Hyzic, the Hydra homolog of the zic/odd-paired gene, is involved in the early specification of the sensory nematocytes

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Summary

Cnidaria are the first class of organisms in animal evolution with a nervous system. The cnidarian Hydra has two types of neuronal cell, nerve cells and nematocytes. Both differentiate from the same pool of pluripotent stem cells. Yet, the molecular regulation of neural differentiation in Hydra is largely unknown. Here, we report the identification of Hyzic, a homolog of the Zn-finger transcription factor gene zic/odd-paired, which acts as an early neural effector gene in vertebrates. We show, that Hyzic is expressed in the early nematocyte differentiation pathway, starting at the level of interstitial stem cells. Expression of Hyzic is restricted to the proliferative stages of nematoblasts. Hyzic acts before and possibly directly upstream of Cnash, a homolog of the proneural bHLH transcription factor geneachaete-scute, and of Nowa, an early nematocyte differentiation marker gene. Hyzic may determine stem cells to differentiate into nematocytes. Our data are consistent with a role of Hyzic in inhibiting nematocyte differentiation, by keeping committed nematoblast cells in the cell cycle. A similar role has been demonstrated for Zic genes in vertebrates. Our results suggest, that genetic cascades of neural development may be conserved from Hydra to vertebrates, indicating that the molecular regulation of neural development evolved only once.

Key words: Hydra, Nematocyte, Neural development, Neuronal differentiation, Zic, odd-paired, achaete-scute, Nowa

Introduction

The evolution of nervous systems in animals provided them with a major advantage, as they could sense their environment and actively respond to it. Comparisons between vertebrates and insects have revealed a great deal of conservation in the genetic control of neural development in Bilateria. In Drosophila and vertebrates the secreted factor Short gastrulation (Sog)/Chordin specifies the neurogenic region by inhibiting Bmp signaling (Sasai, 1998). In this area, the proneural bHLH transcription factor Achaete-Scute defines epithelial cells that are competent to differentiate into neurons. From these competent cells, the neuronal precursor cells are determined by the mutual inhibition of the membrane-bound neurogenic proteins Delta and Notch (reviewed by Artavanis-Tsakonas, 1999). The high conservation of the molecular components in neurogenesis in Bilateria suggests that the common ancestor of Bilateria used the same set of genes to regulate neurogenesis. Yet, not all genes seem to be conserved between insects and vertebrates. In Xenopus the Zn-finger gene zic was identified in a screen for downstream targets of Chordin (Mizuseki et al., 1998; Kuo et al., 1998). zic turned out to be upstream of the earliest known proneural gene, the bHLH transcription factor neurogenin, which belongs to the achaete-scute/atonal class of genes (Jarman et al., 1993; Jan and Jan, 1994; Gupta and Rodrigues, 1997).

Zic genes form a small family of Zn-finger transcription factors closely related to the Gli-family (Aruga et al., 1996a). Both are members of the superfamily of Krüppel-like Zn-finger genes, which all have a conserved H/C link in common (Schuh et al., 1986). So far, five Zic genes have been isolated from mouse, and six Zic genes have been isolated from the frog, of which three, zic1, zic-1 and opl, are allelic to each other (Aruga et al., 1994; Aruga et al., 1996a; Aruga et al., 1996b; Nakata et al., 2000). The sequences of these paralogs are highly similar to each other, indicating that they arose by recent duplication events. In vertebrates, all paralogs have overlapping expression patterns and appear to act cooperatively in neural and neural crest induction (Aruga et al., 1996a; Nakata et al., 1997; Nakata et al., 1998; Nakata et al., 2000; Brewster et al., 1998; Nagai et al., 1997; Salero et al., 2001). Zic genes are expressed in the dorsal neuroectoderm, as well as in the dorsal somitic mesoderm (Aruga et al., 1996a; Grinblat and Sive, 2001; Nakata et al., 1998; Rohr et al., 1999). Analysis of Bmp and chordin mutants in zebrafish showed that the dorsal restriction of zic2 expression requires Bmp signalling and the Bmp antagonist Chordin, suggesting that Bmp2/4 inhibits Zic expression ventrally (reviewed by Mayor and Aybar, 2001; Rohr et al., 1999). By contrast, Sonic hedgehog, suppresses Zic expression in the ventral neural tube and adaxial mesoderm (Rohr et al., 1999). Although Zic genes have a conserved role in neurogenesis in all vertebrates, Zic-related genes in lower chordates, the ascidians, appear to have additional functions in muscle development, and are required for the formation of the notochord and anterior mesenchyme (Nishida and Sawada, 2001; Satou et al., 2002; Imai et al., 2002; Wada and Saiga, 2002). Strikingly, a role in neural development for the Drosophila homolog odd-paired (opa) has
not been reported. Instead, *opa* acts as a pair-rule gene in epidermal segmentation (Beneyt et al., 1994), and also has a role in the formation of midgut constrictions mediated by the visceral mesoderm (Cimbora and Sakonju, 1995). Hence, it is not clear whether the proneural function of Zic in vertebrates is a differentiation gene of all interstitial cells, a cell type exclusively found in Cnidaria. Nematocytes contain a large single vesicle with a large capsule, the nematocyst. Nematocysts are extrusive organelles, i.e. they can discharge and evaginate a tube or filament, often attached with poisonous proteins. Most nematocytes are located in the tentacles, where they are used by the animals to catch their prey. Nematocytes are in contact with the environment via a cnidocil apparatus, a chemosensory structure resembling the hair sensillum of the inner ear, and electrophysiological studies have demonstrated that they have all the properties of sensory cells (Hausmann and Holstein, 1985; Brinkmann et al., 1996). Furthermore, in the freshwater polyp *Hydra*, nematocytes and nerve cells both derive from the same pool of pluripotent stem cells (David and Gierer, 1974; David and Murphy, 1977). Hence, nematocytes are regarded as a second neuronal cell type in Cnidaria.

