Proc. Indian natn Sci Acad. **B69** No.5 pp 853-860 (2003)

Current Opinion

From Cells to Behaviour: the Embryology of the Insect Nervous System

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(Received on 12 July 2003; Accepted after revision 8 September 2003)

There have been great advances in our knowledge of the way in which the nervous system develops in the last few decades. We now begin to understand how nerve cells are made, how growing axons are guided and how connections are formed. However at least one great area of neuronal development remains relatively unexplored: the development of the circuitry underlying movement and the maturation of coordinated patterns of locomotion in embryos. The insects, and the fruitfly *Drosophila* in particular, have been influential in shaping many of the advances made in our understanding of the developing nervous system. Three things ensure this influential role: small numbers of cells; the power of genetics to reveal underlying mechanisms; the conservation of mechanism from flies to humans. These same virtues can be applied to the question of how information for particular patterns of behaviour can be laid down in the developing nervous system. Our recent work with the *Drosophila* embryo reveals an underlying organisation to the developing motor circuitry of the fly which encourages the view that understanding how such circuitry is specified genetically and assembled embryonically may be a feasible project. One important simplification that emerges is that, just as morphology is shaped by a conserved, phylotypic body plan, so the circuitry that underlies behaviour may be assembled from a conserved "wiring diagram" that is robustly built into the embryonic nervous system.

Key Words : Insect nervous system, Embryology, Behaviour, *Drosophila*, Locomotion pattern

In 1996, Martin Raff wrote an editorial in Science, entitled "Neural Development: Mysterious No More?". The substance of his article was that progress in understanding the fundamentals of brain development had been so rapid, that we might soon be able to write down the main principles that underlie the growth and differentiation of the nervous system. Our newly acquired insights into the way the brain develops are part of an extraordinary flowering in the understanding of basic developmental mechanisms that has occurred in the last twenty years. Unexpectedly perhaps, given that their nervous systems are so obviously different from ours, the insects, especially the fruitfly, *Drosophila melanogaster*, have been influential in shaping many of the advances that have been made. In this article I shall argue that three things ensure this influential role for the developing insect nervous system: small numbers of cells; the power of genetics to reveal underlying mechanisms and the conservation of mechanism from fly to man. I shall discuss these before considering what seems to me to be at least one great area of neural development that remains relatively unexplored.

First the question of cells. When I began working with the insect nervous system in the 1960s and 70s, it was well known that invertebrate neuroscientists had the advantage of working with nervous systems that consisted of relatively small numbers of unique cells. In their experiments they could return again and again to the same cell or constellation of cells and ask questions about their structure and function in the neural network. Of course there is an underlying developmental question here– what is the nature of the processes that ensure with such reliability that the same cell with the same characteristics (such as

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neurotransmitter, electrical properties, branching pattern and connectivity) is consistently generated at the appropriate position in the network in every individual of the species? In fact this question encapsulates many of the fundamental questions that we have to solve if we are to understand how to build a neural network. Quite by chance (or almost – a percipient colleague, Ian Meinertzhagen, suggested that I ought to), I decided to look at the progenitor cells in the embryo of the locust (we happened to have a culture of them) that generate these arrays of identified neurons. These progenitors, neuroblasts, had been largely neglected since the 19th century, but it was known that they behaved like stem cells, dividing repeatedly to contribute small groups of immature neurons to the embryonic nervous system. It was easy to show, with more modern methods, that there were segmental sets of neuroblasts in the embryo, that there were 60 + 1 cells in each set, and that, like the neurons they produced, the progenitors were individually identifiable and reliably present in every embryo (Bate 1976a). This of course meant that we (like our neuroscientist colleagues) could go back again and again to the same cell and its progeny and ask, not functional, but developmental questions. How many neurons do you produce? What kinds of neurons? And the most important question of all: how does each cell that you generate gain access to the additional information that it requires to make it different from other, neighbouring cells, so that it becomes a unique, differentiated neuron at the appropriate position in the developing network?

