Development of the zebrafish nervous system: Mechanisms of cellfate specification and axonal pathfinding in the central nervous system and periphery

Sudipto Roy

The triumph of organic evolution on earth is manifested by the complex structural organization of the human brain. The understanding of its precise neuronal connectivity, that is the basis of the intricate and manifold functions it performs, is an endeavour of the highest intellectual order. Interconnections between neurons in the brain and in the spinal cord, and between neurons and their nonneural targets elsewhere in the periphery, occur through a stereotyped pattern of developmental mechanisms that unfold during morphogenesis of the embryo, and is an overt manifestation of an elaborate and complex programme of gene activity.

Due to the relative inaccessibility of the early mouse embryo to developmental manipulation, our knowledge of the mechanisms that pattern the mammalian brain is poor and obscure. On the contrary, progress in our understanding of the mechanisms that generate precise patterns in the nervous system of invertebrates has been more commendable. What developmental neurobiologists interested in vertebrate neurogenesis lacked until recently was an ideal single species with which cellular, molecular and genetic analysis could be carried out on individually identified neurons and their associated cells as they develop and generate a complex nervous system.

Fortunately, the situation is not so hopelessly frustrating now. The introduction of the zebrafish by Streisinger as a model for the analysis of vertebrate development has turned out to be extremely fortuitous. The zebrafish embryo is quickly being established as an excellent vertebrate embryo for studying the mechanisms of development with a combination of genetic, molecular and cellular techniques. One problem that is being fruitfully addressed is how growth cones of developing axons navigate to find their targets in the central nervous system (CNS) and periphery. The inherent clarity and simplicity of the early embryo, the ability to identify, ablate or transplant single cells, combined with the availability of mutations, have attracted the attention of a growing number of investigators, resulting in a steady stream of high quality literature over the last few years. The picture of vertebrate neurogenesis is thus rapidly metamorphosing, and it is hoped that lessons learnt from studies in the fish will hold good for more complex systems like mammals and humans.

Origin of the nervous system in the zebrafish, *Brachydanio rerio*

Spawning zebrafish females lay as many as two hundred eggs per week, that are fertilized externally. The zygote then begins its programme of cell divisions which are very regular initially, giving rise to a stereotyped pattern of cells called blastomeres (Figure 1). This precision and synchrony in the early cleavage divisions of the zebrafish embryo, apparently suggested that invariant patterns of cell divisions or cell lineage could be instrumental in the allocation of cells to distinct fates; a mechanism so crucially important in the nematode, *Caenorhabditis elegans*. Labelling single blastomeres with vital dyes, and following them through gastrulation, has shown that cell lineage in the zebrafish embryo is indeterminate, and the fate that a cell ultimately takes up depends upon its position in the gastrula[1].

The nervous tissue of the zebrafish is nonclonal in origin. Position and cell–cell interactions appear to be the major players in the patterning of the nervous system in this organism. Genetic mosaic analysis on the pigmented retinal epithelium has shown that this tissue arises by the inter mixing of the progeny of many different ancestral cells. During gastrulation, cells that will give rise to the nervous system are segregated from cells that give rise to other tissues, by moving to separate positions within the developing embryo. Moreover, for cells that are determined to contribute to the nervous system, there is a distinct correspondence between their location at the commencement of gastrulation, and the region of the nervous system their progeny will subsequently colonize.

Specification of neuronal fate

In the nervous system of the developing zebrafish embryo specification of neuronal fate occurs in two temporal waves. Those that are specified in the first were are called 'primary' neurons. These cells are distinct from the later developing 'secondary' neurons in their early allocation, large size and small numbers. These 'primary' or 'pioneer' neurons generate a simple scaffold of axonal projections and neuronal circuitry that form a template for elaboration of the adult nervous system. The simple circuitry established by these 'primary' neurons—'primary' sensory, interneuron and motoneuron—is responsible for the early swimming responses of the fry. Not only are the 'primary' neurons developmentally distinct, but they are separable from the secondary neurons by genetic criteria also. For instance, in embryos homozygous for the neural degeneration mutation, *ned-1*, the 'primary' neurons are viable, while the 'secondary' neurons are selectively eliminated after they have developed.