The molecular regulation of neuronal differentiation in *Hydra* is largely unknown. So far, only one of the genes involved in bilaterian neurogenesis has been isolated from *Hydra*, the bHLH transcription factor gene *achaete-scute* homolog *Cnash* (Grens et al., 1995). Interestingly, *Cnash* is not expressed in nerve cells or nerve cell precursors, but in cell clusters that give rise to nematocytes, consistent with the idea that nematocytes are a neuronal cell type.

We now report the isolation and characterization of a homolog of the *zipopa* gene in *Hydra*. We show, that it is also expressed in the nematocyte lineage, and that it acts upstream of *Cnash* and *Nowa*, a differentiation gene of all nematocytes (Engel et al., 2002). First expression can be detected at the level of stem cells and is restricted to the proliferative stages of nematocyte differentiation. It is therefore the earliest marker gene for the differentiation of a neuronal cell type in cnidarians. The evolutionary implications are discussed.

Materials and methods

**Animal culture**

For all experiments, polyps of the *Hydra vulgaris* strain Basel, the temperature-sensitive mutant strain sf-1 of *H. magnipapillata* (Sugiyama and Fujisawa, 1978; Terada et al., 1988) and the sexually active *H. vulgaris* strain AEP (Martin et al., 1997) were used. Animals were kept at 18°C in mass cultures as described previously (Technau and Holstein, 1996), and animals used for experiments were starved for 24 hours beforehand.

**Elimination of interstitial cells**

To eliminate interstitial cells, the temperature-sensitive mutant strain sf-1 was cultured at 26°C for up to three days (Sugiyama and Fujisawa, 1978; Terada et al., 1988). Alternatively, the wild-type strain Basel of *H. vulgaris* was treated for up to three days with 10 mM hydroxyurea (HU). In both cases animals were fed daily during the treatment. Maceration analysis (David, 1973) confirmed that 95% of all interstitial cells had disappeared after one day (data not shown).

**Cloning of *Hyzic***

A full-length clone of *Hyzic* (GenBank Accession Number AA436645) was isolated by screening a *Hydra* cDNA library with the degenerate oligonucleotide ACNCAAYCWGGWGARAARCCWTT, directed against the conserved H/C link (THTGVKPF) of Krüppel-like Zn-finger proteins (Schuh et al., 1986). Hybridization was carried out following standard protocols (Sambrook et al., 1989). Briefly, hybridization was carried out overnight at 42°C. Filters were then washed twice at room temperature in 6xSSC/0.05% Na-Pyrophosphate (Na-PPi) for 15 minutes, once in 3 M TMAC at room temperature, twice in 3 M TMAC at 42°C, twice in 3 M TMAC at 47°C, and once in 6xSSC/0.05% Na-PPi for 15 minutes at room temperature, before being developed by autoradiography. Double-positive clones were isolated by the Lambda Zap II in vivo excision system (Stratagene) and sequenced. As the isolated clone did not contain stop codons in all three reading frames at the 5' end, we performed 5'RACE using the Gene Racer kit (Invitrogen). We isolated a short RACE clone encoding an additional 119 bp with stop codons in all the reading frames. Accordingly, the first AUG in the clone putatively encodes for the translation start site.

