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TOPICAL REVIEW

Central nervous system regeneration: from leech to opossum

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A major problem of neurobiology concerns the failure of injured mammalian spinal cord to repair itself. This review summarizes work done on two preparations in which regeneration can occur: the central nervous system of an invertebrate, the leech, and the spinal cord of an immature mammal, the opossum. The aim is to understand cellular and molecular mechanisms that promote and prevent regeneration. In the leech, an individual axon regrows successfully to re-establish connections with its synaptic target, while avoiding other neurons. Functions that were lost are thereby restored. Moreover, pairs of identified neurons become re-connected with appropriate synapses in culture. It has been shown that microglial cells and nitric oxide play key roles in leech CNS regeneration. In the opossum, the neonatal brain and spinal cord are so tiny that they survive well in culture. Fibres grow across spinal cord lesions in neonatal animals and *in vitro*, but axon regeneration stops abruptly between postnatal days 9 and 12. A comprehensive search has been made in spinal cords that can and cannot regenerate to identify genes and establish their locations. At 9 days, growth-promoting genes, their receptors and key transcription molecules are up-regulated. By contrast at 12 days, growth-inhibitory molecules associated with myelin are prominent. The complete sequence of the opossum genome and new methods for transfecting genes offer ways to determine which molecules promote and which inhibit spinal cord regeneration. These results lead to questions about how basic research on mechanisms of regeneration could be 'translated' into effective therapies for patients with spinal cord injuries.

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After the central nervous system of a leech has been cut in two, axons regenerate and the animal can move normally again. Similarly, frogs can see again after their optic nerves have regrown to the tectum after injury. By contrast, in adult mammals, although peripheral sensory and motor nerve fibres can reach skin and muscles, axons of the spinal cord never grow across a lesion. What mechanisms promote axonal outgrowth and synapse formation by some injured nerve cells but not others? This problem is of interest not merely to neurobiologists, but to cell biologists, molecular biologists and, of course to clinical neurologists, who have to deal with hemiplegic and paraplegic patients.

In this review we describe a strategy for addressing these problems in central nervous systems that can and cannot regenerate. In preparations that do exhibit successful regeneration and restoration of function, numerous questions arise. How are damaged axons stimulated to grow across a lesion, how do they find their way, and how accurately do they recognize their appropriate targets? Such problems have been studied in detail in the leech nervous system. It will be shown that a great advantage of the leech (*Hirudo medicinalis*) is the wealth of information about the structure, properties, connections and functions of the individual nerve cells that make up its nerve cord. Thus, the axon of one particular sensory cell can be followed as it reconnects with a particular motor cell (Elliott & Muller, 1983; Nicholls, 1987).

By contrast, adult mammalian spinal cord is a vastly more complex structure, in which no regeneration occurs and glial scars form after injury. Numerous attempts have been made over the years to overcome failure of outgrowth in adult rat and mouse spinal cords by implanting bridges for axons to grow along (Aguayo *et al.* 1991; Bregman *et al.* 2002; Raisman, 2007; Bunge, 2008; Fawcett, 2008), by neutralizing molecules that block growth (Maier &

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Schwab, 2006; Cafferty & Strittmatter, 2006), and by testing candidate molecules to determine whether they promote regeneration (Salie & Steeves, 2005). A mammal that offers one the possibility of studying neurons that can and cannot regenerate after spinal cord injury is the newly born opossum *Monodelphis domestica*. A stage of development has been defined at which axonal outgrowth across spinal cord lesions fails abruptly. Hence, in the same population of neurons one can search for differences in genes that are up- or down-regulated at the time when regeneration stops being possible.

Regeneration of connections in leech nervous system

Why use the leech to study regeneration? Historical perspective. The leech represents a favourable preparation in which to analyse in detail the mechanisms by which a nerve cell grows, reforms connections after injury, and restores functions that were lost. Over centuries anatomical and behavioural studies were made on leeches because they were routinely applied to treat (or mistreat) patients; accordingly, the leech and its nervous system were objects of interest to biologists and medical practitioners (Payton, 1981). Eventually the bleeding of patients ceased to be an acceptable attempt at therapy for diseases such as hypertension, epilepsy and haemorrhoids (except of course in alternative or new age medicine).

