Copy cats go natural

Cloned mammals have bred naturally for the first time, researchers reported last month, but faced the most trying of times. Two endangered African wildcat clones have each given birth to a litter, a total of eight kittens in all. The first five kittens were born on July 26 to Madge, a clone of the wildcat Nancy. The second litter, consisting of three kittens, was born on August 2 to Caty, also a clone of Nancy. The father of both litters is Ditteaux, a clone of the African wildcat Jazz, who made headlines when he was born as the result of the transfer of cryopreserved embryos to a domestic cat.

"By improving the cloning process and then encouraging cloned animals to breed and make babies we can revive the genes of individuals who might not be reproductively viable otherwise, and we can save genes from animals in the wild," said Betsy Dresser, director of the Audubon Center for Research of Endangered Species, where they were born.

She said skin samples of a long-dead but genetically valuable animal, if properly preserved, could be cloned to create a genetic match of the animal. These genes could then be introduced back into the population through natural breeding.

"The goal is to use whatever tools we can to help boost these populations," she said.

But the Audubon Center is in New Orleans and the announcement was made 10 days before Hurricane Katrina struck and at the time of going to press there was no news on how the center had fared.

Kitten issues: Researchers announced the birth of African wildcats from cloned parents in New Orleans, days before the hurricane struck. (Photo: Audubon Center.)

Primer

Regeneration of the adult central nervous system

Lauren C. Case and Marc Tessier-Lavigne

The human nervous system is an extraordinarily complex structure, and the things that we value most in life, such as cognition and being able to sense and interact with the external world, depend upon its proper wiring. Neurons transmit information along their axons, elongated processes analogous to cables, which connect their cell bodies to target cells. The nervous system can be divided into two distinct divisions, the central nervous system (CNS), which comprises the brain and spinal cord, and the peripheral nervous system (PNS), which consists of the peripheral nerves that innervate the body. One of the ways in which these two compartments of the nervous system markedly differ lies in their capacity to regenerate after injury. In stark contrast to the PNS, where severed axons often will heal and successfully navigate back to their original targets, injured CNS neurons exhibit a burst of stymied growth but ultimately fail, with their axons stalling out and forming distinctive large endings dubbed 'retraction bulbs' that fail to transverse the site of injury (Figure 1). These morphological changes are thought to reflect regeneration failure. Although axotomy can cause neuronal death, many neurons will survive months to years after injury, particularly if their axons have been severed far from their cell bodies.

Clinically, the CNS's inability to re-wire after injury is exemplified by patients who have suffered a spinal cord injury. Although damage to the spinal cord causes general trauma, killing neurons and supporting glial cells at the site of injury, this disorder can be viewed as primarily a re-wiring

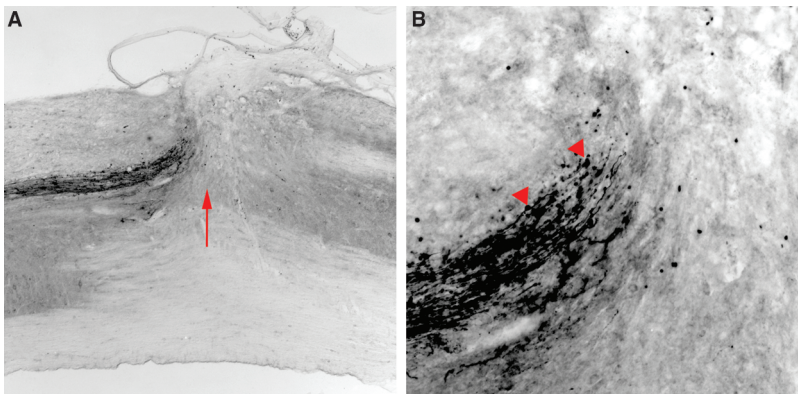

Figure 1. In the adult vertebrate CNS very few, if any, axons regenerate past a lesion site.
(A) In this sagittal view of an injured mouse spinal cord, where rostral is left and dorsal is top, neurons that project from the brain to the spinal cord have been labeled with a dye (black) that allows them to be visualized. Eight weeks after injury, axons remain stalled out near the site of transection (red arrow). (B) A higher magnification image shows characteristic retraction bulbs (arrowheads) at the tips of these axons.

problem as most pathological symptoms, including numbness and paralysis, reflect the permanent interruption of the flow of information from ascending sensory and descending motor tracts. Injuries to the lower spinal segments generally render patients unable to walk or control their bladder and bowels, while injuries to the cervical levels of the spinal cord can cause additional deficits including the loss of arm movement and an inability to breathe without a respirator. In addition to obvious quality-of-life concerns, the CNS's failure to rewire has enormous clinical ramifications for such patients, as reduced mobility often precipitates fatal complications including pneumonia, pulmonary emboli and septicemia.