**Phylogenetic analysis**

The Maximum Likelihood program TREE-PUZZLE (Schmidt et al., 2002) was used to perform a phylogenetic analysis. JTT was used as substitution model, the rate heterogeneity parameter alpha of the gamma distribution was calculated from the data set. 10,000 replica were calculated. Similar tree topologies were obtained by using full-length protein sequences or the Zn-finger domain alone.

**Single and double whole-mount in situ hybridization**

Whole-mount in situ hybridization using single, FITC- or DIG-labeled RNA probes was carried out as described previously by Greens et al. (Grens et al., 1995), except that NBT/BCIP (Roche) was used as a substrate for staining. The double-labeling in situ hybridization procedure was carried out according to the protocol described by Hansen et al. (Hansen et al., 2000). The *Hyzic* probe was labeled with FITC, the *Nowa* probe was labeled either with DIG or FITC, and the *Cnash* probe was labeled with DIG. The first round of staining was always carried out with Fast Red substrate, irrespective of whether the probe was FITC or DIG labeled. Accordingly, the detection of *Cnash* was always carried out with NBT/BCIP as substrate, as *Cnash* expression is weaker than that of *Hyzic* or *Nowa*. All photographs were taken using either a digital Spot Camera (Diagnostic Instruments) or a Hamamatsu Digital Camera (Hamamatsu Photonics), controlled by the MetaMorph and Hipic Software, respectively. Some pictures were false-colored in Photoshop.

**BrdU in situ hybridization double staining on whole-mounts**

Prior to in situ hybridization, polyps were treated with BrdU (5 mM in *Hydra* medium, HM) for 60 minutes, followed by two washing steps in HM and fixation with 4% paraformaldehyde (PFA) overnight. After staining with NBT/BCIP for the labelled RNA probe and destaining for 30 minutes in 100% ethanol, animals were washed in PBS four times for 10 minutes. Then, animals were incubated for 30
minutes in 2 N HCl and washed 4 times in PBS. An anti-BrdU monoclonal antibody (Roche) was added in a dilution of 1:15 in PBS/1% BSA/0.1% Tween 20, overnight at 4°C. Excess antibody was removed by four 15-minute washes in PBS before incubation for 3 hours at room temperature with anti-mouse antibody coupled to ALEXA-488 fluorochrome (Molecular Probes), used in a dilution of 1:400 in PBS/1% BSA. Finally, animals were washed three times in PBS for 10 minutes, mounted in PBS/glycerol (1:9) and examined under the Zeiss Axioskope 2 plus.

Results
Isolation of Hyzic, a Hydra Zic/odd-paired homolog
Using a degenerate oligonucleotide against the conserved H/C link of Krüppel-like Zn-fingers (Schuh et al., 1986), we screened a Hydra cDNA library and isolated a clone that showed a high similarity to the Drosophila pair-rule gene odd-paired and the vertebrate Zic gene family (Benedyk et al., 1994; Aruga et al., 1996a). The isolated full-length cDNA contained 1735 bp, with an open reading frame coding for 415 amino acids (Fig. 1A). The zinc finger domain is located in the C-terminal half of the predicted protein and consists of five C2H2-type zinc fingers (Fig. 1B). As in all other organisms, the first Zn-finger of Hyzic lacks one of the Cysteine residues, indicating that it may not contribute to DNA recognition, because the first Zn-finger of Gli proteins also is unable to bind to DNA (Pavletich and Pabo, 1993). However, the Hyzic Zn-finger contains a Histidine instead of a Cysteine residue in the expected position, and therefore this motif might be able to complex a Zn2+ ion through the CH3-motif and bind to DNA.

Sequence comparison and phylogenetic analysis of the whole protein, or the Zn-finger region, revealed that the isolated Hydra clone clearly clusters with the Odd-paired/Zic protein subfamily of Krüppel-like Zn-finger transcription factors (Fig. 1C). The Hydra Zn-finger domain is up to 80% identical at the amino acid level to vertebrate Zic proteins. We therefore termed the gene Hyzic. The HyZic protein shows virtually no homology outside the Zn-finger domain to the

