

## Abstracts of Oral Presentations

### 1 AMACRINE-SIGNALED LOSS OF INTRINSIC AXON GROWTH ABILITY BY RETINAL GANGLION CELLS

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The central nervous system (CNS) loses the ability to regenerate early during development, but it is not known why. The retina has long served as a simple model system for study of CNS regeneration. Using this model system, we have found that amacrine cells signal neonatal rat retinal ganglion cells (RGCs) to undergo a profound and apparently irreversible loss of intrinsic axon growth ability. Concurrently, retinal maturation triggers RGCs to greatly increase their dendritic growth ability. These results suggest that adult CNS neurons fail to regenerate not only because of CNS glial inhibition but also because of a loss of intrinsic axon growth ability. We have recently found that the amacrine-induced loss of axon growth ability requires gene transcription and we are currently doing gene chips studies to investigate its molecular basis. We hope that understanding the molecular basis of the loss in growth ability will lead to new pharmacological approaches for enhancing the rate of axon regeneration in patients.

### 2 EVIDENCE FOR FUNCTIONAL REGENERATION OF THE VISUAL PATHWAY IN ADULT RATS

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Mature nerve cell axons do not spontaneously regrow within their natural environment unless they are stimulated by external application of growth-promoting measures. In the present study, axonal regrowth and restoration of visual function was studied in adult rats. The optic nerve was completely cut, and its proximal and distal stumps were realigned and sutured back together. During the same surgical procedure, the lens was lesioned in order to induce secondary intraocular inflammation, which is known to strongly support the survival of retinal ganglion cells (RGCs) and to promote axonal regeneration within the distal segment of the optic nerve. The neuroanatomical data showed that cut axons can regenerate over long distances within the white matter of a central nerve like the adult optic nerve, spanning over 11 mm to the chiasm and between 12 and 15 mm to the thalamus and midbrain. Responsiveness of the pupil to light was restored five weeks after injury, thus indicating reinnervation of the pretectal nuclei. Restoration of the ascending pathway between the retina and visual cortex was assured by recording flash visual evoked potentials (FVEPs). As expected, no FVEPs could be recorded during the postsurgical period of axonal growth throughout the optic nerve and tract. FVEPs could be recorded after two months, indicating that synaptic transmission in higher visual areas was established. The recording findings and the restoration of pupillary responses suggest, for the first time, that lentogenic stimulation of RGCs is sufficient to induce the formation of growth cones that can override putative inhibitors at the site of injury.



**3 ACUTE AND DELAYED OPTIC NERVE REGENERATION IN THE ADULT RAT: SLEEPING FIBRES AWAKENED, PUTATIVE CENTRAL NERVOUS SYSTEM (CNS) INHIBITORY SIGNALS IGNORED, DE NOVO SCAR FORMATION INHIBITED AND ESTABLISHED SCARS DISPERSED**

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The axons of retinal ganglion cells (RGC) regenerate in the transected optic nerve (ON) after both intravitreal implantation of a segment of sciatic nerve (ISN), and lens injury (LL). In animals without ISN/LL, RGC axons fail to regenerate. Zonation develops in the lesion within 24h post lesion (pl). Proximal (P) and distal (D) convex membranes, formed by astrocyte processes, circumscribe the wound, defining an astrocyte free zone (AFZ) in which macrophages amass. PAFZ and DAFZ contain the haemorrhagic lesion core. By 24hpl in both regenerating and non-regenerating ON, the largely myelin-free PAFZ, is filled with regenerating axon sprouts which invade the haemorrhagic core. The DAFZ contains the retraction bulbs of the severed distal axon segments. In the non-regenerating ON, scar tissue is deposited from 3/4dpl eccentrically, within the distal wall of the haemorrhagic zone into which few regenerating fibres have penetrated. In the regenerating ON, axon sprouts grow randomly in all zones of the lesion and, by 2/3dpl penetrate the distal astrocyte membrane and thereafter course centrally within the distal ON segment. Axon sprouts in the PAFZ in the non-regenerating ON see little myelin and stop growing in the non-myelinated haemorrhagic core where Semaphorin III, NG2, and EphA2 are expressed. Regenerating axons penetrating the distal ON stump grow unperturbed within myelin debris, reactive astrocytes and NG2-positive synantocytes. NgR is not modulated in the retina or ON by ON transaction, but NogoA, Neuropilin/PlexinA1, EphA4 and Ephrin B2 are down-regulated in both sites.