Current therapies aim to save remaining intact fibers by minimizing secondary damage. The standard treatment often involves stabilization of the spine and administration of methylprednisolone, a steroidal drug that minimizes inflammation and may decrease the number of neurons that die as a result of swelling. After this initial pharmacological intervention, treatment is limited to physical therapy or, more rarely, experimental stem cell transplants. There is great optimism in the field that understanding more about the molecular mechanisms that prevent CNS regeneration will spur targeted therapies that will shift treatment goals from palliative care to regeneration and restoration of function after injury.

Reasons for optimism

Why do many scientists believe that, with appropriate intervention, CNS neurons would be capable of regeneration? One could easily imagine that central neurons are simply hard-wired in such a way that they will not repair under any circumstance. Several lines of evidence argue against this possibility. First, as touched upon earlier, axons in the PNS are capable of regenerating after injury. This is an interesting phenomenon, as many neurons with processes inside the PNS also have an axon or part of an axon within the CNS, yet only the peripheral portions are able to regenerate. These paradoxical growth responses suggest that the neurons' interactions with the two different environments may contribute to their dissimilar regenerative responses and that the PNS, but not the CNS, environment is a permissive substrate for regenerative outgrowth.

Furthermore, numerous comparative studies have revealed phylogenetic differences in the capacity of different species to regenerate. Whereas axons in the CNS of warm-blooded vertebrates (mammals and birds) do not regenerate, those in many lower vertebrates, including newts and salamanders, can regenerate after injury. Very young mammals, birds and certain amphibians are also often capable of substantial CNS repair [1]. These data suggest that the lack of CNS regeneration in vertebrates is the result of a recent evolutionary change, although it is still unclear whether these varied responses are caused by differences in expression of genes that are conserved across these organisms or by the presence of proteins specific to warm-blooded vertebrates.

Although one hesitates to draw too many conclusions from these disparate data, observations of robust regrowth in peripheral nerves, and in the central tracts of young mammals and in adult lower vertebrates, lend hope that the constraints on regeneration involve sufficiently few players that pharmacological interventions might be designed that can stimulate injured tracts to regenerate functionally.

Anatomy of the injury site

CNS axons are normally surrounded by insulating layers of lipids called myelin sheaths, made by a type of cell called an oligodendrocyte. After injury, the severed tips of these neurons encounter a varied terrain as they attempt to regenerate, contacting components present in the intact CNS as well as those unique to the injured CNS. Following traumatic insult, the distal portion of a severed axon will degenerate and the resulting debris from it and its surrounding myelin sheath persists along the lengths of the degenerated tracts until the fragments are cleared by macrophages as well as another type of cell termed microglia.

Although they share many structural similarities, the PNS environment differs from that of the CNS. To begin with, PNS axons are myelinated by Schwann cells rather than oligodendrocytes, glial cells that are morphologically and physiologically distinct from their CNS myelinating counterparts. CNS and PNS injury can also be

distinguished by how quickly degenerating tissue is eliminated. The cleaning-up of axon fragments and myelin debris is many times more efficient in the PNS than in the CNS, a feature probably explained by the involvement of different cellular players as well as differential access to the immune system.

The histology of a lesion varies somewhat depending on the type of injury sustained, but generally in the CNS the epicenter is quickly invaded by a host of cell types, including fibroblasts, vascular endothelial cells and macrophages. Surrounding this area is a zone of glial cells filled with astrocytes, oligodendrocyte precursors and microglia. Astrocytes in this region become markedly hypertrophic, and these 'reactive astrocytes' go on to form a dense network called a glial scar. This particular scarring response is unique to the CNS, as there are no astrocytes in the PNS.

In the field's effort to define what goes wrong after injury, the neurons' intrinsic growth state, the glial scar, myelin debris, and invading cells from the periphery have all in turn been investigated as likely suspects involved in inhibiting CNS regeneration.

Barriers to regeneration

Embryonic and adult neurons differ substantially in their intrinsic growth potential, with their axons switching perinatally from an elongating to a more branching phenotype. This shift in growth ability led researchers to postulate that the intrinsic growth state of adult CNS neurons disfavors regeneration [2]. Although mammalian central neurons generally do not regrow after injury, particular situations have been described which 'precondition' these cells for later regenerative outgrowth. This phenomenon has been documented in a type of bipolar sensory neurons, cells whose peripheral, but not central, branch normally regenerates after injury. However, if the peripheral branch is lesioned before the central branch, the neuron is somehow 'primed' such that the central fiber will now sprout some distance after a CNS lesion, showing that the central branch is indeed capable of regeneration [3,4]. The manipulation of signaling pathways by elevating the level of cyclic (c)AMP can similarly change a neuron's propensity to regenerate. Indeed, CNS regeneration can be enhanced *in vivo* by delivering a cAMP analog or by administering rolipram, which inhibits an enzyme that blocks the breakdown of cAMP [5].

