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The impact of molecular biology on neuroscience

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How our brains work is one of the major unsolved problems of biology. This paper describes some of the techniques of molecular biology that are already being used to study the brains of animals. Mainly as a result of the human genome project many new techniques will soon become available which could decisively influence the progress of neuroscience. I suggest that neuroscientists should tell molecular biologists what their difficulties are, in the hope that this will stimulate the production of useful new biological tools.

Keywords: new tools; conditional knockouts; neuronal cell types; the recording problem

1. INTRODUCTION

In the second half of the 20th century both biology and medicine have been transformed by the spectacular advances in molecular biology. This is especially so since the development of recombinant DNA—the ability to modify defined pieces of DNA and to move them from place to place—plus the techniques of rapid DNA sequencing. This has already led to a better understanding of many medical conditions. Fields such as developmental biology—the formation of a mature organism from the fertilized egg—are advancing rapidly. Even the study of evolution, the ultimate biological problem, has already been influenced by these new tools.

One of the major problems of biology today is discovering exactly how our brains work. This subject has been advancing steadily but more slowly and, in spite of the considerable accumulation of knowledge, still has far to go. When we finally understand scientifically our perceptions, our thoughts, our emotions and our actions—hopefully some time in the 21st century—it is more than likely that our view of ourselves, and of our place in the universe, will be totally transformed, as they have been decisively altered already by Darwin and Wallace's theory of natural selection.

2. THE INFLUENCE OF MOLECULAR BIOLOGY

Molecular biology is likely to influence neuroscience in at least two ways. Present-day biological organisms are the result of an extremely long period of natural selection in a whole variety of past environments. Moreover, as François Jacob (Jacob 1977) has emphasized, evolution is a tinkerer. To an astonishing extent it builds on what is already there, rarely innovating from scratch. The result is that, at all levels, most organisms are unbelievably complex. This is only too clear to molecular biologists, but it is only gradually becoming clear to neuroscientists. Psychologists in particular have a touching faith in Occam's razor, which, however appealing it may seem, is a very unreliable tool in biology. As for theoretical neuroscientists, they too easily believe that because their oversimplified models are so appealing (having been carefully massaged to fit some of the data) they must be true. Molecular biologists have learnt the hard way that a beautiful theory can be quite wrong. I have already dealt with many of these points in my book *What mad pursuit* (Crick 1988), so I will not go over this ground again.

In this paper, I want to have a brief look at what I believe will be the other main influence of molecular biology on neuroscience. This is the provision of new and powerful tools for neuroscientists to use. This is not to suggest that this will be the only source of new tools. In the recent past, new tools have been introduced that owe little or nothing to molecular biology; for example patch clamping (very cheap) and various brain scans, such as positron emission tomography and magnetic resonance imaging (very expensive). The theme of this paper is that the human genome project, the first phase of which will shortly be completed, will provide a great variety of possible new tools. Their impact will be considerably greater than most neuroscientists now appreciate.

Basically the argument is that to understand a complex biological system one must be able to interfere with it both precisely and delicately, probably at all levels, but especially at the cellular and molecular levels. While it is essential to study the behaviour of the whole organism, in a variety of circumstances, both natural and unnatural, in almost all cases a pure black-box approach is bound to fail. Molecular biology will provide new tools for characterizing both the structure and the function of neurobiological systems with much greater precision.

3. THE TOOLS OF MOLECULAR BIOLOGY

It is worth noting what tools molecular biology itself uses. Apart from certain simple methods of physical chemistry, such as sorting molecules by electrophoresis on a gel, the main tools that have powered the dramatic advances associated with recombinant DNA have been

the use of enzymes, for example restriction enzymes. The trick then, is not to use the rather clumsy and inefficient techniques of classical organic chemistry by themselves, but to make use of nature's tools. These work rapidly and very specifically, and do not have to be synthesized. Rather, one explores natural organisms, of one type or another, to discover and extract the required proteins, though it often happens that the techniques of recombinant DNA are used to tinker with them, so they will work even better for the task in hand. Fortunately the fact that two complementary stretches of DNA or RNA can recognize each other in solution makes many techniques possible, which could not be done with proteins.

Another point to notice is that molecular biology advances very fast, partly because there are now so many active molecular biologists. When confronted with a difficulty, in no time at all several groups will come up with a new tool. The tendency in neuroscience (and I'm hoping this will change) is to say, 'Yes, I'd love to have new tools, but will someone else please develop them?'