The most satisfying aspect of studying the development of the early zebrafish embryo, is its optical clarity. With a good dissection microscope, it is possible to observe living embryos and identify single cells progressing through their developmental programme. Identification of individual neurons during embryogenesis has been reported for invertebrates such as the leech and grasshopper. In the zebrafish, the large size, small number and characteristic axonal trajectories of the primary neurons have facilitated their identification as unique

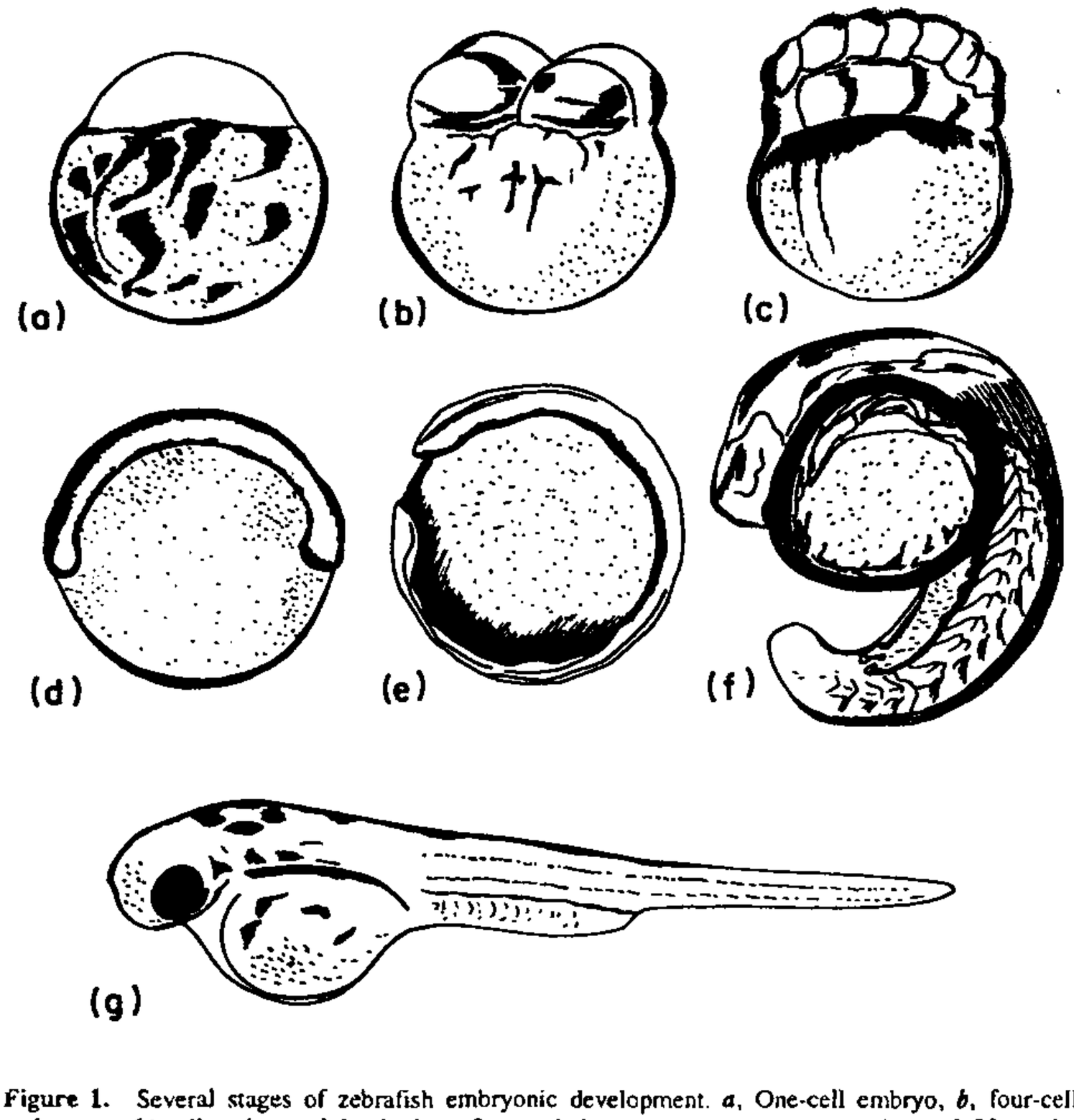

Figure 1. Several stages of zebrafish embryonic development. *a*, One-cell embryo, *b*, four-cell embryo; *c*, 64-cell embryo; *d*, beginning of gastrulation; *e*, one-somite stage embryo; *f*, 20-somite stage embryo; *g*, two-day old hatched embryo.

individuals. It is therefore possible to follow the development of an identified single neuron from birth, through the outgrowth of processes and formation of synaptic connections.

Metamerism in the nervous system

Vertebrates are segmented animals—though this is often not apparent in adulthood, all vertebrate embryos develop metameric structures called somites that give rise to the segmented axial musculature and vertebrae. These somatic muscles or myotomes are innervated by motoneuron pools along the spinal cord, developing in register with the somites. In the zebrafish, the somata or cell bodies of the primary motoneurons can be visualized with an anti-neuronal antibody like Zn-1 mAb. Some other primary neurons, like the VeLD (ventral longitudinal interneuron) primary interneurons, are also segmentally arranged. However, staining with mAb Zn-12 reveals that some primary neurons like the Rohon Beard primary sensory neurons, that lie in the dorsal region of the spinal cord are not metameric in nature.

As in other vertebrates, the hind brain or rhombencephalon in the zebrafish is subdivided into seven segments called rhombomeres. Antibodies are available, that enable the visualization of a complex reiterated pattern of neurons and glial cells, corresponding to the pattern of rhombomeres along the anteroposterior axis. The primary reticulospinal neurons (projections from the reticular formation to the spinal cord) recognized by mAbs Zn-1 and Zn-12 are located ventrally at the centre of each rhombomere, while the Zn-5 mAb identifies the dorsally located commissural neurons. These two groups of neurons are separated by sheets of glial cells, recognized by different antibodies. Like in the spinal cord, primary neurons in the ventral hind brain are also