Moreover, p75 is cleaved into 55 and 25kDA fragments in the retina of ISN rats. No scar is formed in the regenerating ON and when ISN is delayed 8, 10, 12 and 15dpl regenerating axons penetrate the established ON scar and little/or no evidence of the original cicatrix is detectable at 28-35dpl. Evidence that metalloproteinases (MMP) secreted by regenerating RGC axons modulate scarring is supported by up-regulation of MMP1/2/9 and a corresponding down-regulation of TIMP 1/2 in the retina and ON in the regenerating model.

**4 MOLECULAR AND BEHAVIOURAL CORRELATES OF OPTIC NERVE REGENERATION**

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Functional recovery after central nervous system (CNS) damage has several components which, in part, recapitulate development. Death of axotomised neurons must be minimized and axon re-growth to target appropriate tissue triggered. Regenerating axons must then re-establish appropriate functional connections in the brain that are organized as a "topographic map" which faithfully represents the distribution of neuronal cell bodies in the periphery. Here we focus on the molecular basis of topographic map restoration during optic nerve regeneration. The strict topographic representation from the retina to primary visual centres allows the accuracy of axonal regeneration to be assessed. Moreover, the differing capacity for successful optic nerve regeneration within the vertebrate phylum allows anatomical and electrophysiological as well as molecular studies of events underpinning map restoration. In fish, optic nerve regeneration is successful with return of vision. For reptiles, regeneration occurs but projections are inaccurate with axons failing to restore stable topographic maps; as a consequence blindness persists. In adult mammals, although spontaneous optic axon regeneration

does not occur, some axons regrow via a length of peripheral nerve grafted between the eye and visual centres. However, as in reptiles, topography is not re-established. We have investigated expression pattern of molecules associated with restoring topography in regenerating systems. Our results indicate that, as in development, tyrosine kinase Eph receptors and their ligands, the ephrins, are key players. It is also necessary to ensure that a balance of glutamergic excitatory and GABA-ergic inhibitory connections is maintained. A recently completed study in lizard indicates that an appropriate sequence of events can be triggered during optic nerve regeneration by training on a visual task, resulting in the restoration of vision. The result implies that restoration of function within regenerating central projections in mammals may require not only re-expression of developmentally expressed molecules but also appropriate training regimes.

##### **5 PERSISTENT AND INJURY-INDUCED NEURONAL REGENERATION IN THE VERTEBRATE RETINA**

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Persistent neurogenesis occurs in the adult nervous system of myriad vertebrate species including humans. All brains harbor stem cells, although in mammals their contribution to normal neuronal turnover or growth appears limited to specific brain regions. The presence of a reservoir of self-renewing multipotent stem cells forms a common link between the adult brain and all other adult tissues—both are capable of generating differentiated, tissue-specific cells. However, unlikelike neural tissues, the brain of most adult vertebrates has only a limited capacity to replace cells lost to injury. The neural retina of amphibians, birds and fish is an exception. Animals within each of these vertebrate classes has the capacity to regenerate retinal neurons following an injury. Interestingly, the regenerated neurons emerge from distinct cellular sources: the retinal pigmented epithelium (amphibians), Müller glia

(post-hatch chickens), or resident stem cells (teleosts). In amphibians and fish, regenerated retina is structurally and functionally a strikingly accurate recapitulation of the original. Injury stimulates the re-expression of developmental regulatory genes found in the embryonic retina, and at the conclusion of the regenerative neurogenesis, all the characteristic cellular and synaptic elements have been re-formed. In addition, regenerated retina can generate electrical responses with normal waveforms and mediate simple behaviors. In teleosts, cell type-specific lesions can stimulate cell-type specific neuronal regeneration. In birds, retinal regeneration is limited and although acute injury stimulates proliferation of retinal progenitors, only a few differentiate into mature neurons and glia. These studies demonstrate that in some vertebrate classes mature nervous tissue can be repaired by intrinsic cellular mechanisms. Understanding the intrinsic and extrinsic signaling pathways that direct the proliferation, migration and fates of injury-induced progenitors in regenerating retinas may provide new insight into the regulation of persistent and injury-induced neurogenesis in all nervous systems.