It was in the early 1960s that the leech was re-introduced as a novel preparation for neurobiology. Kuffler and Potter wished to study glial cells, the properties of which were at that time unknown. Did glial cells have resting potentials, did they fire action potentials, did they communicate with neurons and if so how? David Potter found in the beautiful drawings of leech ganglia by Gustav Retzius (1891) the ideal preparation for such experiments. Glial cells were large enough to see in the microscope and could be impaled by microelectrodes, while adjacent neurons could be recorded from at the same time. The landmark paper by Kuffler & Potter (1964) served as the stepping stone for subsequent studies of vertebrate glial cells, the properties of which were shown to be similar to those in the lowly leech (Ransom & Sontheimer, 1992). (As Stephen Kuffler said: 'Far from being a round about approach, the use of the leech was in fact a short cut').

As a bonus, work on leech glial cells presented neurobiologists with a finite, simplified nervous system. The ganglia of the leech nerve cord are highly stereotyped and contain only about 400 nerve cells (Macagno, 1980; Muller *et al.* 1981). This constitutes a manageable number of cells in which to determine functional elements of the wiring pattern, as if one were tracing a map of the Paris Metro. In leech ganglia one could hope to explain how an animal behaves, in terms of the way in which its individual identified nerve cells are interconnected. The neural circuits that enable a leech to bend or walk, or to start and stop swimming, have been unravelled in terms of connections between individual sensory cells, interneurons and motor cells (Nicholls, 1987). With such background information it becomes possible to follow events, step by step, while axons regenerate and re-form their connections after an injury.

Microglia, laminin and the outgrowth of leech axons after injury. When all the axons in a segment of the central nervous system of the leech are broken by a cut or a crush, the anterior and posterior parts of the body become disconnected; rhythmical swimming movements no longer spread along the body from head to tail. After a few weeks, regeneration occurs and the leech swims normally again (Nicholls, 1987). When axons at the site of the lesion are stained a few days after the operation, one sees profuse outgrowth toward the next ganglion, a large number of microglial cells, and an accumulation of laminin (Masuda-Nakagawa *et al.* 1993; von Bernhardi & Muller, 1995).

What mechanisms induce the damaged axons to grow? Microglial cells play a key part in this process. These small scavenger cells of the nervous system have a mesodermal origin (they were, as it happens, first described by Del Rio Hortega in the leech). At rest microglial cells are scattered throughout the nervous system. But they immediately migrate towards the site of a crush over long distances. In living preparations, one can observe by video-microscopy that microglial cells residing far from a lesion start to move toward it after a short delay of no more than 3 min at a rate of up to 7 μm min^{-1} (McGlade-McCulloh *et al.* 1989). There is good evidence that molecules thought to be chemo-attractants, such as ATP, are liberated by the injury and that they are responsible for activating the movement of microglia. But does ATP influence the *direction* of movement and cause the cells to accumulate at the site of the injury? Experiments show that nitric oxide, produced at the crush site and by glial calcium waves, is both crucial for directed migration of microglia from hundreds of micrometres away and also acts as a stop signal for them at the lesion. Such effects are mediated by a soluble guanylate cyclase (McGlade-McCulloh *et al.* 1989; Duan *et al.* 2009).

How do the microglial cells that accumulate play a part in regeneration? Activated microglial cells produce laminin, a protein that promotes outgrowth of axons in many types of nerve cells, including identified leech neurons and both developing and peripheral mammalian neurons. Thus, an individual sensory nerve cell that responds to pressure applied to the skin on the dorsal surface of the leech can be identified in a ganglion, plucked out and maintained in tissue culture for days or weeks (Dietzel *et al.* 1986). Over the next day, the axon of that

cell plated on plastic shows limited growth of a few micrometres; by contrast, the same cell plated on a dish coated with laminin will produce multiple branched processes that spread out rapidly, for distances of millimetres. Such outgrowth is blocked by application of antibodies against laminin. In addition, it has been shown in culture that activated microglia change their shape and start to produce laminin (Masuda-Nakagawa *et al.* 1994; von Bernhardi & Muller, 1995). These results provide a framework for understanding how leech neurons sprout at the site of injury, but do not explain how functions are restored.