segmentally arranged. The Zn-1 mAb recognizes individual neurons or small groups of them in seven discrete hind brain regions. Interestingly, these neurons are precisely spaced from each other—the distance between two labelled cells spanning the length of a spinal segment. Some of the identified reticulospinal neurons show characteristic segment-specific differences in morphology and development, suggesting that the different hind brain segments are not equivalent. In invertebrates, diversity among segmentally repeated neuromeres is generated by a later process of rearrangement of axonal trajectories of cells with a common developmental history. It appears from the study on the zebrafish nervous system that regionalization or compartmentalization in the nervous system of vertebrates arises right from the start, with axons in different segments projecting along different pathways.

Axonal pathfinding

The specificity of synaptic connections unfolds in three major steps: pathway selection, target selection and address selection[2]. First, the growing tips of neurons, called growth cones, traverse long distances to find their target region. *En route*, they encounter a series of crossroads called choice points. Surprisingly, the growing axons are able to correctly navigate these pathways in a remarkably unerring way. On reaching their correct neighbourhood, they seek out, contact and innervate their correct target. In vertebrates, the initial pattern of connections made during early embryonic development, is further refined as axon terminals retract and expand to select a specific subset of cells from within the target.

Growth cone navigation has been studied for several types of primary neurons in the embryonic zebrafish, both in the CNS and periphery. In principle, neurons from developmentally staged animals were labelled with antibody or dyefills and the time course of axon outgrowth reconstructed from studies on several individuals differing in their developmental sequence. These studies have revealed that each type of neuron extends a growth cone along a characteristic, cell specific pathway. They are able to reach their respective targets without committing obvious errors *en route*. Since axons extend from cell bodies situated remote from the target tissue, it is possible that

the intervening tissue provides cues for precise path selection. In the zebrafish, the growth cones of the sensory neurons of the lateral line sense organ are an exception: they remain in continuous contact with their targets and comigrate with them to their final destination along the body axis.

