

*Recovery from Spinal Cord Injury: will Genomics and Proteomics of the South American Opossum *Monodelphis domestica* Provide an Answer?*

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Spinal Cord Injury

A severe spinal cord injury is a condition for which there is currently no effective curative treatment. Clinical care of such patients has increased remarkably since the middle of the last century when paraplegics (i.e. people with a complete lesion of the spinal cord in the chest region leading to paralysis and loss of sensation in the upper limbs) survived at best for a year or two, but now have a more or less normal life expectancy. The change for quadriplegics (lesion in the neck leading to complete paralysis and loss of sensation in all four limbs) has been even more dramatic. In the 1950s, such patients barely survived at all. Now they are approaching a normal life expectancy. The incidence (number of new cases per year) is about 16 per million in Australia and 40 per million in the US. Most of the injuries occur in young risk takers, particularly from road accidents. Some patients do show remarkable degrees of recovery for largely unexplained reasons. This makes assessment of therapies extremely difficult, once they become available from animal studies. For the many that do not make much recovery, the burden on the patient, family and carers is prolonged and the lifelong financial cost is enormous (over \$1M for paraplegics, \$2M for quadriplegics).

What is often not realised, except by patients themselves, is that it is dysfunction of the autonomic nervous system controlling bodily functions such as bowel and bladder together with severe chronic pain that are the main problems afflicting many patients, rather than the loss of the ability to stand up and walk. The inability of the spinal cord to repair itself and recover function is in contrast to what happens in most other tissues of the body. For example, if skin, liver or kidney are damaged, those organs have substantial capacity for repair. It was long thought that the reasons why the spinal cord and brain do not show functionally effective repair following injury are because almost all nerve cells are postmitotic and damaged nerve fibres of surviving neurons do not have any capacity to grow again (regenerate). These limitations now turn out to be untrue. The adult nervous system does have regions where precursor or stem cells can give rise to new neurons (1) and damaged nerve fibres (axons) do have some capacity to grow again (2). However, for reasons that are only beginning to be unravelled, neither process seems to result in any functionally useful repair in the spinal cord.

Spinal Cord Regeneration in the Immature Central Nervous System

New axon growth is prevented in damaged spinal cord by the formation of a local scar by accumulating astrocytes (one type of satellite cell in the central nervous system) and by specific mechanisms that inhibit axon growth (3,4,5). The lack of axon growth in response to injury in the adult mammalian spinal cord is in contrast to what happens in injured peripheral nerves (6), in the central nervous system (CNS) of non-mammalian vertebrates (7) and in the immature nervous system of mammals (8). The propensity of the peripheral nervous system to regenerate following injury and a belief [only recently substantiated by experimental evidence (9,8)] that the immature CNS would have a substantial capacity to support regenerative axon growth has led to a huge experimental effort to devise therapies for spinal cord injury that involve implantation of a wide range of cells and tissue into the site of injury.

In experimental animals many such strategies appear to result in some degree of improved function, but it is not always clear whether this is local recovery only within the spinal cord or is recovery controlled from the brain. The former would be of little value to a patient. A secondary problem is that many different injury models and repair strategies have been tried, with very little attempt at replication. As has been the case historically in many fields of medicine, lack of evidence has not inhibited some doctors from trying treatments on patients particularly for severe untreatable medical conditions. In the case of spinal injury there are a few centres where implantation of fetal CNS tissue or specific cell types (e.g. olfactory ensheathing cells, 10) into the site of a spinal cord injury is being tried. The largest series of such operations, in over 300 patients, has been carried out in China. Only seven of these patients have been assessed independently (11). Five had the serious complication of meningitis (infection of the covering layers of the brain), which can be fatal. None was functionally improved. Many of the others were claimed to have improved, but the assessment of the patients was not "blind" and there were no controls, which is a difficult ethical problem, but generally regarded as essential if effectiveness of a treatment is to be established.

Monodelphis domestica as a Model for Spinal Cord Regeneration

Our approach has been a developmental one using neonatal opossums (South American grey short-tailed opossum, *Monodelphis domestica*, Fig. 1). Marsupials have the experimental advantage that the young are born at an extremely immature stage of development. With respect to the spinal cord and brain, a newborn opossum is equivalent to an embryonic day (E) 13-14 rat (12). This means that experiments can be carried out on the equivalent of embryonic stages of brain and spinal cord development in animals that are already outside the mother. This avoids complicated intrauterine surgery on fetuses, such as would be required in rodents. It also means that the mother does not have to be killed at the end of the experiment. In addition, behavioural testing following injury is an essential part of the experimental approach. *Monodelphis* are much better than rodents at and require little encouragement to do key tests, such as swimming and climbing, which demonstrate whether the brain has reconnected to the spinal cord. Compared to Australian marsupials, South American opossums have the advantage that they breed all the year round, have multiple young (litters of six to ten) and are relatively easy to handle and house. They are the size of a small rat (Fig. 1).