##### **6 DEVELOPMENT OF A RETINAL PROSTHESIS TO RESTORE VISION TO BLIND PATIENTS**

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Our research team is endeavoring to build a retinal prosthesis to restore vision to patients with some forms of retinal disease. As proof-of-concept, we have performed electrical stimulation of human retina in 6 volunteers during experiments that lasted hours. In the five blind patients, the electrical stimulation threshold for each patient was linearly related to the severity of visual loss, and the amount of charge required to stimulate the retina in the best cases was near to generally accepted safety levels for iridium oxide electrodes. We were not able to achieve letter recognition. Roughly 50% of the time the volunteers did





not see percepts with a form that matched the pattern of electrical stimulation, although there was 66% “reproducibility” of the form of the induced percepts. One of the volunteers had normal vision—in this special case the electrical thresholds were 25% less than the lowest values for blind patients, but we were not able to achieve better perceptual results with respect to the quality of the perceived images. These results suggest that our lack of understanding of how to effectively stimulate the retina hindered our ability to achieve better quality vision. It is reasonable to assume that experiments of this type performed with a chronically implanted prosthesis might yield more favorable results because of the opportunity for researchers to learn how to communicate with the retina and for the patients to learn how to interpret the artificial stimuli. To this end, our group is nearing completion of our custom-designed, chronically implantable microelectronic device.

#### **7 BONE MARROW STROMAL CELLS (MSCS) AS A POTENTIAL SOURCE FOR NEURAL REPAIR**

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A number of cell types have been used for cell transplantation studies in animal models to remyelinate demyelinated regions of the central nervous system. These include committed myelin-forming cells such as oligodendrocytes and Schwann cells as well as precursor cells derived from either embryonic or adult brain. We report that mononuclear and stromal cells derived from bone marrow can differentiate into myelin-forming cells and form myelin in animal models of demyelination upon direct injection into the lesion or after intravenous delivery. This opens the intriguing prospect of systemic delivery of MSCs to target sites of demyelination and to achieve myelin repair. The mononuclear fraction from autologous

bone marrow was separated on a density gradient and various concentrations of cells were delivered intravenously into either of two lesion models, 1) chemically-induced spinal cord demyelination or 2) a transient cerebral ischemia model. Extensive remyelination of the spinal cord was observed after autologous bone marrow cell delivery. Many of the myelin profiles observed subsequent to bone marrow transplantation are characteristic of peripheral myelin. In the cerebral ischemia model cells were delivered at various times from 3 to 72 hours post-infarction. Reduced infarction size and improved functional outcome was observed at all tested times, but greater improvement was observed at the shortest intervals. While these approaches are experimental in animal models, the prospect of using an expandable and renewable source of cells from bone marrow to repair the demyelinated central nervous system (CNS) or to confer neuroprotection to the infarcted CNS by systemic delivery has implications for future consideration of such an approach in man.

#### **8 THE PROS AND CONS OF OLFACTORY ENSHEATHING CELLS (OECs) IN CENTRAL NERVOUS SYSTEM (CNS) REPAIR**

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Over the last decade, the idea of using cells obtained from the peripheral olfactory system for transplant mediated repair of CNS lesions has attracted considerable interest. Evidence has come from a range of studies in animal models that examine the potential of OECs to either remyelinate experimentally-created demyelinated axons or to promote the repair of spinal cord injury. Schwann cells have also been proposed as a candidate for transplant-mediated repair, as like OECs they have a permissive role in endogenous regeneration after injury. We have shown using mainly in vitro studies that OECs may have advantages over Schwann cells in transplant-mediated repair. For example, Schwann cells do not intermingle well in an astrocyte-rich environment and have been shown to induce characteristics