Fig. 1. Sequence analysis of Hyzic. (A) Schematic of the predicted HyZic protein showing the zinc finger domain located in the C-terminal part. Numbers represent amino acid positions. (B) Alignment of the zinc finger domain of HyZic (Hydra-Zic) with various Zic proteins and Gli proteins from other phyla. The Cysteine and Histidine residues of the C2H2 motifs are boxed in red. Identical amino acids or conservative changes in all sequences are boxed in black; sequences with >80% similarity are boxed in gray. Note, that HyZic contains a number of diagnostic residues of the Zic protein subfamily. (C) Phylogenetic tree of Zic, Gli and Macho zinc finger motifs using the maximum likelihood program TREE-PUZZLE (Schmidt et al., 2002). The analysis is based on CLUSTALW alignments. Numbers are percent statistical support. HyZic clusters showed >90% statistical support to the Zic protein family, when the more derived Ciona sequences were removed from the analysis (data not shown).
other OPA/Zic proteins. Some vertebrate Zic proteins contain an additional conserved motif of 12-14 amino acids at the N terminus, which is absent in the *Hydra* Zic protein.

**Hyzic is expressed in the nematocyte differentiation pathway**

In *Hydra*, the pluripotent interstitial cell lineage can give rise to nerve cells, nematocytes, gland cells and gametes (reviewed by Bode and David, 1978). Quantitatively, the two major derivatives are the two neuronal cell types, nerve cells and nematocytes. Nerve cell precursor cells are committed stochastically in the body column and migrate to the site of their final differentiation, predominantly in the head and the foot (Heimfeld and Bode, 1984a; Heimfeld and Bode, 1984b; Technau and Holstein, 1996; Hager and David, 1997). By comparison, nematocyte differentiation also begins stochastically in the body column, but involves a varying number of synchronous cell divisions, leading to nests of 4, 8, 16 and 32 nematoblasts. At any of these stages, nematoblast nests can undergo a final mitosis and start differentiating the nematocyte capsule (David and Challoner, 1974; David and Gierer, 1974). Hence, nematocytes differentiate as nests of 8, 16, 32 or 64 cells. When the formation of the capsule is completed, the nests separate into single nematocytes. Most of them subsequently migrate into the tentacles and are incorporated in the battery cells.

From whole-mount in situ hybridization (WISH) analysis it was determined that *Hyzic* is expressed in the body column in cells of the ectodermal layer. No *Hyzic* cells are found in the hypostome, the tentacles or the lower peduncle and foot region (Fig. 2A). This expression pattern was reminiscent of that of the *achaete-scute* homolog *Cnash* (Fig. 2B) (Grens et al., 1995), and of *Nowa*, which encodes for a structural protein of the outer capsule wall (Fig. 2C) (Engel et al., 2002). This indicated that *Hyzic* is also expressed in the nematocyte pathway, or in another pathway of the interstitial cell lineage.

A more detailed analysis showed that *Hyzic* is expressed in single, and pairs of, interstitial cells, and in nematoblast nests of 4-16 cells (Fig. 3A-E). No staining could be observed in mature nematocytes. Quantitative analysis revealed that the majority of *Hyzic* nematoblast nests consist of 4 or 8 cells, whereas the majority of *Cnash* and *Nowa* expressing nests consist of 8-16 cells (Fig. 3F). Hence, on average, *Hyzic* expressing nests are smaller by one cell division than *Cnash* and *Nowa* expressing nests.

Besides nerve cells and nematocytes, interstitial stem cells can also give rise to gametes in *Hydra*. Gamete precursors are thought to differentiate from a pool of sex-cell restricted stem cells expressing the germline-specific genes *vasa* and *nanos* (Littlefield et al., 1985; Littlefield, 1991; Nishimiya-Fujisawa and Sugiyama, 1993; Nishimiya-Fujisawa and Sugiyama, 1995; Mochizuki et al., 2000; Mochizuki et al., 2001). We therefore analysed the expression pattern in polyps undergoing gametogenesis. We found that *Hyzic* expressing cells are excluded from areas undergoing oogenesis (Fig. 4) from the earliest stages, when no morphological sign of gamete differentiation is visible (Fig. 4A,B), onwards. Even at late oogenesis stages (stage 5) (Miller et al., 2000), and after formation of the oocyte (stage 7), the egg field does not contain *Hyzic* expressing cells, indicating that *Hyzic* is excluded from the oogenesis pathway. Similar results were obtained during spermatogenesis (data not shown). This further suggests that
Hyzic expressing cells do not rapidly repopulate the former region of egg formation in the body column (Fig. 4C,D). Likewise, Cnash and Nowa expressing cells are also excluded from the area of gametogenesis (data not shown). These results suggest that Hyzic expressing cells do not contribute to gametogenesis, and that nematocyte differentiation and gametogenesis are governed by separate pathways.

