* is expressed as a spot on the body column, where this next bud will form, and continues to be expressed in the apical tip of the developing bud (Hobmayer et al., 2000).

As blocking GSK-3β activity led to a uniform rise in the level of *HyTcf*




**B.** **Capacity for reversing the head activation gradient**

| Graft Type | Graft Number | Head regeneration 2-end | 4-end | None |
|---|---|---|---|---|
| 2-4 | 39 | 36 | 0 | 3 |
| 2-4/4 | 23 | 23 | 0 | 0 |
| 2-4/H | 22 | 2 | 11 | 9 |
| 2-4/ALP-4 | 23 | 1 | 11 | 11 |

**Fig. 9.** Acquisition by the body column of the capacity to produce a signal that sets up the head activation gradient. (A). Diagram of the experiment. (B) Results.

transcription throughout the body column, one might expect a similar rise in *HyWnt* expression. As it is likely that the rise probably occurs unevenly, high levels of *HyWnt* will initially appear in random spots. And, if the rise in *HyWnt* is responsible for, or directly coupled with head organizer formation, then these random spots will form head organizers and begin producing head inhibition. In turn, these organizers will prevent the formation of other head organizers in their immediate vicinity. This could lead to the observed fairly uniform distribution of *HyWnt*-expressing spots, each of which is a putative head organizer region. Each of the spots is similar in size to the one in the hypostome, which is also consistent with each of them being associated with a head organizer.

As these head organizers in the body column continuously produce head inhibition, eventually one would expect rising levels of head inhibition throughout the body column. This was observed in the head inhibition experiment (Fig. 8). The amount of head inhibition produced by a piece of body column tissue derived from an alsterpaullone-treated animal was similar to, if not more than, that produced by a normal head. Plausibly, this reflects the presence of more than one head organizer in the piece of body column used in the transplant, as indicated by the presence of more than one *HyWnt* spot in this piece.

The elevated levels of head inhibition, coupled with an elevated level of head activation in the body column, also provide an explanation for the formation of ectopic tentacles but no complete heads in alsterpaullone-treated animals. In a normal animal, the head organizer in the hypostome produces and transmits head inhibition down the body column, preventing tissue of the body column from initiating head organizer, and hence head, formation. In the alsterpaullone-treated animals, the level of head inhibition is so high due to the multiple head organizers, that complete head formation involving hypostome and tentacle zone does not take place.

By contrast, tentacle formation is controlled by the head activation gradient, and is not affected by head inhibition. The head organizer produces and transmits a signal to the body column that sets up the head activation gradient in the body column in a normal hydra (MacWilliams, 1983b). Tentacle formation occurs above a threshold level of head activation. This is reflected in the commitment of tissue just below and in the tentacle zone to tentacle formation (Hobmayer et al., 1990). In addition, treatment with LiCl raises the head activation level in the body column (L.G. and H.R.B., unpublished), which leads to the formation of ectopic tentacles on the body column (Hassel et al., 1993). The multiple organizers in the alsterpaullone-treated animals most probably produce the signal for head activation, thereby generating a high level of head activation throughout the body column. In turn, this level is probably above the threshold level for tentacle formation, resulting in the large number of ectopic tentacles observed in alsterpaullone-treated animals.

Alsterpaullone also resulted in an elevated level of cell division, as indicated by an increased labeling index. However, it is unlikely that this effect is related to the formation of the ectopic tentacles or the head organizer activity in the body column. The patterning processes in hydra are morphallactic, and hence independent of cell division. For example, isolation of a piece of the body column results in the regeneration of a head at the apical end and a foot at the basal end and the proportions of a normal animal. The same pattern of regeneration takes place in the presence or absence of cell division (Cummings and Bode, 1984).

### Role of the Wnt pathway in the maintenance of the head organizer

Should the Wnt pathway play a central role in the head organizer, it would also provide an explanation for the maintenance of the head organizer in the context of the tissue dynamics of an adult hydra. In an adult hydra, the tissues are in a steady state of production and loss. Cells of all three cell lineages in the body column are continuously in the mitotic cycle. These are the epithelial cells of both the ectoderm and endoderm, as well as the interstitial cells of the interstitial cell lineage (David and Campbell, 1972; Campbell and David, 1974). To maintain the size of the animal, tissue is displaced apically into the head and basally onto the foot from the body column, and eventually sloughed at the extremities (Campbell, 1967). Since the hypostomal tissue is continuously displaced toward its apex and lost, the head organizer must also be in a steady state of production and loss.

The canonical Wnt pathway is known to act as a positive feedback loop in *Drosophila* (Heslip et al., 1997). If it acted in a similar manner in hydra, it could be involved in maintaining the head organizer. The Wnt signal produced by the head organizer in the tip of the hypostome could stimulate neighboring cells just basal to the tip by blocking GSK-3β and raising the nuclear level of β-catenin. In addition, *HyPKC2*, a hydra *PKC* homolog that is expressed in the apical half of the hypostome (Hassel et al., 1998), may augment this activity, as PKC is known to inhibit the activity of GSK-3β (Goode et al., 1992). Subsequently *HyWnt* would be transcribed in the neighboring cells, completing the positive feedback loop. Hence, the Wnt positive feedback loop would be continuously active, which would maintain the head organizer in the context of the continuous displacement toward, and loss of tissue at, the apex of the hypostome. This would also be consistent with the continuous expression of *HyWnt* and *HyTcf* in the hypostome (Hobmayer et al., 2000).

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