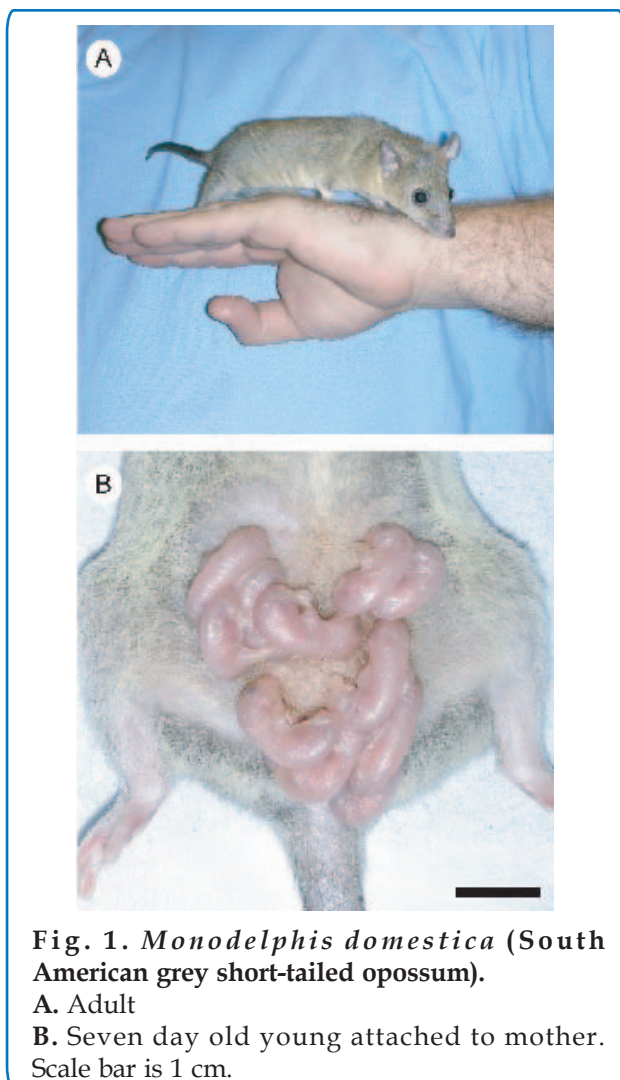


Fig. 1. *Monodelphis domestica* (South American grey short-tailed opossum).

A. Adult

B. Seven day old young attached to mother.

Scale bar is 1 cm.

If the spinal cord of a one to two week old opossum is cut completely across in, for example, the mid-thoracic region, new axons begin to grow across the lesion and the animals show a considerable degree of normal locomotor (walking, climbing and swimming) activity by the time they have grown up (13, 14). Their autonomic nervous system function also appears to be substantially normal, although we have not so far studied this directly.

The locomotor recovery in *Monodelphis* has also been shown in the related opossum species the North American opossum *Didelphis virginiana* (9). If the lesion is made after about three to four weeks of age, no axons grow across the lesion and there is no functional development below the level of the lesion. The functional development following a lesion at one week of age could be occurring because of the re-establishment of local neural circuits that become connected to axons descending from the parts of the brain involved in controlling limb movement and other functions. However, our evidence is that it is more likely to involve growth of new axons from the injured ones, the cell bodies of which originate in the parts of the brain involved in locomotor and sensory control such as the brainstem and midbrain. This we have demonstrated by injecting the spinal cords of opossums, that have recovered from a spinal transection made when they were one week old, with a fluorescently labelled molecule that is taken up by the axons below the lesion and transferred back to the cell bodies of origin of the axons in the brain (15). This is illustrated in Fig. 2. In the left panel FluoroRuby dye, injected into the lumbar spinal cord one day before transection, only labels those brainstem neurons that have axons extending down the spinal cord to the injection site (red). The right panel illustrates some of the brainstem neurons that have been back-labelled with FluoroRuby. By examining the spinal cord at different times after injury, the growth of labelled axons across the injury site can be followed. These axons can be labelled with a second labelled dextran (e.g. Oregon Green, not illustrated). Brainstem neurons retrogradely labelled with both markers are those that regenerated axons across the injury. Brainstem neurons that are labelled with the second marker are those that grew axons across the injury for the first time (i.e. were not yet present at the time when the cord was injured).

Of the axons present in the thoracic spinal cord at the time when a complete transection was made, about 50% regenerate new axons that cross the lesion (8). It is proving difficult to determine their contribution to the functional recovery seen in the older animals, because in addition to the axons regenerating from the injured ones, about ten to 20 times as many axons grow across the lesion as a part of normal development. These axons were not yet present at the site of the lesion when it was made.