typical of hypertrophy in astrocytes, namely an increase in astrocyte area and up-regulation of molecules inhibitory to axonal outgrowth, including chondroitin sulphate proteoglycans. Further work has demonstrated a role for the adhesion molecule N-cadherin in orchestrating the differences between the two glial cells. Other recent developments for the use of OECs in transplant-mediated repair have been (a) Pure OEC preparations are less good at mediating CNS repair when compared to cell preparations that contain added meningeal cells. (b) OECs may not promote reconnection of the dorsal root fibres after rhizotomy from studies using electrophysiological and immunohistochemistry methods. These studies will be discussed in relation to the use of OECs as candidates for CNS repair.

#### 9 LAMINA PROPRIA-DERIVED OLFACTORY ENSHEATHING CELLS IN SPINAL CORD REGENERATION

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Olfactory Ensheathing Cells (OECs) provide a permissive glial environment for neurogenesis of olfactory receptor neurons and regeneration in the olfactory system. Since OECs do this throughout a lifetime, there has been considerable interest in using them to promote axonal repair and regeneration in other regions of the adult nervous system, in particular the spinal cord. Lamina propria-derived olfactory ensheathing cells (LP-OECs) from olfactory mucosa, which are an accessible source in adult humans, have thus become prime candidates for autologous human transplantation to repair spinal cord injuries. Our lab is exploring a number of avenues of LP-OEC action in vivo and in vitro. When mouse LP-OECs expressing green fluorescent protein (GFP) are transplanted directly into rat and mouse rubrospinal tract lesion sites they demonstrate minimal migration, but significantly attenuate scar formation, reduce cavity formation, promote host Schwann cell invasion and form a novel glial environment to promote regeneration. In this model, profuse

axonal sprouting is seen, where multiple axons sprout across the lesion site into the distal cord, including serotonin- and tyrosine hydroxylase-positive axons, but rubrospinal axons responded less robustly. When LP-OECs are introduced rostral and caudal to the site, a differential axonal and glial response is seen. In contrast to previous studies, when LP-OECs are transplanted in an attempt to bridge the dorsal root entry zone following dorsal root avulsion, sensory neuron regeneration is not successful. So what underlies the differential abilities of OECs to enhance regeneration and CNS-PNS boundary formation in some cases, but not others? We have found, for example, that OECs lose their potency for promoting neurite outgrowth in embryonic dorsal root ganglia and neuronal survival with age in culture, which could have a profound influence on the behaviour of OECs when transplanted. I will review some of the confounding data currently in the field and discuss how some of the data from our lab and others may be reconciled and extended in order to utilize OECs in a more targeted strategy to promote spinal cord regeneration.

#### 10 SCHWANN CELLS AND OLFACTORY ENSHEATHING GLIA FOR REPAIR OF THE INJURED SPINAL CORD

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In the United States, each year the lives of 10,000 persons changes dramatically due to a spinal cord injury resulting in paralysis. Many of these people are below the age of 30 and face a lifetime of disability and life-threatening complications. Following an injury to the adult spinal cord, nervous tissue is lost progressively and regeneration of axons is poor. Consequently, descending motor axons and ascending sensory axons will be permanently interrupted, which results in partial or complete loss of voluntary movement and sensory perception in arms and/or legs. In some cases, human spinal cord injury results in complete disconnection of the spinal cord ends. In most cases, however, damage to the human spinal





cord is the result of a contusion injury. It is imperative to develop effective transplantation paradigms to limit nervous tissue loss, promote axonal regeneration and improve functional recovery in the injured cord. The reparative abilities of Schwann cells (SCs) and olfactory ensheathing glia (OEG), alone or combined, have been studied extensively in both a complete spinal cord transection and in a contusion model. OEG grafts integrate well with the spinal tissue, and result in tissue sparing and axonal regeneration/sparing. In some paradigms motor improvements following grafting has been reported. The observed improvements in motor outcome are variable between paradigms. Combining SC or OEG grafting with neuroprotection strategies, administration of neurotrophic factors, or the obstruction in the function of axonal growth inhibitors in the spinal tissue may provide means to further improve motor outcome. Such combination strategies may ultimately result in axonal regeneration from grafts into the spinal cord, which will enhance the changes for formation of synaptic connections and improving motor outcome.