Another kind of question could also be asked by looking at the early outgrowth of axons. Pioneer neurons, cells first postulated by Ross Harrison (1910), could be identified in the form of early differentiating cells that laid out nerve pathways both in the periphery and in the central nervous system (Bate 1976b, Bate & Grunewald 1981). These pathways formed as the pioneers put out axons which were the first to navigate through the virgin territory of the early embryonic nervous system. Wigglesworth had noted years before that the preferred substrate for a growing axon was another nerve (Wigglesworth 1959), and this was confirmed by the observation that the pioneers laid out a framework or scaffold of early pathways that were subsequently followed by the axons of later differentiating neurons. Now we could ask questions about axon guidance with these identified cells. How do the pioneers find their way? What are the cues to which their growth cones respond? How do other axons find the pioneer pathways? How do these later axons discriminate between alternative pioneer pathways and select one along which to grow?

Some crucial experiments were possible with the locust embryo, using a combination of laser ablation and cell manipulation. For example, Corey Goodman and colleagues showed that a mechanism intrinsic to the neuroblast was capable of generating a sequence of different neuronal fates and that this, together with positional specification of differences between neuroblasts and signalling between sibling progeny cells was sufficient to explain how each neuroblast could contribute a unique clone of cells to the developing nervous system (Doe et al. 1985). The same lab was able to show that there were real differences between the alternative pioneer pathways established in the early nervous system and that the growth cones of identified neurons reliably chose to extend along specific elements of the axon framework. If the appropriate pathway was ablated, then growth cones that would normally grow along it stalled or grew in aberrant and unpredictable ways (e.g. Bastiani et al. 1986). This was an important advance, because it indicated, in general terms, how a complex three dimensional neuronal branching pattern could be specified and reliably generated in the course of embryogenesis. The essential requirement is that an initial set of pathways is established by pioneering axons which presents a set of alternative substrates for subsequent nerve growth. The growth cones of later differentiating neurons now explore this framework, choosing to grow over particular elements and making turns at "choice points" where different elements of the framework overlap, so presenting alternatives to the growing tip of the axon. Of course, as each new axon grows out, it itself contributes to the axon scaffold, so that, in principle, the framework, and the number of alternatives for growth at choice points increases

with time as the developing network becomes more complex.

However, while this phase of work with the large embryos of insects like locusts was influential, it suffered like many earlier developmental experiments, in that it could indicate rules and principles but it could not reveal the underlying mechanisms. We knew that neuroblasts generated a sequence of neuronal fates through a series of divisions, but what did this mean in terms of molecular determinants and their segregation from stem cell to progeny? We knew that axons could choose between alternative pathways and generate complex and predictable branch patterns, but how did they choose and what was the molecular nature of the differences between nerve pathways that the experiments had revealed?

The resolution of this difficulty came from two sources, one of which has had revolutionary consequences for our understanding of developmental mechanisms in general while the other provided a much smaller change in our appreciation of how the nervous systems of different insects (and their near relatives, the crustacea) are put together. First the revolution. In the 1980s, Christiane Nüsslein-Volhard and colleagues carried out a series of mutageneses and screens in *Drosophila* looking for embryonic lethal phenotypes (e.g. Nüsslein-Volhard & Wieschaus 1980). They reasoned that since the components of developmental mechanisms are encoded in the genome, it would be possible to identify the genes concerned by screening for embryonic lethal mutations with characteristic developmental phenotypes. The results of this painstaking, labour intensive endeavour were triumphantly successful in identifying many of the essential genes required for *Drosophila* development. It became possible to clone these genes, to sequence them and identify their products and to dissect the nature of the machinery of which they formed a part. Further than that, by homology, the same genes were shown to be present in other organisms including ourselves and have been shown in many instances to have remarkably similar functions.