That recovery from injury in the immature spinal cord is not a peculiarly marsupial phenomenon is shown by an old, little-known experiment in Italy by Migliavacca in 1928, who showed that cutting the spinal cord in E15 embryonic rats could be followed by recovery once the animal had been born and allowed to grow up.

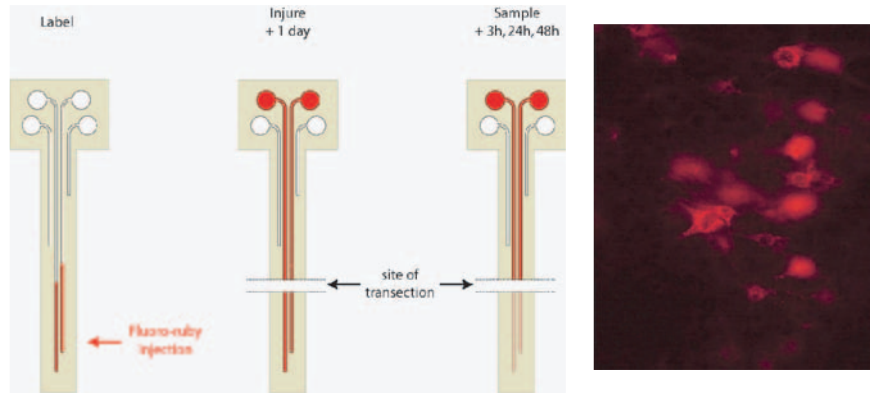


Fig. 2. **Left:** FluoroRuby dye, injected into the lumbar spinal cord one day before transection, only labels those brainstem neurons that have axons extending down the spinal cord to the injection site (red). **Right:** brainstem neurons back-labelled with FluoroRuby. By examining the spinal cord at different times after injury the growth of labelled axons across the injury site can be followed.

Development of *M. domestica* Microarrays and Proteomics

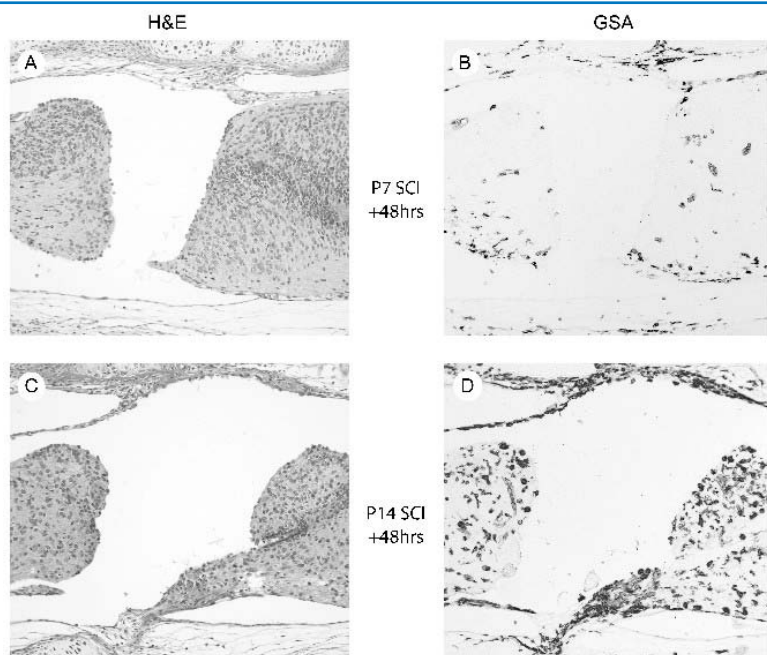
Our quest, which is only just beginning, is to understand the molecular and cellular changes that occur between an age when axon growth and functional recovery occur (e.g. one week of age) and a stage when it does not (e.g. one month of age). At the cellular level we are using morphological methods including immunocytochemistry and electron microscopy to define differences in cellular distribution in spinal cords injured at one, two and four weeks of age. One clear-cut age-related difference lies in the immune response at one and two weeks of age. Following injury to the cord at one week of age there is only a minimal accumulation of microglia and macrophages; while accumulation is much greater in animals with cord lesions made at two weeks of age (Fig. 3).

At the molecular level we are using microarray analysis and are about to start parallel proteomic analysis to try to determine which genes and proteins may be critical in determining the successful repair in animals with injuries to their spinal cords in the first week or so of life. A technical problem with microarray analysis at this stage of the project is that there are no

specific *Monodelphis* arrays available. This will change over the next year or two, because of an NIH-supported program to sequence the *Monodelphis* genome (Dr P. Samollow, personal communication). However, we have been able to make a start using mouse arrays from Superarray (Bethesda, Maryland, www.superarray.com) designed for specific gene clusters, for example, for inflammatory or growth factor genes. The problem with using interspecies arrays is that we are likely to miss some genes of interest because of lack of cross-hybridisation and we may be misled by false positive hybridisations. Fortunately there is now sufficient information available from the *Monodelphis* genome project for it to be possible to prepare RT-PCR primers to some of the *Monodelphis* genes, that in screening against mouse arrays appear to be significantly up- or down-regulated, compared with uninjured controls or cords injured at different ages. Preliminary results from the mouse arrays, which we are currently validating with RT-PCR, support the finding from the cellular studies that the local immune response following spinal injury is significantly different, even when there is only a week between the ages at which the lesions were made (one and two weeks).