Mechanisms of growth cone guidance in the developing zebrafish nervous system

Since neurons send out axons that reach out for their targets over a heterogenous mass of intervening tissue, and furthermore since they do so in a relatively invariant way, it is certain that the exploring growth cones are able to sense some guidance cues advertised by the intervening tissue and extracellular matrix as well as attractants and repellants emanating from target and nontarget tissues respectively. In the zebrafish, a number of potential mechanisms that may play a role in axons guidance have been investigated. These include (i) interactions between primary neurons themselves, (ii) intervening tissue, (iii) extracellular matrix, (iv) electrical activity of the target, and (v) absence of the target.

Interactions between primary motoneurons

It is possible to specifically ablate single identified motoneurons by focusing a laser microbeam onto the somata of these cells. The remaining neurons can then be labelled with intracellular dye injections and their axonal trajectories observed. When such experiments were done on primary motoneurons, it was found that these cells do not interact with each other while reaching out to their targets. In the absence of specific primary motoneurons the remaining ones were able to innervate their targets comfortably. Time lapse cinematographic observations on live embryos, with neurons labelled with rhodamine dextran or the lipid soluble dye 1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Di-I) showed that in every segment of the trunk, the primary motoneurons extend their growth cones in an invariant sequence, and these extend direct to the appropriate muscle field without making mistakes. Another interesting observation in zebrafish is that motoneurons do not compete with each other for target innervation. This is borne out by the fact that, when one of the neurons that grow closely together and innervate neighbouring muscles is ablated, the remaining neuron does not encroach into the target field of the other.

Intervening tissue

The floor plate of the nerve cord of vertebrates has been implicated as a possible site where growth cones of CNS axons make specific pathway choices[3]. In the zebrafish spinal cord, the growth cones of two classes of inter neurons CoPA (primary commissural inter neuron) and VeLD exhibit cell-specific turns near the ventral midline of the cord in the floor plate region (Figure 2). Both the CoPA and VeLD neurons first project growth cones towards the ventral midline. On reaching this site, the VeLD growth cone turns posteriorly and ipsilaterally, while the CoPA growth cone crosses over to the other side and projects anteriorly and contralaterally. These specific turns which axons of certain neurons make at the floor plate are probably due to chemotropic signals from a single row of ventral midline cells. In the *cyclops* (*cyc-1*) mutation the midline cells are missing and both VeLD and CoPA growth cones follow aberrant pathways on reaching the floorplate.

This effect of the *cyc-1* mutation can be reproduced by laser ablating the floor plate midline cells. A homologous mutation in the fruit fly *Drosophila*, orthodenticle, eliminate certain midline cells resulting in aberrations in the axons that pioneer the posterior commissure[4]. However, the midline cells appear to be superfluous in the spinal cord since both CoPA and VeLD neurons are able to project their axons normally in their absence. This suggests that redundant cues exist and guidance from the basal lamina or the underlying notochord or both may be crucial. Neuroepthelial cells lining the superficial surface of the central nervous system may also be involved in providing guidance cues to growing axons. This has been found in the epiphysial neurons in the zebrafish brain (also for Rohon–Beard neurons in the Japanese medaka fish spinal cord). It is possible that spatially restricted subsets of the endfeet of neuroepthelial cells may express molecules which promote or direct axonal out-growth differentially. Occurrence of specific 'guide post' cells as in several insects have not been documented yet; nevertheless we should keep our minds open for their role in axon guidance in vertebrates too[5].