Now for the smaller, but nonetheless important change in our thinking. While the mutageneses and screens were going on in the fly, we began (heavily influenced by the work of Nüsslein-Volhard) to wonder why we were working on the locust and not *Drosophila*. If we wanted to understand how genes made brains, then it seemed that we were working with the wrong insect. The apparent difficulty in abandoning all the work done on the locust and moving to the tiny embryo of the fly (which would surely be very different) disappeared, when we began to look in detail at the embryonic nervous system of the fly and other insects. Strikingly, we found that the same progenitor cells, early differentiating neurons and axon pathways could be found in all the species we looked at, including *Drosophila*. We had stumbled upon what seems to be a common building plan for insect nervous systems, and with minor adjustments for crustaceans too (Thomas et al. 1984). The conservation was remarkable, so that despite being separated by millions of years of evolutionary time, the same individual embryonic neurons could be identified in *Drosophila* and the locust, derived from the same progenitor cells, making the same choices with their outgrowing axons and as we now know in some cases, expressing the same genes. The important point was that all the cellular detail and experimental evidence accumulated in the locust could be transferred to the fly. Now we could begin to ask the question, what are the genetic and molecular mechanisms underlying the developmental processes we observe?

For example, the neuroblasts: these cells are interesting in their own right as well as the repeated, asymmetric, stem cell mode of division which contributes families of immature neurons to the developing brain. In the last few years there has been rapid progress in understanding the cell and molecular biology of asymmetric division and the unequal partitioning of substances between dividing cells using the *Drosophila* neuroblast as a model. But for the developmental neurobiologist, the more interesting question is undoubtedly the nature of the elusive mechanism, intrinsic to the neuroblast, that reliably generates a sequence of different neuronal fates. Everything we have learned in recent years tells us that the characteristic features of different neurons will be dictated by highly specific patterns of gene

transcription. It comes as no surprise therefore (although it represents a considerable advance in our knowledge of the system), to discover that each neuroblast transiently expresses a very specific sequence of transcription factors. The neuroblast moves through the sequence, in time with the clock of its cell division, so that progeny neurons are indelibly marked by the expression of a particular transcription factor according to their birth order in the lineage (Isshiki et al. 2001). What is more surprising perhaps is to find that all neuroblasts and their progeny express the same sequence of factors, despite the fact that each neuroblast contributes a different, highly specific set of neurons to the developing nervous system. Thus the sequence of gene expression now revealed seems to reflect birth order in the lineage rather than the specific characteristics of the cells concerned. It is very likely that the effect of this orderly sequence of gene expression is to open for business transcriptional programmes that are appropriate to early or late parts of the lineage. These programmes are specific to particular neuroblasts and depend on their position in the overall array of progenitor cells. In this view, there is an intersection of transcriptional control mechanisms in which distinctive programmes set for each progenitor by its position are progressively revealed as neuroblasts pass through a common sequence of cell division and gene expression. The outcome of this coupled programme of division and gene expression is to produce a roughly layered structure in the developing nervous system, in which early born neurons lie furthest from the neuroblasts and are marked by the expression of "early" transcription factors, while late born neurons lie closer to the progenitor layer and express "late" genes. There is an obvious echo here of the layered structure of the vertebrate nervous system. Although it seems increasingly likely that the neuroblast is very much an insect speciality (Stollewerk et al. 2001), it will be extremely interesting in the next few years to see whether in vertebrates there is an analogous intersection of transcriptional control between factors representing the general, layered structure of the developing brain and specific regulators that reflect the tangential, regionally specialised organisation of the nervous system.

Genetic and molecular questions could also be asked for the growing axons that lay out the first pathways in the developing nervous system. These pathways form a ladder-like framework consisting of longitudinal axons running up and down the nerve cord on either side of the midline with two transverse commissures in every segment where axons cross the midline, linking the two halves of the system together. Almost all nervous systems are similarly bilaterally symmetrical, with mirror image halves connected by axons that cross the midline. But the midline presents an important paradox when considered from the point of view of axon guidance mechanisms. The paradox is this: by definition two equivalent cells on either side put out axons that grow towards the midline, meet at the midline and grow past each other in opposite directions. The puzzle is to understand what sort of molecular mechanism allows cells with identical patterns of gene expression to grow in opposite directions at the same point. The mystery has been solved by the use of genetic screens to identify genes that are required for axons to cross the midline – exactly the same stratagem used earlier by Nüsslein-Volhard and colleagues to find genes essential for more generalised patterning mechanisms in the embryo (Seeger et al. 1993). In the event there turn out to be two major phenotypes in such screens: one where few or no axons cross the midline and the opposite phenotype where many too many axons cross. Formally, the failure to cross phenotype could represent loss-of-function mutations in a gene encoding an attractant to the midline, while the opposite phenotype of too many axons crossing could be caused by mutations in a gene encoding a repellent. Indeed we now know that there are attractants in the form of proteins called Netrins and a repellent, a protein called Slit, that are produced by the cells of the midline. However the original phenotypes recovered in the genetic screens identified genes that code for a receptor for the repellent, Roundabout (Robo) and a gene *commissureless* (*comm*) whose product regulates the trafficking of the Robo receptor to the axon surface. Simply put, axons expressing Robo on the surface are repelled and do not cross the midline, while mutations in *robo* remove the receptor and