**Hyzic expression does not overlap with Cnash and Nowa expression**

The nearly identical expression patterns of both transcription factor genes Hyzic and Cnash, and that of the early differentiation marker Nowa, in the same cell lineage, prompted us to carry out double in situ hybridization, in all possible gene combinations, to determine whether or not the expression patterns of the three genes overlap. However, we were unable to detect an overlapping expression of Hyzic with Cnash and Nowa, respectively (Fig. 5A,B). By contrast, Cnash and Nowa show substantial overlap of expression (Fig. 5C). Quantitative analysis revealed that 60% of all Cnash and Nowa expressing cell clusters express both genes, although 20% clearly express Cnash alone, and 10-15% either express Nowa alone or in combination with a weak Cnash expression (Fig. 5D).

**Hyzic is expressed in proliferating nematoblasts**

There are two possible explanations for the non-overlapping expression of Hyzic with Nowa and Cnash: either these genes mark two distinct, parallel subpopulations of the nematocyte differentiation pathway, or they could represent two temporally separated subpopulations of the same lineage. To test this, we performed pulse-labeling with the thymidine analog BrdU and determined, by double staining, the fraction of Hyzic, Cnash or Nowa expressing cells, that are BrdU positive. We found that a large fraction of the Hyzic cell clusters have incorporated BrdU, and hence are in S-phase of the cell cycle (Fig. 6A,D). By contrast, neither Cnash nor Nowa expressing cells are positive for BrdU (Fig. 6B-D). This clearly shows, that Hyzic is expressed in proliferating nematoblasts, while Cnash and Nowa are expressed in non-proliferating cell clusters, presumably after the last mitosis, which marks the begin of capsule differentiation. This fits the fact that Nowa encodes the major protein of the outer wall of the nematocyte capsule and is known to be a nematocyst differentiation gene (Engel et al., 2002). The partially overlapping expression of Cnash and Nowa suggests that these genes are expressed in slightly different time windows. In a continuous BrdU-labeling experiment, we found that the first BrdU-labeled Cnash-expressing cells arise after 24
hours, and that almost 60% Cnash-expressing cells are BrdU-labeled after 48 hours (Fig. 6E). By comparison, no BrdU-labeled Nowa-expressing cells could be detected at 24 hours, and only 20% were labeled at 48 hours (Fig. 6E). Thus, during nematocyte differentiation, Hyzic is expressed in early proliferating nematoblast nests, followed by Cnash and later Nowa, after a final mitosis. Interestingly, the pulse-labeling experiments also revealed proliferating cell clusters that do not express Hyzic (see Discussion). These clusters are defined as nests of 2^k cells with BrdU-positive nuclei, that are in close proximity. About 70% of the BrdU-labeled 4- and 8-cell clusters express Hyzic. Thus, the ~30% of 4- and 8-cell clusters that do not express Hyzic could represent either precursors of another stem cell differentiation pathway or, alternatively, reflect a variable temporal onset of Hyzic transcription in these stages (see Discussion).

To further confirm the successive onsets of expression of Hyzic, Cnash and Nowa, we analysed their expression in animals depleted of interstitial stem cells. In Hydra, stem cells can be eliminated in wild-type strains by treatment with the S-phase blocking agent hydroxyurea (HU) for up to three days; alternatively, stem cells can be eliminated in the temperature-sensitive mutant sf-1 by shifting the temperature to 26°C for up to three days (Sugiyama and Fujisawa, 1978; Terada et al., 1988). We performed both types of experiments with very similar results. Upon HU treatment, the number of Hyzic expressing cells was drastically reduced after 12 hours, and virtually no Hyzic expressing cells could be detected after 18 hours (Fig. 7A-C,G). By contrast, the number of Cnash and Nowa expressing cells was virtually unaffected within the first 48 hours. Only after a HU treatment of longer than 48 hours (up to 72 hours) could a significant reduction in number of nematoblast nests positive for Cnash and Nowa be observed (Fig. 7D-F,G). This clearly confirms that Hyzic is expressed very early in the nematocyte pathway in proliferating cells, starting from the single cell, while Cnash and Nowa are expressed in a closely following manner (Cnash first, Nowa second) after last S-phase of the differentiating nematocytes.

**Discussion**

The first Krüppel-like Zn finger Zic gene from the simple metazoan Hydra

We isolated the first zic homolog from Hydra. The Zn-finger domain of the HyZic protein is highly conserved (up to 80% identical to vertebrate Zic proteins), but lacks the conserved N-terminal motif found in some organisms (e.g. Aruga et al., 1996a). The phylogenetic analysis shows that it clearly clusters with the Zic proteins but not with the related Gli proteins. This suggests that Zic genes encode an ancient family of Zn-finger proteins that are highly conserved during animal evolution.