#### **11 AN EARLY ANTI-INFLAMMATORY STRATEGY MARKEDLY REDUCES AUTONOMIC DYSREFLEXIA AND CHRONIC PAIN AFTER CLIP-COMPRESSION SPINAL CORD INJURY IN RATS**

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A significant degree of the post-traumatic tissue damage and subsequent neurological deficits that occur after spinal cord injury (SCI) are due to secondary reactive processes including the release of chemokines, cytokines, free radicals and proteases, the peroxidation of lipids and the influx of neutrophils and macrophages. A selective early anti-inflammatory treatment may diminish the destructive aspects of this response and limit secondary injury. We used an early anti-integrin interven-

tion consisting of a monoclonal antibody to the CD11d subunit of the Cd11d/CD18 integrin delivered intravenously at 2, 24, and 48 h after clip compression SCI. This antibody treatment attenuated neutrophil (~80%) and hematogenous macrophage (~40%) infiltration into the injured cord by blocking the interaction between vascular adhesion molecules and this cell surface integrin. Within the first 72 hr after SCI the treatment decreased ED-1 expression by 70% (estimating macrophage infiltration), myeloperoxidase activity by 40%, lipid peroxidation by 60%, iNOS expression by 50%, cyclooxygenase-2 expression by 50%, protein nitration by 70% and protein oxidation by 70%. At six weeks to three months after this treatment, the locomotor BBB score improved from 8 to 11, demonstrating the ability to weight-bear rather than simply sweep the hind legs, increases in arterial pressure due to autonomic dysreflexia were reduced by 50% and the development of chronic pain was reduced by 50%, correlating with significantly greater myelin and neurofilament within and near the lesion. In summary, intravenous treatment with the anti-CD11d antibody promotes white matter sparing by limiting oxidative damage to the spinal cord and results in generally improved neurological recovery. This early anti-inflammatory strategy could be an important foundation that precedes later regenerative treatments.

The research was supported by the ICOS Corporation, Canadian Institutes of Health Research, Ontario Neurotrauma Foundation and Heart & Stroke Foundation of Ontario.

#### **12 INTRASPINAL SPROUTING AND NEUROPATHIC PAIN FOLLOWING DORSAL RHIZOTOMY**

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Descending monoaminergic axonal systems (serotonergic, noradrenergic and dopaminergic) have complex roles in nociceptive signaling. In the absence of injury, these are thought to have primarily an anti-nociceptive role,

inhibiting pain transmission directly at the level of the primary afferent terminal, the second-order projection neuron, or indirectly via inhibitory interneurons. However, these systems may also mediate descending facilitation, enhancing pain signalling at these same loci. We have investigated changes in monoaminergic transmitter content following cervical dorsal root injuries in rats and mice, as a clinically relevant model of brachial plexus avulsions, which often produces severe intractable phantom-like spontaneous pain, as well as enhanced evoked pain in humans. In both rats and mice, cervical rhizotomy induces serotonergic and (to a lesser extent) noradrenergic sprouting in the spinal cord dorsal horn. Since myelin-derived proteins (such as NogoA, oligodendrocyte myelin glycoprotein and myelin-associated glycoprotein) have been shown to inhibit injury-induced and spontaneous plasticity of spinally-projecting axons, we examined sprouting in Long-Evans Shaker (LES) rats, which lack central nervous system (CNS) myelin in adulthood, following dorsal rhizotomy. We also used a soluble Nogo receptor (sNogoR) in normally-myelinated LE rats to pharmacologically antagonize myelin inhibitory proteins. Since the Nogo receptor is thought to signal through p75, we characterized monoaminergic sprouting in p75 null-mutant mice. Finally, since p75 has been shown to inhibit regeneration through activation of the small GTPase Rho, we used a specific antagonist of a downstream effector of Rho (Rho-kinase), Y-27632. In all cases, we found that serotonergic and noradrenergic sprouting was enhanced following dorsal rhizotomy. These results support the myelin-NogoR-p75-Rho model of axonal inhibition within the adult CNS, and suggest pharmacological routes to enhance plasticity of descending monoaminergic systems following dorsal rhizotomy. Further experiments are underway to characterize the behavioural consequences of cervical dorsal rhizotomy and pharmacological antagonism of myelin signalling.