(a) *Older techniques*

First let us look briefly at a few useful techniques that have been around for some time. These have come mainly from biochemistry and depend on the active transport of material up and down the axons. For tracing pathways one of the first was the use of radioactive amino acids. It is worth noting that this could have been introduced at least ten years earlier, but nobody appears to have thought of it then. This was quickly followed by the use of horseradish peroxidase and similar molecules. A more recent method, which showed which types of pyramidal cell project to a certain part of the macaque cerebral cortex, was used by John Morrison and his colleagues (de Lima *et al.* 1990). This involves injecting the recipient cortical area with fast blue, which is transported retrogradely; then after a delay, sacrificing the animal, making slices of the projecting cortical areas and injecting any blue nuclei in the slice with Lucifer yellow to display the shape and location of each projecting neuron. They found that at least eight distinct types of pyramidal cell contribute to the pathway from the higher (ventral) levels of the visual system to the prefrontal region near the principal sulcus. Such is the systematic neglect of neuroanatomy that nobody else appears to have used this very useful technique. If sufficient funding were provided there is no reason why the whole of the large-scale connectivity of the macaque monkey should not be worked out by this or similar methods.

(b) *Recent techniques*

There are a number of topics that are sufficiently well known that I am not going to dwell on them, such as using various types of immunoglobulins to label where different proteins occur, or the localization of messenger RNA (mRNA). These techniques will no doubt be improved. Instead I will give you a few examples of some more recent ones, to give a flavour of some of the things that are already happening, before going on to what one would like to happen. We already have methods of telling where a neuron sends its axon, using the transport of material up and down the axon, which of course for ethical reasons can only be done on animals, not human beings. Can this be done sequentially? Can you put something in a particular neuron so that the label passes to the neurons synapsing on the first set, and so on in stages?

This has been done using herpes simplex virus. For a recent example of this see the paper by Hoover & Strick (1999). You need the right virus for the right job; that is, for the right neural system and the right animal. Not each virus need be herpes simplex type 1. This virus multiplies as it travels and in this case is transported retrogradely. If you choose the right virus, or handicap the virus in some way, it does not kill the cell. You inject the virus in one part of the animal and wait for about two or three days. For other animals you wait four to five days or four to seven days. Comparing the results you can see the label moving along from one set of neurons to the next. This has been done with great success in the basal ganglia pathways. At the moment there are problems with using it in the cortex, because of the complexity of its wiring.

Another recent technique uses green fluorescent protein. It was used to see which synapses were active (Miesenböck *et al.* 1998). Here again the authors had to alter the design of the amino-acid sequence of the green fluorescent protein to make it more sensitive to pH. When a synaptic vesicle was released, the fluorescent protein was exposed to a less acid pH, and this changed the fluorescent response.

Another technique uses wheat germ agglutinin (WGA). This method of course is already known, but the new twist by Yoshihara and colleagues (1999) used the cDNA for it. The disadvantage of using WGA itself was that it often gave an inflammatory response, so it was not ideal, but by using the cDNA, which is delivered in a special way, one can get over that. They used a truncated version of the cDNA, which made it work better. In this case the WGA is transported anterogradely. They have traced pathways in the mouse cerebellum and olfactory pathways, and also in the Drosophila visual system.

A technique that is already well known, so I will mention it only in passing, is gene knockouts (or knockins) in mice. Mice are used because they are cheap and breed rapidly, since several generations are needed. The disadvantage is that usually the gene is knocked out at a very early stage in development. It is known that this can lead to difficulties. For example, if a young child who is right-handed and normally has language on the left has severe damage at an early age on the left-hand side, language will usually develop on the right. One often gets compensating changes to damage as the organism develops.

It is possible to kill special types of neurons by the use of a photoactivatable dye. This was done by Nirenberg & Cepko (1993) on various types of cells in the mouse retina. They genetically engineered the animals so that their cells contained the gene for the non-toxic enzyme β-galactosidase, controlled by a promoter specific for a particular type of retinal cell. They then added the dye (linked to a sugar moiety), which was taken up by all cells. However, the dye was released in detectable amounts only in those particular cells that contained the β-galactosidase. These cells could then be photoablated with the aid of a sensitizing agent. They also tried out the

technique on neurons in mouse cerebral cortex and in zebrafish embryos.

(c) *Conditional techniques*

Recall that we already have two techniques of reversibly silencing small volumes of neural tissue: the use of GABA, or a GABA analogue, and cooling (see the discussion by Payne *et al*. 1996). What we lack is the ability to do this rapidly for a particular neuronal type. What is really needed is something with regional specificity, which acts on only special types of neuron and also has temporal specificity. Tonegawa and his colleagues (Wilson & Tonegawa 1997) were lucky enough to find a way of doing this using one of the NMDA-type of glutamate receptor genes, the R1 gene, which, it so happens (at least in the part of the brain they were interested in), is only expressed in CA1 pyramidal cells of the hippocampus. Moreover, it does not usually start its action until the third postnatal week. So here is a case where there was some control of regional and temporal specificity. The results were really interesting. The animals were deficient in the water maze test and the place cells (using multi-unit recording) still functioned, although they had a broad specificity. With the knockout, the multi-unit recording showed a loss of coordinated firing.