**Hyzic is involved in nematocyte differentiation in Hydra**

As nematocytes are sensory cells, they are regarded as a specialized neuronal cell type that derives from the same pluripotent stem cell population as nerve cells (David and Gierer, 1974; Bode, 1988). Cell cloning and labeling experiments have established that cell clusters of 4 cells or larger usually differentiate into nematocytes (David and Gierer, 1974; David and Challoner, 1974; David and Murphy, 1977; Hager and David, 1997). Our analysis shows that Hyzic is, to date, the earliest marker of nematocyte differentiation, starting at the level of the interstitial stem cells. Hyzic expression is detected in the proliferative stages, and includes single and pairs of interstitial cells (1s+2s), which are part of the interstitial stem cell pool. Interestingly, the S-phase labeling index (40%) is significantly lower than would be expected from previous measurements of the cell cycle parameters of nematoblasts (Campbell and David, 1974). This could indicate either an underestimation of the cell cycle length of nematoblasts, or that Hyzic not only marks this population but also includes other more slowly cycling cells or cells that have exited the cell cycle. Consistent with the early expression, we find no overlapping expression with the differentiation marker Nowa (Engel et al., 2002), which codes for a major wall protein of the nematocyte capsule, H22, a protein that is recognized by the monoclonal antibody H22 (Kurz et al., 1991). Nowa and
H22 expression can be strongly detected after terminal mitosis (Engel et al., 2002). Nowa mRNA is downregulated in mature nematocytes, but the protein can still be detected.

Surprisingly, we also do not find an overlap of expression of Hyzic with the achaete-scute homolog Cnash (Grens et al., 1995), another gene involved in nematocyte differentiation. This gene is expressed in nests of differentiating nematocytes, but was also found to be expressed in interstitial cells, as shown by maceate in situ hybridization (Grens et al., 1995). Hence, Cnash expression is expected to span the time window from proliferating interstitial cells resembling stem cells until differentiating nematocytes with developing capsules (Grens et al., 1995). Although we can not completely rule out the possibility that the difference between the findings in this paper and those of Grens et al. (Grens et al., 1995) is due to different sensitivities in maceate and whole-mount in situ hybridization, our results show, that Cnash expression is restricted to non-proliferating stages of the nematocyte differentiation pathway. Several lines of evidence support this. (1) Following pulse labeling with BrdU, no labeled Cnash cells could be detected, and the first BrdU-positive Cnash cells appeared between 24 and 48 hours of continuous labeling (Fig. 6E). (2) Elimination of the proliferating interstitial cell population in the mutant strain sf-1, or in HU-treated wild-type animals, did not affect the number of Cnash expressing cells during the first 48 hours, whereas it strongly affected the number of proliferating Hyzic cells (Fig. 7G). (3) Cnash shows substantial overlapping expression with Nowa (Fig. 5). (4) The average cluster size of Hyzic expressing cells is smaller by one cell division compared with Cnash and the non-proliferating Nowa cells, suggesting that Hyzic cells have gone through a final mitosis and turned on Cnash expression (Fig. 3F). (5) We recently isolated a JNK gene that is also expressed in the nematocyte differentiation pathway. Expression of this JNK gene overlaps both with Hyzic and with Nowa, thereby bridging Hyzic expression and Cnash/Nowa expression (I. Philipp, B. Hobmayer and T.W.H., unpublished). Hence, we conclude that Hyzic is expressed early, primarily during the proliferative stages, while Cnash is expressed later, after the final mitosis.

Does Hyzic mark an independent sublineage?