Extracellular matrix

The tip of a navigating growth cone sends out long cytoplasmic extensions called filopodia. These palpate the surrounding environment and act as feelers to sense attractive and repellant cues. Growth filopodia inspect the extracellular matrix (ECM) by interacting with ECM molecules. Hence ECM molcules could play a role in growth cone guidance. It has been found that zebrafish primary motoneuronal growth cones contact laminin-rich areas of the embryo and avoid

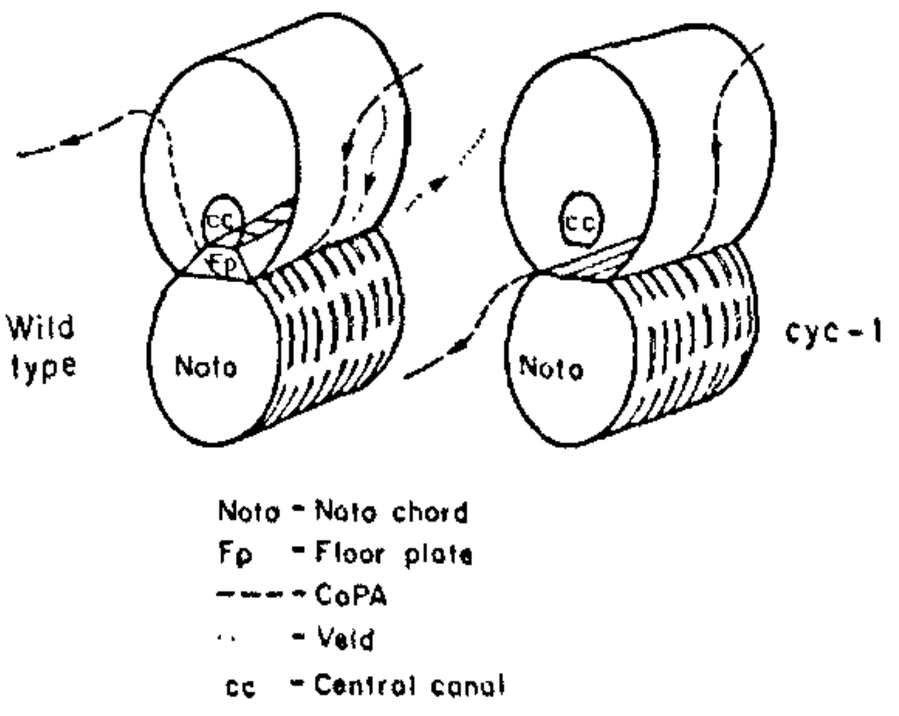

Figure 2. cyc-1 deletes the floorplates (Fp), a single row of cells below the central canal (cc). This results in misprojections of the CoPA and VeLD neurons (only aberrant trajectory of CoPA axon is shown).

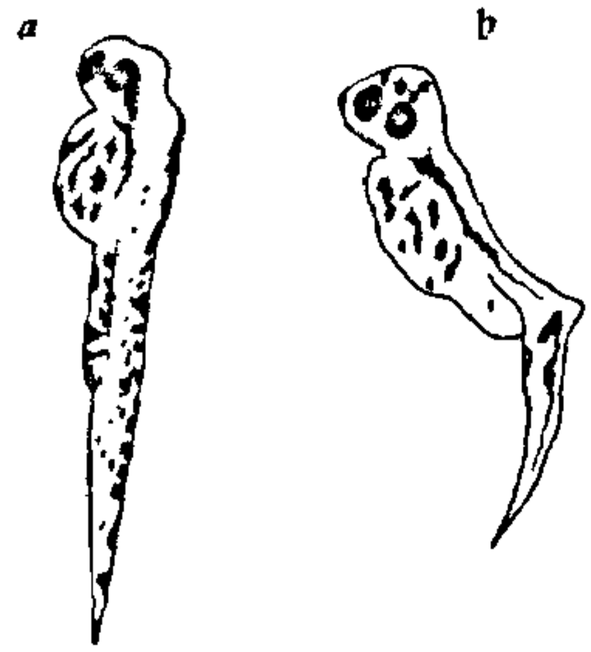

Figure 3. Phenotype of the spadetail (*spt-1*) mutation. *a*, Wild type embryo. *b*, Spadetail mutant embryo.

fibronectin-rich areas such as segment borders. When fibronectin is injected into ectopic locations along the laminin-rich region, growing axons avoid these regions, as though the presence of the fibronectin prevents them from carrying out normal pathfinding. The normal occurrence of fibronectin along the segmental borders could be to prevent motoneurons from adjacent segments invading each others territory. Neuronal receptors for ECM molecules are believed to be one of the three major groups of cell-surface glycoproteins: integrins, Ca^{2+} independent cell adhesion molecules (CAMS) of the immunoglobulin superfamily, and Ca^{2+}-dependent CAMS or cadherins[6, 7]. Importance of cell surface galactosyl transferase has been implicated in neurite outgrowth at the growth cone in mouse and chick neurons. Similar mechanisms may be operative in the zebrafish. However, the signal transduction mechanisms that ensue upon receptor–ligand interaction are unknown. Zebrafish axonal growth cones may be a useful system to investigate this problem.