Formation of specific synaptic connections by regenerated leech axons after injury. For regeneration, while a prerequisite is that axons should grow, this in itself is not enough. If the nervous system is to carry out functions after regeneration has occurred, axons that have regrown must make connections with appropriate targets. Alternatively, after regeneration leeches might swim again as a result of novel, 'incorrect' connections that subsequently become modified by experience. It has been possible to determine the accuracy with which leech neurons reconnect to their original targets, by making use of known synaptic connections between identified nerve cells.

For example, the sensory cell that responds to pressure on the skin (mentioned earlier) sends an axon to the neighbouring ganglion, where it makes a direct synaptic connection onto a motor cell that produces shortening of the animal. If this sensory cell axon is cut or broken it grows back to the next ganglion in about 2 weeks. There it restores the original connection to the particular motor cell that produces shortening but avoids countless other targets on the way (Jansen & Nicholls, 1972; Wallace *et al.* 1977; Elliott & Muller, 1983).

Another example of extraordinary specificity is provided by a small cell, known as the S cell. In each ganglion in the ventral nerve cord of the leech there is only one such S cell. Its large axon spreads anteriorly and posteriorly toward the neighbouring ganglia. Midway between one ganglion and the next, the S cell axon terminates in a synaptic connexion specifically with the axon sent by its brother S cell (Muller, 1979). Although the role of these cells has not been completely elucidated, it has been shown that the S cell chain must be intact for the animal to perform a complex behavioural act known as 'sensitization'. (This is the basis of the hypersensitivity and acute awareness of the environment that one feels after being shocked by a sudden, loud bang.) Similarly in the leech, when a mild stimulus is applied to its skin, it normally elicits only a weak motor response. If, however, a strong diffuse shock is given to the nervous system immediately before the weak test stimulus, the motor response becomes greatly amplified (Sahley *et al.* 1994). S cells have been shown to participate in sensitization by several experiments, including the following. If the axon of the S cell in one segment is selectively severed, with no damage to other neurons, the leech will respond as before to the mild stimulus applied to the skin. But after the break in the chain of S cells, a strong shock no longer gives rise to sensitization. Over days, the severed axon of the damaged S cell grows, reaches the axon of its brother from the next ganglion and then reconnects to it, without connecting to any other axons. After it has reconnected, a strong shock applied before the test stimulus once again gives rise to sensitization (Burrell *et al.* 2003). This experiment demonstrates the way in which precise regeneration by a single cell can restore a complex behavioural function that had been lost after damage.

Together, experiments made on leeches have revealed cellular and molecular mechanisms by which growth of axons across a lesion is accomplished and have demonstrated the precision with which individual neurons can become reconnected. What remains completely unknown in both invertebrates and vertebrates is how a growing axon recognizes its correct target and forms a synapse on it, while ignoring all the other potential partners.

Regeneration of spinal cord in neonatal opossum

Why use the opossum to study regeneration? The starting point for these experiments was the idea that lesions of the spinal cord could perhaps be repaired at an early stage of development, before myelin has formed and while connections are still being made. Since regeneration cannot occur in adult mammals, one could hope to define the transition time during development at which regeneration fails. The principal reason for using opossums (non-placental mammals) is that they can breed in captivity and are born in an extremely immature state; the newly born animals correspond roughly to 14 or 15 day mouse or rat embryos. The pup is so tiny that its central nervous system can be dissected out in its entirety and maintained in culture for periods of days or weeks. In isolated preparations, reflex activity continues, neurons continue to be born, and the structure remains normal in appearance, with minimal cell death (Nicholls *et al.* 1990).

After the spinal cord is cut or crushed, even in a dish with tissue culture medium, many damaged fibres start to regenerate within a day or two (Nicholls & Saunders, 1996). *In vitro*, axons stained by the fluorescent dye DiI can be followed by video-microscopy as they grow across and beyond the lesion. By 5 days, there is profuse outgrowth in preparations after injury, over distances of more than 1 mm. This result is of importance since it provides a rapid and reliable assay for testing whether particular molecules or experimental procedures affect regeneration.

Similar results have been obtained in spinal cords of mouse embryos maintained in culture (Saunders *et al.* 1992).