Fig. 3. Sagittal sections through *Monodelphis* spinal cord 48 hours following injury (SCI) at postnatal day seven (A and B) and 14 (C and D). Hematoxylin and eosin staining.

A and C show tissue morphology. Microglia and macrophages were detected using *Griffonia simplicifolia* (GSA) lectin histochemistry (B and D). Note the greater accumulation of these cells in animals injured at two weeks of age (P14).



From our preliminary results it is apparent that many cytokines and chemokines are up-regulated, and a few are down-regulated in both seven day (P7) and 14 day (P14) old animals, within three hours of injury. However, individual inflammatory molecules appear to be affected to a different extent at the two ages. For example, the cytokines TGF- β_1 , TGF- α , IL-6 and chemokines Ccl3, Ccl20, Ccl25 and Cxcl13 in P7 animals were upregulated two to eight times more following injury than in P14 animals following injury. In contrast, Ccr7 was upregulated to a greater extent at P14 than at P7, whereas IL2 and IL20 were downregulated in P7 animals but barely so in P14 animals following injury. The plan is to extend these studies to later times after injury using additional gene arrays and eventually to screen against a *Monodelphis*-specific array. These gene expression studies will be complemented by parallel proteomic studies to confirm that the protein products of the up- or down-regulated genes are indeed present at different levels. It is already clear that the microarrays will provide a plethora of data that may be difficult to interpret, let alone utilise to develop novel therapies for spinal cord injury. However, the aim would be to determine the key upstream regulatory genes and manipulate these in such a way as to return the adult spinal cord to a more youthful stage when it would be able to produce a functionally useful regenerative response. Neonatal *Monodelphis* is currently the only mammalian system in which sufficient regeneration occurs following spinal cord injury for it to be possible for the biological processes involved to be studied directly.

This is a long-term strategy, but seems worth pursuing since it is clearly a different approach from all of those currently being tried in adult animals, none of which have yielded an effective therapy for patients even after 20 or more years of research. So the answer to the question posed in the title of this article is: not yet, but perhaps in the not too distant future.

References

1. Rietze, R.L., Valcanis, H., Brooker, G.F., Thomas, T., Voss, A.K., and Bartlett, P.F. (2001) *Nature* **412**, 736-739
2. Cajal, S.R. (originally 1913-1914) in *Cajal's Degeneration and Regeneration of the Nervous System* (1928: May, R.M., translated; 1991: DeFelipe, J., and Jones, E.G., eds), Oxford University Press
3. McKerracher, L., and David, S. (2004) *Nat. Med.* **10**, 1052-1053
4. Schwab, M.E. (2004) *Curr. Opin. Neurobiol.* **14**, 118-124
5. Buchli, A.D., and Schwab, M.E. (2005) *Ann. Med.* **37**, 556-567
6. Sunderland, S. (1991) *Nerve Injuries and their Repair: a Critical Appraisal*, Churchill Livingstone
7. Nicholls, J.G. (1987) *Magnes Lecture Series Vol. 2*, Sinauer
8. Fry, E.J., Stolp, H.B., Lane, M.A., Dziegielewska, K.M., and Saunders, N.R. (2003) *J. Comp. Neurol.* **466**, 422-444

9. Wang, X.M., Terman, J.R., and Martin, G.F. (1998) *J. Comp. Neurol.* **398**, 83-97
10. Feron, F., Perry, C., Cochrane, J., Licena, P., Nowitzke, A., Urquhart, S., Geraghty, T., and MacKay-Sim, A. (2005) *Brain* **128**, 2951-2960
11. Dobkin, B.H., Curt, A., and Guest, J. (2006) *Neurorehab. Neur. Rep.* **20**, 5-13
12. Saunders, N.R., Adam, E., Reader, M., and Møllgård, K. (1989) *Anat. Embryol.* **180** 227-236
13. Saunders, N.R., Deal, A., Kitchener, P., Knott, G.W., Nicholls J.G., and Smith T.J. (1998) *J. Neurosci.* **18**, 339-355
14. Saunders, N.R., and Dziegielewska, K.M. (2000) in *Degeneration and Regeneration in the Nervous System* (Saunders, N.R., and Dziegielewska, K.M., eds) Harwood Press
15. Migliavacca, A. (1928) *Neonati. Boll. Soc. Med-Chir.* 1147-1151