Keeping in mind that intrinsic factors play an important role, classic experiments by Aguayo and colleagues [6] argue that environmental cues also ultimately dictate whether or not an adult neuron will regenerate after injury. In a clever set of experiments these researchers used segments of PNS nerve to connect rats' spinal cords with their brain stems. Remarkably, they observed that many CNS axons grew out from the spinal cord more than three centimeters into this peripheral nerve graft. This experiment was particularly exciting because it showed that, in a suitable environment, CNS neurons are capable of extending processes as adults. This recognition galvanized efforts to determine which molecular differences between the PNS and CNS environment makes the latter inhospitable for the re-growth of adult neurons. While the CNS may lack certain positive factors present in the PNS [7], much attention has focused on molecules present in the CNS that actively block regeneration.

The glial scar

Several lines of evidence suggest that the glial scar contributes to regeneration block [8]. When dissociated adult neurons were injected into rats that had sustained CNS injuries, researchers observed that many of these transplanted cells grew considerable distances, hindered only when they approach the glial scar. The addition of growth factors similarly increased the ability of CNS axons to grow, but they still stalled out at the scarred region. After traumatic insult, a number of extracellular molecules of the chondroitin sulfate proteoglycan (CSPG) family are elevated in the scar tissue, leading researchers to propose these molecules as candidates for mediating the inhibitory activity of the scar.

Consistent with this possibility, the application of chondroitinase ABC (chABC), an enzyme which selectively degrades CSPGs, leads to clear regeneration *in vivo* following various types of CNS lesion [8,9]. This negative effect of CSPGs on regenerative outgrowth may reflect a direct effect on the axons. Alternatively, as these highly charged molecules bind many molecules, it is conceivable that neurons are inhibited by distinct factors that associate with CSPGs. As chABC treatment has been shown to be a robust and reproducible method of increasing CNS regeneration, this line of research should prove exciting to follow in coming years as the molecular players are delineated.

Inhibitory proteins within CNS myelin

As previously mentioned, the clearance of myelin debris is extremely slow within the adult CNS. As these remnants persist for weeks and months after injury, the possibility was raised that residual myelin contains factors that actively prevent injured neurons from regenerating. Numerous *in vitro* experiments are consistent with this hypothesis; for example, dissociated neurons are impeded from extending axons when plated on either purified myelin extracts or substrates of myelin-rich CNS tissue [10,11]. Various approaches that target myelin lead to some regeneration *in vivo*; examples include experiments where animals were first irradiated in order to impair the formation of myelin-producing oligodendrocytes, or immunized with myelin extracts [11].

Over the past decade three prominent myelin-derived inhibitors have been identified: myelin associated glycoprotein (MAG), Nogo-A, and oligodendrocyte myelin glycoprotein (OMgp) [12]. Surprisingly, although these three inhibitors are structurally distinct, work from the last several years

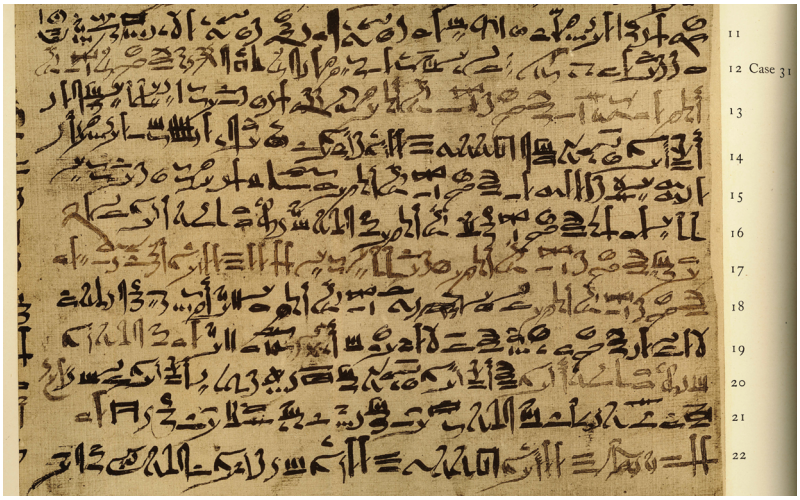

Figure 2. The Edwin Smith surgical papyrus.

The earliest known reference to CNS injury is from an Egyptian text written about 2500 B.C. [24] "When you examine a man with a dislocation of a vertebra of his neck, and you find him unable to move his arms, and his legs…Then you have to say: a disease one cannot treat". Courtesy of the New York Academy of Medicine Library.

has suggested that all three bind a receptor complex containing the Nogo receptor (NgR), the low affinity neurotrophin receptor ($p75^{NTR}$) and/or the $p75^{NTR}$ relative TROY [13]. Signaling through this receptor complex is thought to inhibit neurite outgrowth by activating RhoA and ROCK, molecules known to regulate cytoskeletal dynamics [14]. Hopes remain high that this striking convergence of inhibitory signaling pathways may allow for simultaneous targeting of multiple inhibitory constituents of CNS myelin.