This is just a start, but we need more. We need conditional knockout genes in mice; some method whereby we can turn a gene on and off in a mature animal, by some signal or another. There are a number of groups that have developed mechanisms for doing this (Furth *et al*. 1994; Shockett *et al*. 1995; Baron *et al*. 1999). The one that I know best is that by Ron Evans and his colleagues at the Salk Institute (No *et al*. 1996). This uses ecdysone, actually muristerone (an ecdysone analogue). The molecular biology is such that when you give muristerone it will turn on the gene. When you dilute it out the gene turns off. This is another example where the experimentalists had to tinker with it to get it to work better. One has to ask: What is the ratio between on and off? This ratio was originally only about three. When they tinkered with the molecular biology intelligently, the ratio became a thousand to one.

It would be extremely useful if one could give something to an animal so that a particular type of neuron was conditionally turned off—that is, temporarily prevented from firing. How could one do that? There are a number of possibilities. Johns *et al*. (1999) have done it by activating a particular potassium channel. This produces a more negative resting potential, making it more difficult for the neuron to fire. Admittedly this was done on neuronal cultures of the rat superior cervical ganglia, and admittedly it was rather slow, because to turn the neurons on again you have to dilute out the ecdysone signal and also the protein of the potassium channel. But after 36 hours the neurons were firing again. This is not an ideal system, but it is a beginning. It has not yet been done on an intact animal.

The trouble with most of the methods so far, especially with the conditional knockouts, is that, for the moment, they can be used only on mice. If one is interested in the visual system, as I am, the mouse is not the ideal animal. The olfactory system might be a better choice. What one would like are methods that could be used on a monkey. This is much more difficult, but there is very strong effort being made medically to develop viral vectors to cure various disorders in people's brains. Although the viral vectors used up to now have not been very successful (because there has not been enough basic work on the nature of the vectors) nevertheless it is likely there will be substantial progress in the future. How fast that will come along, I do not know. Naturally with my interest in monkeys I would like to see such techniques develop very rapidly.

(d) *Pharmacology*

There is one other approach that is apt to be left on one side because it is not quite so glamorous, and that is the pharmacological approach. See, for example, the article by Izquierdo & Medina (1998) making the case that pharmacological methods are often more trustworthy than inferences from brain damage. The trouble with most pharmacological agents is they are not specific enough. What does molecular biology have to do with this? Molecular biological techniques are being used to produce more specific pharmacological agents. This can be done at an immense rate by using combinatorial chemistry to synthesize large numbers of very similar compounds. These can then be tested on isolated cells that have been engineered to have the particular receptor you are interested in, so that you can select a molecule that is very specific for that type of receptor. It is worth remembering that we can hope to have more specific pharmacological agents, and of course, in the case of the monkey you have the opportunity to inject them locally. Incidentally there will probably be a number of reagents of this sort that will be difficult to use on human beings, but are not so difficult in monkeys, because one does not have to worry so much about long-term side-effects.

4. POSSIBLE FUTURE TOOLS

The above gives a few glimpses of what is already going on. Let us now ask not what tools are available now but what tools will be needed in the future. Naturally everyone will have their own wish list. I can only outline a few items from my own.

It is, of course, foolish to predict in detail exactly what tools will become available, as one can see all too clearly by recalling people's past predictions, but one can sometimes spot major trends in what lies ahead. In our case, it is the human genome project and its consequences. Before long almost the entire human genome will have been sequenced, together with the genomes of some other animals and plants, and of numerous micro-organisms. This is only the first phase. In the next phase the structure and activity of the coded proteins will be explored, and in the third phase their many interactions and the mechanisms that regulate their rate of production (and destruction). Probably in the first phase the control regions of many genes will be identified. It is these control regions that are of special interest to us.

(a) *Cell types*

As I have already mentioned, I believe that to understand how our brains work (or the brains of other animals) it will be necessary to study the fine molecular and cellular details. To many neuroscientists one

pyramidal cell is just like another. I, on the contrary, believe that it is important to distinguish the many types (and probably subtypes) of pyramidal cells. One can often see that two pyramidal cells look quite different. They may occur in different cortical layers, have quite different patterns of dendritic and collateral axonal arbors, and project to quite different places in the brain. It is now accepted that in the retina it is important to make these fine distinctions. In fact Cajal described many distinct types of amacrine cells there, just from the exact sublayer they were in and their appearance (Cajal 1995).