Because Hyzic and Cnash expression do not overlap, there is still the possibility that the two genes mark two separate sublineages. In fact, Hydra have four different types of nematocytes: stenoteles, holotrichous isorhizas, atrichous isorhizas and desmonemes (Holstein et al., 1990), and one could imagine, that Hyzic is expressed only in one or few of these nematocyte subtypes. At present, conflicting reports exist about whether commitment to the different subtypes occurs early or late during nematocyte differentiation (Fujisawa and David, 1981; Fujisawa and David, 1982; Shimizu and Bode, 1995). Most Hyzic-expressing cell clusters consist of 4 or 8 cells, and we also detected few single interstitial cells expressing Hyzic. Interestingly, in BrdU pulse-labeling experiments, we also found nests of 4 and 8 cells that did not express Hyzic (Fig. 6D). This could mean either that Hyzic marks only one particular subpopulation of nematoblasts, or that the onset of Hyzic expression is not sharply defined to a
particular stage. We favor the second interpretation for the following reasons. (1) Pulse-labeling experiments reveal that up to 30% of all BrdU-positive cell clusters (4 or 8 cell clusters) do not express Hyzic. Thus, if the Hyzic-negative cells represented a second independent lineage, we would expect that it makes up one third of all developing nematocytes. This is highly unlikely. All nematocyte precursors express Nowa (Engel et al., 2002), which encodes H22, a protein of the outer nematocyst wall. Nowa expression temporally overlaps with that of Cnash, which is consistent with the finding that Cnash is expressed in the great majority of all differentiating nematocytes (Grens et al., 1995). Furthermore, Cnash/Nowa expression and Hyzic expression are bridged by the expression of the JNK gene (see above). Hence there is no evidence for a second major subpopulation besides the Hyzic cells. (2) At any cluster size, only a certain fraction keeps proliferating, while the other fraction undergoes a final mitosis and starts differentiating into nematocytes (see Fig. 8). Thus, if Hyzic is turned on sharply at the transition between the 2-cell and the 4-cell stage, we would expect less Hyzic-expressing 8-cell clusters, and even less 16-cell clusters, and so on. However, we found only a few 1s+2s, many 4-cell clusters, and almost as many 8-cell clusters, expressing Hyzic (Fig. 3F); at the 16-cell cluster size the number drastically decreases. It is an obvious possibility that Hyzic expression remains after the division into the 8-cell cluster, irrespective of whether this cluster remains in the cell cycle or whether it enters the differentiation pathway. This would be consistent with the comparatively low BrdU-labeling index. However, this should lead to an overlapping expression with Nowa, which is turned on in all differentiating nematocytes immediately after the final mitosis (Engel et al., 2002). However, we never observed such an overlap. This can be best explained by a continuous supply of proliferating 4- and 8-cell clusters from the pool of Hyzic-negative cell clusters, compensating for the loss of Hyzic expressing clusters by differentiation. We therefore propose that the onset of Hyzic expression is not sharply defined and can occur between the 2-cell and the 8-cell stage.

In line with this, we recently isolated another Zn-finger gene that is expressed in all interstitial cells (1s+2s) and a few nests of 4 and 8 cells. Expression of this gene does not overlap with that of Hyzic, accounting for the BrdU-positive/Hyzic-negative cell clusters (D.L. and U.T., unpublished). Although these results are consistent with a rather late commitment to the different subtypes of nematocytes (Fujisawa and David, 1981; Fujisawa and David, 1982), we can not completely rule out an early commitment (Shimizu and Bode, 1995).

A possible role for Hyzic in nematocyte differentiation

Although all attempts to knockdown the Hyzic transcript by RNAi failed (data not shown), our data are consistent with the view that Hyzic marks the commitment to the nematocyte differentiation pathway. The variable onset of Hyzic expression suggests that the commitment can occur in a prolonged phase during proliferation of the interstitial cells. Whether the Hyzic-negative cell clusters still have stem-cell like properties, and whether they can still differentiate into neurons, is unclear. It

Fig. 8. The role of Hyzic in neuronal differentiation. (A) Schematic of the stem cell proliferation, nerve and nematocyte differentiation pathway in Hydra [modified from David and Gierer (David and Gierer, 1974)]. Determination of stem cells to neuron or nematocyte differentiation can occur at any proliferating stage from 2 to 8 cells (white circles in the middle). The probability of a determination event is represented by the thickness of arrows (the thicker the arrow the more likely the event). After determination to nematocyte differentiation, nematoblasts undergo a variable number of cell divisions leading to nests of 2, 4, 8, 16, and 32 cells. At any of these stages, nematoblast cells can undergo a final mitosis and enter the differentiation program, which is mainly characterized by the development of the nematocyst (shown by the increasing black color). The red labeled circles represent Hyzic-expressing proliferating cells already committed to the nematocyte pathway. The onset of Hyzic expression is not sharply defined, so that additional proliferating nests could be detected that might also give rise to nerve cell precursors or enter the nematocyte pathway at later stages (see also Fig. 6D). Post-mitotic differentiating nematocytes express Cnash and Nowa, as indicated by the blue circles. (B) Genetic pathway leading to nematocyte differentiation. It is unclear whether Chordin also acts upstream of Hyzic in Hydra. (C) Genetic regulation of neural differentiation in vertebrates, showing that at least some homologous genes are involved in neural differentiation in both Hydra and vertebrates.
is well established that most neurons differentiate in pairs after a final mitosis from individual interstitial cells (Hager and David, 1997; Heimfeld and Bode, 1984b; Holstein and David, 1986). However, some differentiation of 4 and 8 neurons from single migrating interstitial cells has occasionally also been observed (Heimfeld and Bode, 1986; Hager and David, 1997), indicating that they underwent one or two additional rounds of mitosis. Yet, a neuronal differentiation from the cycling Hyzic-negative cell clusters would require a breakdown into single cells, because 4- and 8-cell clusters were never observed as intermediate stages in nerve cell differentiation. Furthermore, committed precursor cells of the interstitial cell lineage might exist that have differing capacities for proliferation, allowing for the regulation of amplification rounds (Heimfeld and Bode, 1986). Thus, the likelihood to differentiate into neurons decreases with the cluster size, whereas the probability to differentiate into nematocytes increases with the cluster size (see Fig. 8A). If this was the case, we would expect to find genes that are expressed in the non-committed interstitial cells and in few nests of 4 cells and 8 cells (see above).