Knockout mice for many of these molecules, including Nogo, MAG, NgR and $p75^{NTR}$ have been generated, and mutant animals have also been assessed for their potential to regenerate after injury. Although some lines demonstrate evidence of increased regeneration, the absence of a robust regenerative phenotype in others suggests that redundancies in myelin cues or synergy with other inhibitory factors ultimately prevent successful regrowth ([15,16] and references therein). In general, the relative contributions of each of these individual factors to regeneration block remains unclear. Future analysis of various double and triple knockout animals should be instructive.

Guidance molecules as contributors to regeneration block

Other factors that are strong candidates for contributing to regeneration block are members of the netrin, semaphorin, ephrin and slit families of axon guidance molecules. Many of these proteins instruct neurons' directional choices during embryonic development through repulsive or inhibitory actions on axons [17]. Aspects of successful regeneration recapitulate developmental events, and the potent ability of some of these molecules to repulse embryonic axons underscores the possibility that they inhibit regeneration in the adult. Although many axon guidance molecules are downregulated when development is complete, some of them remain expressed in the adult, and others are reexpressed following injury in components of the glial scar, in myelin, or in gray matter [18,19].

The contributions of these molecules to regeneration block are only now starting to be defined. In one recent study [20], striking regeneration was observed following spinal cord injury in mice mutant for an ephrin receptor, EphA4. Various members of the semaphorin family of molecules are highly expressed after injury, and recent *in vitro* work has shown that one of these proteins, Sema5A, likely contributes to the regeneration block of neurons which project from the retina to the brain [18,21]. Many of these repulsive axon guidance ligands and their receptors are expressed in patterns consistent with their playing a role in the regeneration block; in particular, netrin-1, Sema4D and ephrinB3 have all been shown to be expressed by CNS myelin, and evidence has been obtained that Sema4D and ephrinB3 contribute to the inhibitory activity of myelin *in vitro* [19,22,23]. It will be important to ascertain to what extent they contribute to the observed stalling out of axons after injury.

Conclusions

Despite the passage of forty-five centuries since an anonymous ancient Egyptian described the catastrophic effects of CNS injury [24] (Figure 2), it wasn't until the last 20 years that we have begun to understand some of the molecular events that contribute to regeneration block. As researchers continue to define and characterize the critical barriers to regeneration, it will be interesting to assess to what extent recovery is possible. Many investigators have been pondering and started to tackle critical 'next-step' questions. Once past the lesion site, do regenerating fibers successfully navigate back to their former target cells? If so, do they form functional synapses? Are treatment strategies that repair a clean knife-swipe (cut) lesion pertinent to the complex contusion (crush) injuries that comprise the vast majority of human cases? Will therapies be limited to recent injuries, or might people benefit who have sustained spinal cord damage years or even decades before?

This is clearly a time of great excitement and hope — and a belief that understanding the molecular processes that prevent regeneration could shift treatment options from palliative care to targeted restorative therapies. Although it may prove difficult to completely recover lost function, successfully encouraging neurons

to grow just a spinal segment or two could translate into dramatic quality-of-life improvements. For instance, short-distance restoration of spinal circuitry could allow patients with cervical injuries to breathe independently without a respirator, or those who have sustained lumbar injuries to increase mobility and regain bowel and bladder function. The field of CNS regeneration is alive and bursting with potential; the next decade holds the promise of exciting progress.

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Requirement for high-level processing in subliminal learning

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We are constantly learning new things as we go about our lives, and refining our sensory abilities. How and when these sensory modifications take place is the focus of intense study and we report here that even subliminal learning, which occurs without awareness of what is learned, requires high-level processing.

Some researchers have proposed that sensory plasticity can only take place on features a person attends to [1,2], but others have shown sensory improvements can occur for unattended features [3,4]. In the latter case, subliminal motion vectors were learned when they were temporally correlated with the targets of the subject's task [3]. This led to the view that successful recognition of the task-targets triggers a diffuse learning signal that enables learning of features temporally correlated with the task-targets. We have directly tested this proposition to ascertain what level of processing is required for this subliminal learning.

We used the attentional blink paradigm [5]: an imbalance in identification accuracy of two masked targets presented in rapid succession; the first target is seen but the second not. The attentional blink is mostly studied within the context of a rapid serial visual presentation (RSVP). For example, in our experiment, participants were trained on the identification of two target digits (T_1, T_2) presented within a series of distractor letters (Figure 1). Each stimulus is presented for 100 ms, and subjects must hold