It is of course well known that not all pyramidal cells in the cerebral cortex look the same and that they do not all project to the same place, but little effort has been made to count the number of distinct types, even in one cortical location. Rough estimates show that the number is unlikely to be as few as 20, and could possibly be as many as 50 or more. It is not made easier by the absence of any exact definition of cell type. The obvious thing to say is that in addition to the broad morphological features, a cell's type will, at bottom, depend on which genes are acting. This is probably too glib, as there may be changes in gene action due to such processes as synaptic modification or production (for example, associated with learning).

Thus a twofold approach is required: first, to describe the broad morphological features mentioned above; second, to find which genes are active in one type of neuron but not in another. Eventually one will want to know the distribution of the various ion channels and other key molecules in any particular type of cell (including, of course, spiney stellates and the many different types of inhibitory neurons, and other neuronal types on other brain regions) in order to explain exactly how each neuron handles the incoming information. The first step, however, is to try to distinguish between the various neuronal types.

How can one find out which genes are expressed in which pyramidal cell (or other cells)? One way is to try various known genes by seeing if the relevant mRNA or protein is present. With luck, one might hope to hit on a specific marker for that particular type of neuron. Another way is to compare the mRNAs expressed by several distinct types of pyramidal cells.

Given a sufficient number of two types of neurons, it is possible to discover which of the more abundant mRNA molecules are expressed in one type but not the other. (It would be most useful if this technique could be made to work using single neurons.) Moreover, this need not, in the first instance, be done in the intact brain, but in a suitable brain slice. Such information, as it accumulates, should enable us to arrive at a more precise idea of what one means by neuronal type or subtype. Moreover, it will enable one to see whether there are one or more control regions which are active just for that particular cell type and also which transcription factors are involved. This information will be needed if one wishes to turn the firing of that type of neuron on and off selectively, as discussed above.

(b) *The recording problem*

It is one of the peculiar features of most modern neurophysiology that the experimentalist usually knows from, say, which cortical area he or she is recording, but seldom knows which type of neuron he or she is listening to, and often not even from which cortical layer he or she is recording. This is especially true for most work on alert monkeys. It is common for the experimentalist to record that, say, 25% of the neurons studied behave in a particular way, 37% in a different way and a further 15% in a third way. There is no indication where these different sets of neurons are sending their information, let alone exactly what type of neuron they are. This is not science but rather natural history. Rutherford would probably have called it stamp collecting.

Neurophysiology, especially on alert monkeys, is difficult enough, and one can sympathize with the experimenters, since at the moment there is no easy method of finding where the recorded neuron projects. It should be possible to discover in which cortical layer, or sublayer, the neuron lies, but this is seldom attempted; nor are new substances being invented which could leave a fairly permanent mark to label the electrode's position. But in the long run it will be essential to know which type of neuron the electrode is recording from. This problem deserves immediate and serious attention.

A major first step, then, is to identify the many different types of neuron existing in the cerebral cortex and other parts of the brain. One of the next requirements (as discussed above) is to be able to turn the firing of one or more types of neuron on and off in the alert animal in a rapid manner. The ideal signal would be light, probably at an infrared wavelength to allow the light to penetrate far enough. This seems rather farfetched but it is conceivable that molecular biologists could engineer a particular cell type to be sensitive to light in this way.

Most modern theories of brain action stress the firing (in one way or another) of not single neurons but groups of neurons. One particular example of a possibly significant group is all the neurons in some small location that project to one place, and especially if they are all of one type. One way-out suggestion is to engineer these neurons so that when one of them fires it would emit a flash of light of a particular wavelength. The experimenter could then follow the firing of that group of neurons alone.

(c) *Architectonics*

What other problems are there? It is difficult to trace neural pathways long distances in humans, since this depends on active transport up or down the axons, and this we cannot do on humans. However, something could be done for architectonics. Architectonics means looking at the kinds and distribution of different types of neurons, especially in the cerebral cortex: what neurons occur in what layer, whether there is a large layer 4 or a small one, etc. This is the kind of thing K. Brodmann did to sort out the different cortical areas. He used rather few criteria, but the human genome project will give us many more specific markers. This type of technique, with reservations about getting the materials fresh, can be done on both monkeys and humans. In five years or so it will be possible to do quite detailed comparisons between the monkey brain and the human brain or any other brain for that matter (e.g. chimp brain).

5. CONCLUSION

There are undoubtedly very many other problems in neuroscience that might yield to new molecular biological techniques. I have only touched on a few of them. The point I want to stress is that neuroscientists should scan molecular biology for appropriate techniques but, most important, they should ask their molecular biology friends for new tools. They should tell them what their difficulties are and what they want to do. Once the word gets round that a certain type of problem exists it is surprising how often someone has a bright idea of how to solve it. So, don't be shy—ask! After all, exactly how our brains work is of vital interest to us all, so why shilly-shally.

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