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### 13 GROWTH AND GUIDANCE FACTORS MODULATING NOCICEPTIVE AFFERENT SPROUTING WITHIN THE ADULT SPINAL CORD

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Spinal cord injury or nerve growth factor application to the dorsal horn causes abnormal pain through undefined mechanisms. One potential mechanism is aberrant sprouting of calcitonin gene-related peptide (CGRP) and substance-P (SP) sensory afferents within the spinal cord. To examine if NGF-induced sprouting of these peptidergic axons contribute to abnormal pain, we over-expressed GFP or NGF using recombinant adenoviruses. Injections of NGF/Adenovirus (Ad), but not GFP/Ad, into adult lumbar spinal cords induced robust sprouting of CGRP(+) fibers and led to the development of thermal hyperalgesia, mechanical allodynia, and touch mediated vocalization within 2 weeks. Since NGF could contribute to abnormal pain independent of sprouting, semaphorin3A (Sema3A) was used to inhibit sprouting without affecting other NGF-mediated events. During spinal cord development, Sema3A acts as a chemorepulsive factor to restrict CGRP(+) fiber growth to lamina I & II. For these experiments, we constructed an adenovirus encoding Sema3A which demonstrated excellent in vitro chemorepulsion that was dependent on the dose of NGF. To examine sprouting within the spinal cord, we co-injected sema3A/Ad or GFP/Ad with NGF/Ad at either a high (> 10 fold above physiological conc.) or low concentration (< 8 fold above physiological conc.). Co-injections of GFP/Ad with either NGF/Ad dose resulted in robust sprouting of both CGRP(+) and SP(+) fibers and abnormal pain. Behaviorally, these animals showed thermal hyperalgesia, mechanical allodynia and touch mediated vocalization. Co-injection of Sema3A demonstrated statistically significant reductions in sprouting to normal densities, but only at the lower concentration of NGF/Ad. Interestingly, only reductions in mechanical allodynia were observed in these animals. These findings demonstrate 1) the molecular interplay between chemoattractive and chemorepulsive





factors can govern sprouting, 2) sprouting only partially contributes to NGF-mediated pain mechanisms, and 3) chemorepulsive factors may be useful as a potential therapy for controlling aberrant sprouting that results in abnormal function.

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#### 14 NOVEL ELECTRICAL STIMULATION TECHNIQUES FOR RESTORING LIMB FUNCTION

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In the absence of spinal cord regeneration after injury, techniques such as functional electrical stimulation (FES) have been developed to restore some of the lost motor functions. In this talk, I will describe existing FES systems for restoring hand and leg function after spinal cord injury (SCI). I will also present a novel FES technique, intraspinal microstimulation (ISMS), focused on restoring function by directly activating spinal circuitry through indwelling microelectrodes. We have shown in experiments conducted in intact adult cats with chronically implanted electrodes that ISMS is safe, feasible and can generate synergistic limb movements which remain stable for long periods of time. No signs of discomfort are seen even when ISMS in the ventral horn of the lumbosacral enlargement generates powerful limb movements, some capable of lifting the animals' hindquarters. More recently, we applied ISMS in chronically spinalized cats and found that modulated, fatigue resistant weight-bearing stepping can be induced using patterned stimulation paradigms. Furthermore, non-patterned, or tonic, low-level ISMS delivered simultaneously through 2–4 adjacently located electrodes in the lumbosacral enlargement can elicit rhythmic locomotor activity resulting in weight-bearing stepping of the hindlimbs. This suggests that low-level tonic ISMS can recruit local neuronal elements involved in the generation of rhythmic locomotion. In contrast to the non-load bearing locomotor activity elicited by epidural stimulation of the spinal cord in cats and humans

with complete SCI, tonic ISMS is capable of evoking weight-bearing stepping. I will discuss the use of various ISMS patterns to modulate neuronal membrane properties to reduce spastic hypertonus after spinal cord injury. I will then conclude by showing examples of the added benefits obtained by combining various therapeutic approaches, e.g., pharmacology, locomotor training and ISMS, in restoring functional walking after SCI.