In the dish, it has been shown that regenerating sensory fibres labelled by horseradish peroxidase become reconnected to motoneurons. After axons in the spinal cord are completely severed in a neonatal opossum, which is still attached to the mother but anaesthetized during surgery, they grow over long distances and form functional connections. These are precise enough for the animal to walk, climb up a rod, and swim in a co-ordinated manner that cannot be distinguished from normal by behavioural tests such as the BBB test (Saunders *et al.* 1998). Hence, regeneration of connections does occur successfully in mammalian spinal cord provided that the animal is young enough. Double staining or video microscopy in living preparations show that both regeneration of cut axons and newly grown uninjured axons sprout across the lesion.

Age dependence of capacity for regeneration. A cut-off point for regeneration occurred between 9 and 12 days. Lesions were made in cervical segments of spinal cords of 9- and 12-day-old opossums in culture. As described above, 9-day-old cords regenerated reliably. By contrast 12-day-old preparations showed no regeneration in the cervical region. However, in the lumbar cord, which was less mature, regeneration continued to be possible until about 17 days. Development proceeds rapidly at these ages: glial cells start to appear at about 6 days of development; myelin and associated inhibitory molecules at about 11 days (Varga *et al.* 1995; Terman *et al.* 2000).

Changes in gene expression at stages when regeneration can and cannot occur. In neonatal opossums, it was possible to assess how gene expression changes in spinal cords as they stopped being able to regenerate. The aim of these experiments was to provide a comprehensive list of candidate molecules that might play a part in stimulating or preventing regeneration. To this end, RNA was extracted from cervical spinal cords of animals aged 9 days and 12 days, with and without lesions (Mladinic *et al.* 2005). The correlation of expression with the ability to regenerate was extended by examining RNA extracted from lumbar segments at 12 days (which can still regenerate). While such experiments may seem conceptually and technically simple, they are fraught with difficulties. Vast numbers of genes are expressed by a nerve cell (approximately 20 000) and of these presumably very few can be expected to play a part in regeneration. Hence, to find the essential genes that have changed their expression is like searching for a needle in a haystack (except that one does not know the shape of the needle or how many of them there are). A major challenge is to separate genes that are regulated developmentally from those that change their expression as a result of injury.

Two main methods were used to see which genes are changed. First, PCR analysis was performed for a series of subtractions to reveal which genes were up- or down-regulated. Subtraction 1: 12 day cervical RNA was subtracted from 9 day; in principle this could display those genes that might promote regeneration. Subtraction 2: subtraction of 9 day cervical RNA from 12 day could display those genes that might prevent regeneration. To narrow the search, two further subtractions were made. Subtraction 3: cervical 12 day RNA from 12 day lumbar RNA, which in theory could resemble the results of subtraction 1. Subtraction 4: 9 day lumbar from 12 day cervical.

A second, quite different method was used to test the validity of the results obtained by PCR subtractions. RNAs from different regions of spinal cord at different stages of development were analysed on microchips with nucleotides obtained from a related marsupial (the tammar wallaby) (Brennan *et al.* 2007). In addition, gene expression was compared with and without lesions (M. Mladinic, E. A. Del Bel & M. R. Digby, unpublished observations). The results obtained by both methods were surprisingly similar and revealed numerous genes that, from the literature, would be expected to promote or prevent regeneration. Table 1 shows some promising candidate genes that were up- or down-regulated. For example in spinal cords that can regenerate, genes coding for laminin receptors and growth-promoting molecules such as β-thymosin are prominent; in spinal cords that cannot regenerate, the genes for reticulon, myelin basic protein and semaphorin receptors, which tend to block axon outgrowth, are expressed.

There are, however, serious limitations to drawing firm conclusions from these results. Thus, PCR subtractions and microchip analyses are not quantitative. Accordingly, for a number of promising genes, such as those that produce growth or prevent it in other systems, quantitative measurements were made using Northern blots, RT-PCR, and real time PCR. In addition, *in situ* hybridization and histochemical staining were used to determine at what sites the genes were expressed within the spinal cord. A satisfying result was that for the genes selected, quantitative tests confirmed the up- or down-regulation seen by subtractions or microchip analysis.