Importance of target activity

One of the mechanisms that orchestrates the formation of connections between motor neurons and their targets is dependent on the activity of neurons and their targets. Westerfield and his associates have investigated the role of activity in pathfinding and synapse formation by zebrafish primary motoneurons by blocking muscle activity pharmacologically and genetically. Embryos were allowed to develop under conditions that blocked Na^+ channels or Ach receptors; it was found that muscle innervation in these embryos was perfectly normal. In zebrafish, the *nic-1* mutation results in removal of nicotinic Ach receptors. Embryos homozygous for the *nic-1* mutation do not respond to the administration of Ach or its agonist nicotine, nor are their muscles appreciably labelled with α-bungarotoxin. In *fub-1* mutants, the ability of muscles to contract is blocked because the muscles are defective mechanically[8]. Despite these defects, the mutant embryos have proper innervation. The ultrastructure of neuromuscular junctions in such embryos appears normal. These findings suggest that neuro-transmitter-evoked muscle activity is not pertinent in pathfinding and synapse formation. Studies in amphibians and birds have shown that motoneurons often make exuberant branches in target regions and later eliminate redundant projections. This results in mononeural innervation of muscle fibres. However, muscle fibres of the zebrafish like other fishes and unlike other vertebrates are polyneurally innervated. Though some degree of synapse retraction does occur, competitive interactions between different motoneurons (as in other vertebrates) do not seem to be operative[9].

Absence of target

Neuron–target interaction is crucial for target recognition and innervation. Embryos afflicted with the *spadetail* mutation *spt-1*, have aberrant development of primary motoneurons (Figure 3), some of these neurons extend growth cones out of the spinal cord but the axons that ultimately form are morphologically aberrant. A concomitant observation is that *spt-1* mutation also have severely anomalous segmental muscle morphology. This mutation appears to act on prospective trunk muscle cells, preventing their proper migration during gastrulation. It has been observed that muscle pioneers appear to be absent in *spt-1* mutants[8]. These cells differentiate earlier than all other muscle cells at the location where the horizontal septum forms in each myotome, and just where the growth cones of the primary motoneurons normally pause during their outgrowth. Interestingly, the location of the apparent stalling of motor axons in *spt-1* corresponds to where the muscle pioneers should have been. Muscle pioneers have previously been implicated as players in motoneuronal patterning. Recently however, another mutation, *ntl-1*, has been characterized, in which muscle pioneers are also missing, but in which, unlike *spt-1*, motor axons do not halt at the site of the horizontal myoseptune. This suggests other factors besides muscle pioneers are responsible for furnishing guidance cues.

In order to dissect the possibility of the *spt-1* mutation affecting both the nerves and the muscle or any of the two, individual primary motoneurons were transplanted between mutant and wild type embryos. These experiments showed that the mutation acts autonomously in the mesoderm and by altering properties of the target environment, it brings about aberrations in axonal trajectories.