What could be the role of Hyzic in nematocyte differentiation? Because it starts being expressed in interstitial cells, it might commit stem cells to the nematocyte pathway. Interestingly, in mice, Zic1 is expressed in proliferating neuronal precursors, but inhibits terminal neuronal differentiation (Aruga et al., 2002a). Consistently, Zic1 positively regulates levels of Cyclin D, and inhibits the mitosis inhibitors p27 and p16 (Aruga et al., 2002b). Hence, overexpression or ectopic expression of Zic1 leads to an expansion of dorsal neurons due to extended rounds of proliferation (reviewed by Bally-Cuif and Hammerschmidt, 2003). This is very similar to the situation in Hydra, where Hyzic might keep cells in the cell cycle that are committed to the nematocyte pathway, thereby ensuring that large numbers of nematocytes can differentiate. Hydra has a turnover of several thousand nematocytes per day; hence, proliferation of nematocyte precursors is primordial in order to amplify the number of nematocytes.

Evolutionary considerations

Neural development is largely conserved from insects to vertebrates on the molecular level (reviewed by Sasai, 2001). The Bmp antagonist Sog/Chordin defines the neurogenic region high up in the molecular hierarchy. As a result, proneural genes of the bHLH family (achaete-scute, atonal/neurogenin) are activated, which further activate the neurogenic genes Notch and Delta. In vertebrates, the zinc finger gene Zic, is activated downstream of Chordin and upstream of neurogenin (Mizuseki et al., 1998; Kuo et al., 1998); hence, it acts very early in the hierarchy of neuronal differentiation, in particular in the differentiation of the neural crest. Yet, the Drosophila homolog, odd-paired (opa) is not involved in neuronal differentiation at all. It rather has a pair-rule function in segmentation and in midgut constriction (Ingham et al., 1988; Benedyk et al., 1994; Cimbora and Sakonju, 1995). Thus, it is unclear, whether the function of vertebrate Zic genes in neural differentiation is ancestral or newly adopted. Interestingly, we also recently identified a Hydra Sog/Chordin ortholog (F. Rentzsch and T.W.H., unpublished), and the situation in a member of an outgroup to the Bilateria, such as the cnidarian Hydra, is therefore of great significance for this question.

In vertebrates, Zic is downstream of Chordin, and epistatic to the proneural bHLH genes achaete-scute and neurogenin (Mizuseki et al., 1998). In zebrafish, as well as in the lower chordates Amphioxus and Ciona, homologs of the Zic family are also expressed in specific domains of the dorsal mesoderm, in particular in parts of the somites (Gostling and Shimeld, 2003). On the basis of these results, the mesodermal expression has been proposed to reflect the ancestral situation for all chordates (Gostling and Shimeld, 2003). Although the role of Zic in this tissue is not understood, it could reflect the influence of Chordin in dorsal compartments of the embryo. In Hydra, because Hyzic is expressed in the proliferating stages of a neuronal cell type, the nematocytes, we propose that the ancestral function of Zic was in neuronal differentiation. Furthermore, like in vertebrates, Hyzic acts upstream of the proneural bHLH gene Cnash, an achaete-scute homolog (Fig. 8B,C) (Grens et al., 1995). This suggests that components of the gene cascades regulating the differentiation of neuronal cell types are conserved from Hydra to vertebrates.

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