Comparisons of gene expression were also made in 9 and 12 day cords that had been lesioned previously (M. Mladinic, E. A. Del Bel & M. R. Digby, unpublished observations). While the results largely overlapped with previous results obtained by PCR subtractions, a few genes were found that changed differently after injury in cords that could and could not regenerate. For example after injury, apoptosis and cell survival-related genes were up-regulated at P8 compared to P12. Of interest was the appearance after injury of genes related to Alzheimer's disease and other neurodegenerative disorders (Table 1).

Table 1. Selected genes expressed differentially in opossum spinal cords at 8 days (regenerating) and 12 days (non-regenerating) revealed by PCR analysis and by microarray cDNA technology

	Selected clones in regenerating spinal cord	Selected clones in non-regenerating spinal cord
Transcription factors	Transcription factor NRF Transcription factor 7-like 2	Signal transducer and activator of transcription 3 Pax-6
Myelin-related proteins		Reticulon 4 (NOGO) Reticulon 3 Myelin basic protein
Protein processing	Ribosomal proteins	
Signal regulatory proteins	RAS-family GTP-binding protein RAP-1B GTP-binding RAB2 protein Phosphatidylinositol 3-kinase, regulatory subunit 1 Mitogen-activated protein kinase	Calmodulin 1 Rho GTPase activator DLC1 Cyclin-dependent kinase 4 Ephrin receptor B4
Cytoskeleton and cell adhesion	β-Catenin Tenascin Neuron navigator 3 α-Spectrin Laminin receptor 1 N-CAM-140/-180	Sepmaphorin 3A receptor Reelin
Apoptosis	SON DNA binding protein Cofilin 1	Annexin 1 Annexin 6 BAT3 Death inducer-obliterator 1 Nuclear factor of κB-cells inhibitor, α
Neurodegenerative disease-related	Amyloid β precursor protein binding protein 1	Amyloid β (A4) precursor-like protein Amyloid β (A4) precursor protein-binding Gelsolin

These results suggest that a single 'magic molecule' may not account for successful or unsuccessful regeneration, since many changes occur in genes associated with the immune and vascular systems, during the period when regeneration stops being possible.

These experiments set the stage for testing whether a candidate molecule does influence regeneration in injured spinal cords. In principle, one would expect a candidate molecule detected in spinal cord at 9 days to promote growth when expressed in the cervical region of the spinal cord from a 12-day-old opossum. Conversely, a candidate gene from a 12-day-old cervical cord might prevent growth when expressed in a 9 day cord. There remains the possibility however, that a gene identified as being changed between 9 and 12 days might be associated with normal development rather than regeneration. Fortunately, in the isolated opossum spinal cord, the assays for regeneration are rapid (5 days), reliable and easy to perform. Recent experiments provide grounds for optimism regarding the practicability of such assays: the complete opossum genome has been sequenced (Mikkelsen *et al.* 2007) and techniques have been developed for introducing genes into the opossum central nervous system by electroporation (E. Puzzolo, unpublished observations).

Discussion

What can one conclude at this stage about mechanisms that prevent regeneration in adult mammalian spinal cord? One frequent assertion is that outgrowth of axons does not occur because it is prevented by inhibitory molecules. This seems unlikely to be the complete explanation, since unmyelinated fibres fail to grow across lesions. Moreover, blocking the actions of inhibitory molecules results in at best sparse outgrowth. Another assertion is that the glial scar is responsible. This too seems unlikely, since scars develop late, long after fibres have had the opportunity, but failed, to grow across the lesion.

A major reason for our trying to make a comprehensive list of genes that might promote or prevent regeneration was the idea that multiple molecules and mechanisms might be involved. For example, in adult mammalian spinal cord, some neurons might not be stimulated by injury to produce axonal outgrowth, quite apart from axons being inhibited from growing through the site of a lesion. An obvious pitfall in the approach outlined here is the way in which candidates were selected. We can only identify genes already known to play a part in other systems, and then only those that show large changes in

expression. Nevertheless, although important genes may have been missed, the isolated opossum nervous system offers a uniquely rapid and reliable bioassay for testing directly those that seem promising.