Genetics of neural fate specification

Genetic analysis of neuronal development and cell fate specification in the nervous system has been well investigated in *Drosophila*. It has been shown that two classes of genes—the proneural (*Achaete-scute complex*) and the neurogenic genes (*Notch family*) are involved through complex interactions in specifying cell fate in the fly nervous system. Genetic analysis of vertebrate neuronal development is still in its infancy. Nevertheless, homologues of *Drosophila* neural genes have been identified in vertebrates and it is assumed that they may be involved in similar functions. Studies in zebrafish have revealed that several homeobox-containing genes are expressed in the nervous system. For instance, when an antibody that recognizes the homeodomain-containing region of the *Drosophila* segment polarity gene *engrailed* was used to study expression in zebrafish embryo, it was found to stain cells surrounding the mesencephalon/rhombencephalon boundary. Moreover, expression was detected in developing somites, localized to 3–4 indentified muscle cells. Interestingly, during axonal arborization, the growth cones of primary motor neurons pause for several hours after contacting these cells, implicating that they could be involved in growth cone guidance. Another homeobox gene, *XIIIbox1* has been found to show graded expression pattern in Rohon–Beard primary sensory neurons along the antero-posterior axis in specific regions of the body. Outside these restricted domains, the cells do not

show expression of this gene. This is a seminal observation, since it suggests that cells can differentially express regulatory genes in a position-dependent manner and that positional information may regulate the expression patterns of some homeobox genes.

Epilogue

The zebrafish has a glorious future ahead. With the isolation of newer mutations and development of molecular techniques for cloning genes rapidly, a lot more will be learnt about the riddles of vertebrate development; the development of the nervous system in particular. Several laboratories have been successful in generating stable lines of transgenic fish. This raises the possibility that we may soon have 'insertional mutagenesis' and enhancer detection methods refined in zebrafish. A simple sequence repeat map of the entire zebrafish genome may soon become available[10]. Simple sequence repeats have been shown to be abundant and quite polymorphic between different zebrafish strains. Such a molecular map will be a boon in mapping mutations and may provide starting points for chromosomal walks and jumps. Unfortunately however, the zebrafish has 25 chromosomes and 10^9 bp of DNA; demanding several years of hard work before a map may become available. Be it as it may, it is time we welcome this novel developmental paradigm to our own laboratories, considering the exciting avenues it promises.

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Sudipto Roy is in the National Centre for Biological Sciences, TIFR Centre, Indian Institute of Science Campus, P. O. Box 1234, Bangalore 560 012, India.

COMMENTARY

Need for genetic and molecular biological research in Indian fish

T. J. Pandian, S. Mathavan and L. A. Marian

Many countries have taken up a conscious policy decision to participate in genome analysis programmes, i.e. the molecular mapping and sequencing of the genetic material in human and other organisms. For instance, China has embarked on a 15-year project on the sequencing of the entire rice genome, under the direction of G. F. Hong of the Shanghai Institute of Biochemistry. The anticipated benefits of this project are expected to be much greater than their present investment. Realizing the importance of gene therapy and the possible accruing economic benefits, the Americans and French have gone ahead with human genome project; their findings are to be patented, and may not be available to a country like India. Hence, there have been many arguments for and against launching an Indian human genome project with earmarked funds (e.g. J. Gowrishankar, *Curr. Sci.*, 1993, **63**, 705; P. M. Bhargava and Lalji Singh, *Curr. Sci.*, 1993, **65**, 663). A massive human genome programme is not only cost-intensive but involves many other issues, including ethical and social problems.

Much of our understanding of basic human biology comes from research work on organisms, which do not involve the practical, ethical and social problems of experimenting on human being. Such surrogate organisms used in research are few in number and the most widely used ones are the bacterium *Escherichia coli*, yeasts, nematode worms, fruitflies, zebrafish, clawed toads and house mice (P. Little, *Nature*, 1993, **366**, 204). However, most of these surrogate organisms do not possess all the specialized functions of mammals, and the genome of mouse is as large as human with much of nongenic DNA, such as repeats, pseudogenes and other noncoding sequences, which make the technology of genome analysis difficult, and laborious as well as cost-intensive. Therefore, Sydney Brenner and his associates (including an Indian young scientist, at the Institute of Molecular and Cell Biology, National University, Singapore) made search for a better surrogate vertebrate, which has a 'small and perfectly formed genome, with small introns and high relative content of exon information'; hence, sequencing a part or even all this component genome would be an effective way of discovering vertebrate genes and provide relevant access to the human genome'.

Brenner is already known for introducing the surrogate nematode worm *Caenarhabdsis elegans*. Undertaking elegant