these axons are now free to cross. In a normal embryo Comm alters the trafficking of the Robo receptor in axons that will cross the midline so that Robo no longer reaches the growing tip of the axon but is targeted for degradation instead (Keleman et al. 2002). Thus the elements of a machine that guides axons towards the midline begin to emerge, with a post-translational control of the receptor for Slit allowing some to cross and others to be repelled.

Most elements of this machine are present in vertebrates as well, where they also act at the midline to allow some axons to cross and there has been a remarkable synergy in the unravelling of the molecular details in the two systems. For example, our understanding of the important issue of why axons first grow towards the midline, only to grow away from it after crossing was dramatically improved by Stein and Tessier-Lavigne, working not with flies, but with the frog, *Xenopus* (Stein & Tessier-Lavigne 2001). In the frog as in the fly, commissural axons grow towards the midline because they are attracted to Netrin and insensitive to Slit. But as they cross, their response changes radically and Stein and Tessier-Lavigne could show directly that they are now repelled by Slit and indifferent to Netrin. This change at the midline depends not only on the upregulation of Robo responsiveness to Slit, but on a direct interaction between Robo and the receptor for the attractant Netrin. The two receptors interact through their cytoplasmic domains, with the result that as the Robo receptor becomes active, the Netrin receptor is silenced, and the response of the growing tip of the axon is switched from positive to negative. Studies such as these are important because they reveal a dynamic quality to the way in which axons are guided as they grow. Far from having a fixed repertoire of behaviour, rapid changes in the internal machinery by which a growing axon senses its environment can radically alter its response to the cues that organise the wiring of the brain. The cross talk that this elegant analysis reflects between discoveries made in the fly and work with nervous systems more similar to our own, is just a hint of the extent to which the evolutionary conservation of genes and their products illuminates our understanding of the way the brain is built.

Finally, I want to consider what for me is still the great unsolved mystery of brain development. As Mark Ridley (1986) has pointed out "the simplest definition of behaviour is movement, whether it is the movement of legs in walking, wings in flying, or heads in feeding……in a more accurate sense therefore animal behaviour consists of a series of muscular contractions". We know very little about how the neural circuits that orchestrate these highly significant muscle contractions are specified genetically and assembled in the course of development and I think it is worth asking why this should be so.

The first person to carry out an experimental analysis of how circuitry is built into the brain was Roger Sperry. He worked with the visual system of the frog and was able to show for the first time that the connectivity of the system depended on the regulated growth of axons to form a map of the visual world (reviewed in Sanes et al. 2000). By demonstrating that the development of the brain, like any other organ, was a conventional embryological problem which could be understood at a cellular level in terms of cell growth and differentiation, Sperry ushered in the fruitful era we are now living in where many of the fundamental questions relating to the growth and connectivity of nerve cells have been solved. But Sperry worked with the sensory system and while our knowledge of connectivity in the sensory system and how it develops has increased hugely, we still know very little about the motor system. The reasons for this are not difficult to find. There is an obvious organisation to the growth of sensory systems in that they differentiate to form a map of sensory endings in the central nervous system that reflects characteristics such as the visual field in the periphery. Thus is it is relatively straightforward to interpret the results of experiments that address developmental questions in terms of axon growth and targeting to form an organised map.