#### 15 VERTEBRATE LIMB REGENERATION AND THE ORIGIN OF LIMB STEM CELLS

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Salamanders are unique among tetrapod vertebrates in their ability to regenerate their limbs as adults. As a consequence, salamander limb regeneration has been extensively studied by several generations of biologists, and there is a wealth of cellular and molecular data that have provided valuable insights into the mechanisms controlling tissue and organ regeneration. Recent advances in molecular biology, bioinformatics and functional analysis have stimulated a renewed interest in regeneration that has led to exciting new opportunities to understand regenerative mechanisms, and ultimately to stimulate regenerative responses in humans. Numerous studies have demonstrated the critical role of interactions between connective tissue fibroblasts and nerves in the control of growth and pattern formation during regeneration. Nerves provide critical factors that allow fibroblasts to dedifferentiate and form a blastema, and in turn, fibroblasts generate a blueprint that directs the pattern of growth of nerves in the newly formed limb. In order to investigate the nature of these interactions we have developed a Step-Wise Model for limb regeneration that allows for the experimental dissection of limb regeneration into discrete parts, each of which can be studied independently of the others. Progress in this endeavor is being accelerated by the availability of an EST database for regenerating axolotl limbs, and by advances in techniques for somatic cell transgenesis.



## 16 MSX GENES AND BMP SIGNALING IN MAMMALIAN DIGIT REGENERATION

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The regeneration of digit tips in mammals, including humans and rodents, represent a model for organ regeneration in higher vertebrates. We had previously characterized digit tip regeneration in fetal and neonatal stages of digit formation in the mouse and found that regenerative capability correlated with the expression domain of the *Msx1* gene. Using the stage 11 (E14.5) digit we now show that digit tip regeneration occurs in organ culture and that *Msx1*, but not *Msx2*, mutant mice display a regeneration defect. Associated with this phenotype we find that *Bmp4* expression is down-regulated in the *Msx1* mutant digit and that mutant digit regeneration can be rescued in a dose-dependent manner by treatment with exogenous BMP4. Studies with the BMP binding protein NOGGIN show that wild type digit regeneration is inhibited without inhibiting expression of *Msx1*, *Msx2* or *Bmp4*. These data identify a signaling pathway essential for digit regeneration in which *Msx1* functions to regulate BMP4 production. We also provide evidence that endogenous *Bmp4* expression is regulated by the combined activity of *Msx1* and *Msx2* in the forming digit tip, however we discovered a compensatory *Msx2* response that involves an expansion into the wild type *Msx1* domain. Thus, while both *Msx1* and *Msx2* function to regulate *Bmp4* expression in the digit tip, the data are not consistent with a model in which *Msx1* and *Msx2* serve completely redundant functions in the regeneration response. These studies provide the first functional analysis of mammalian fetal digit regeneration and identify a new function for *Msx1* and BMP4 as regulators of the regenerative response.