Another question that arises from results such as those obtained in leeches and opossums is: 'What biological advantages accrue to mammals from not being able to repair the central nervous system after injury?' Although no answer can be given, one possibility is that failure is the penalty paid for having a highly plastic nervous system. A frog, which can regenerate its optic nerve and can see again, never learns to correct its behaviour. If the eye that has been removed is inserted upside down the animal sees again, but always jumps for a fly in the wrong direction (Attardi & Sperry, 1963). An adult owl can never adjust its vision if a prism is placed over its eyes (Knudsen, 2004). But we can. Perhaps in mammals, sprouting by axons is permitted so as to mediate plasticity and adaptation to novel stimuli. But only over short distances of less than a millimetre. One can speculate that perhaps such growth has to be limited, by growth inhibitory molecules, to prevent chaotic connections from being formed in the brain.

Another unanswerable question concerns the relevance of work on leeches or opossums for therapies in hemiplegic patients. The last sentence in many papers on spinal cord regeneration in animal 'models' often contains a type of mantra that states: 'The results presented here will help to provide a therapy for patients with spinal cord injuries. . . .' (or words to that effect). One hopes that this will indeed turn out to be true. But true or not, what is completely lacking is a time scale. And this is of vital importance for patients with spinal cord injuries. If a patient with cancer is told by the doctor that a cure is just around the corner, and it fails to appear, the patient is disappointed and probably angry at having been misled. For a patient with a new spinal cord injury, the situation is far more serious. To face the reality that repair will not happen is a prerequisite for training the patient to lead the fullest possible life in spite of the disability. The Paralympics and countless individual examples show what can be achieved, with immense effort, after spinal cord lesions. If such a patient is given false hopes (and this happens in newspapers, on TV and in clinics) and hears 'The cure is just around the corner!', there is a natural temptation to wait and put off the hard work of rehabilitation. While effective treatments may well be in the offing, they will almost certainly be no use for those patients with old lesions that occurred, say, one or two years earlier. In one's fondest dreams it is hard, at present, to imagine a cure that would enable an *atrophied* spinal cord to regain functions. An analogy for thinking about the development of a new therapy is the repair of a watch. An unskilled person challenged by a broken (mechanical) watch needs to understand the functions of each moving part, and then diagnose exactly where the problem lies. If asked on what day the watch will work again, the reply would surely be that the exact day and time cannot be foretold, until the point is reached when only one last part needs to be replaced. The spinal cord is millions of times more complicated than a watch; we do not know how it is put together in the first place, and we seem to be far from knowing how to repair the broken pieces. It is reasonable to hope for therapies in the long run since, in principle, stem cells could provide replacements for damaged tissue (Daniela *et al.* 2007). What cannot be said is *when* this will be practicable, for what types of patients, or from what type of research the cure will arise.

The approach outlined in our experiments on leeches and opossums is therefore directed not toward the clinic, but toward understanding mechanisms that promote and prevent regeneration. The animals are not 'models'. Rather they provide preparations for a worthy task – increasing our natural knowledge of basic physiological functions.

Personal note by J.G.N.

Stephen Kuffler used to say at the time that Ken Muller and I were in David and Torsten's group within the Department of Neurobiology, 'Remember, John, *these* are the good old days!' He was right. For one thing, there were the wonderful experiments being made on vision; for another, there was the feeling of friendship and collegiality. What a pleasure to hear David talk about music and to teach bright undergraduate students with him at Harvard in the evenings (Bio 166). What a pleasure to go to the theatre with Torsten and to take a trip with him to Mayan ruins in Central America (where we nearly bought a small parcel of land on a ruin).

But David and Torsten added an important extra dimension to my life. Since I have always been incapable of doing administrative work of any sort, I paid my dues to the universities where I worked by teaching neurobiology, including visual system, as well as I could. I also taught and still teach for IBRO in many poorer countries (under Torsten's auspices). And still today, in 2009, students all over the world, in India, China, Peru, Paraguay, Vietnam, Nigeria, Uganda, Iran, Jordan and Pakistan (to name a few out of many more places) become fired up and passionately keen to learn about neurobiology, whenever they hear for the first time about Torsten and David's classical work on visual cortex, which has been added to, but never surpassed in all these years.

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