Motor circuitry appears not to be like this. The so called central pattern generating circuits that drive rhythmic movements such as walking lie hidden away within the central nervous system, often as relatively inaccessible ensembles of neurons. We know that the output of such circuitry depends on the connectivity and electrical characteristics of these

cells, but unlike the sensory system, there is no obvious simplifying principle to the connectivity which would facilitate understanding how the circuitry is specified and assembled in the course of development. If we could discover such a principle it would greatly assist us in exploring these important unsolved questions.

Recently we have decided to begin such an analysis, once again using our favourite organism the fly. We think *Drosophila* is a good organism to work with despite its small size (and we make things worse by working with the embryo). It takes just 21 hours from fertilisation to make a fully functional nervous system which drives the simple locomotor patterns of the *Drosophila* larva. To our undoubtedly biased eyes, to translate the necessary inherited information into the neural circuits needed for larval movement in just 21 hours, is as great an achievement as the similar processes that take place over much longer times to generate the circuitry that underlies our own patterns of movement. More to the point, in *Drosophila*, we should be able to get at the molecular mechanisms involved and the way in which they are regulated to produce biologically adaptive outcomes. Two discoveries are already simplifying our task. First, we find that if we block the function of the sensory system throughout embryonic development, the motor circuitry still develops normally (Suster & Bate 2002). This is important because it shows us that the central pattern generator and the motor output that it produces can develop without sensory feedback. It is also a useful simplification: it shows that the development of the motor system is likely to be an autonomous property of the neurons that comprise the central pattern generator and allows us to focus our future analysis on these cells and their properties.

The second advance is that when we consider the organisation of the embryonic motor circuits, we find that the dendrites of the motorneurons (which are the input elements on which the endings of the pattern generating neurons will be distributed) are indeed arranged to form a well organised map of the muscles that they innervate (Matthias Landgraf ms. in preparation) . By labelling each motorneuron individually we find that their dendrites are specifically partitioned within the nervous system in such a way as to represent centrally the position and orientation of their target muscles. Thus, just as the sensory system is organised to form a map of the periphery, so the first layer of the motor circuitry is similarly organised to form a map of the muscle field. With this simplifying principle in mind we can begin to ask how neuronal differentiation and axon growth are regulated to produce this stereotyped pattern of connectivity.

I will end with a speculation that arises naturally from our interest in motor systems. As developmental biologists we are familiar with the idea that the embryology of particular kinds of organisms such as vertebrates is constrained by the fact that all members of the group conform in early development to a phylotypic body plan (see e.g. Wolpert 1998). Such embryonic body plans are highly conserved and the plan of a vertebrate is very different from that of an insect. Since morphology necessarily arises by the elaboration and modification of these characteristically different, highly conserved body plans they have great explanatory value in understanding the variety and detail of animal forms. We could of course apply the same thinking to neural circuitry. In the embryo of an insect such as *Drosophila* we find a way of organising locomotor circuitry which suggests that we are dealing with the wiring diagram for a peristaltic, crawling animal in which circular and longitudinal muscles act as antagonists. In contrast we know that most vertebrate embryos pass through a phase when their first movements are of a sinusoidal fishy kind and this suggests that their locomotor system begins with a very different kind of wiring diagram. In our view, actual motor circuitry may arise by the elaboration and modification of such simple, highly conserved embryonic circuit diagrams. If this is so, then the identification of these early circuits will, we believe, have great explanatory value for understanding how the variety and detail of mature circuitry is specified and assembled.

It is probably fitting that, as Martin Raff began by questioning whether we had any more to learn

about neural development , he should be allowed to end with a (for me) far more exciting comment "An alternative view is that this feeling that understanding is just a few steps away is a recurring and necessary delusion that keeps scientists from dwelling on the extent of the complexity they face and how much more remains to be discovered".

Acknowledgements

I am grateful to Helen Skaer for her comments on a draft of this article and to Matthias Landgraf and K. VijayRaghavan for many useful discussions. MB is a Royal Society Research Professor and work reported here was supported by a grant from the Wellcome Trust.

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