## 17 DETERMINING THE MOLECULAR BASIS FOR CELLULAR PLASTICITY DURING NEWT LIMB REGENERATION

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Newts and other salamanders have the remarkable ability to regenerate lost structures and injured organs, including their limbs, tails, spinal cords, retinas, lenses, optic nerves, jaws, and heart ventricles. These regenerative processes require an extraordinary degree of cellular plasticity that is observed in only a few vertebrates, such as the salamanders. For example, following the initial wound healing phase of newt limb regeneration, internal limb cells residing within approximately 2 mm of the amputation plane begin to dedifferentiate and migrate distally to form a pool of multipotent and committed progenitor cells known as the regeneration blastema. The blastemal cells later redifferentiate to form all of the cell types that comprise the internal tissues of the regenerated limb. We have previously shown that proteins present in regenerating newt limbs, but absent in nonregenerating limbs, can induce both newt and mouse myotubes to dedifferentiate (McGann et al., PNAS 98:13699-13704, 2001). In an effort to identify the genes that encode these dedifferentiation-initiating proteins, we have performed differential display analyses between regenerating and nonregenerating newt limbs and have cloned and sequenced the entire coding regions for more than 130 of the up-regulated newt genes. We are currently performing both in vitro and in vivo assays to elucidate the biochemical, cellular, and biological function of these genes during the initial stages of limb regeneration.





### 18 MOLECULAR DETERMINANTS OF SUCCESSFUL REGENERATION IN THE CENTRAL NERVOUS SYSTEM (CNS) OF ADULT ZEBRAFISH

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Successful axon regeneration in the CNS of zebrafish is accompanied by increased expression of growth-promoting cell recognition molecules, such as the L1-related immunoglobulin superfamily molecule L1.1. After a complete transection of the spinal cord in adult zebrafish, expression of L1.1 is transiently increased in axotomized supraspinal neurons with spinal axons, which correlates with axon regrowth, and in non-axotomized spinal interneurons, which may correlate with plasticity of spinal-intrinsic circuitry. In contrast, spinal neurons with axons ascending to the brain and supraspinal neurons with descending axons in certain brain nuclei show a low capacity to regrow their axons and to up-regulate L1.1. To analyze the function of L1.1 during regeneration, anti-sense morpholinos to L1.1 were applied to a spinal transection site. Morpholinos were retrogradely transported into axotomized neurons in the brain. L1.1 immunoreactivity was reduced in these supraspinal neurons and in the spinal cord gray matter at a distance of up to 4 mm around the lesion site for at least 6 weeks. Animals treated with L1.1 morpholino showed significantly decreased numbers of supraspinal neurons with regenerated spinal axons (33% of control groups) at 6 weeks post-lesion. Videotracking of freely swimming fish 6 weeks after a spinal transection revealed that L1.1 morpholino treated fish swam shorter distances and exhibited a lower average and maximal swim velocity than lesioned control fish. These observations suggest that up-regulation of L1.1 expression is a necessary component of axon regrowth from supraspinal neurons with spinal axons and for functional recovery after a spinal cord lesion. L1.1 dependent plasticity of intraspinal

circuitry may also contribute to functional recovery after injury.

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### 19 THE NEURAL RECOGNITION MOLECULE L1 AND FUNCTIONAL RECOVERY IN MAMMALS

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Recognition molecules of the immunoglobulin superfamily are mediators of important developmental events, such as neural proliferation and migration, neurite outgrowth and fasciculation, synaptogenesis and synaptic plasticity in the adult. More recent studies have shown that these molecules are also involved in regeneration after injury in the central and peripheral nervous systems of mammals. My presentation will focus on the neural cell adhesion molecule L1 which has been shown to overcome inhibitory cues that predominate in the adult central nervous system of mammals. When infused in soluble L1 form as L1-Fc dimer close to the lesion site immediately after contusion injury, L1 enhances functional recovery of hindlimb movements of adult rats. Some regrowth of axons of the corticospinal tract is detected, but does not correlate with the degree of functional recovery. L1 also beneficially enhances neuronal differentiation: Exposure of neural stem cells to L1 in cell culture increases their potential to differentiate into neurons and into particular neurotransmitter specific phenotypes. Furthermore, expression of L1 in neural stem cells enhances their ability to migrate from the injection site in the substantia nigra of one hemisphere to the other hemisphere and to differentiate into tyrosine hydroxylase positive dopaminergic neurons in an MPTP Parkinson's lesion paradigm in aged mice. Injection of these stem cells into mice ectopically expressing L1 in astrocytes under the control of the mouse glial fibrillary acidic protein promoter results in synergism of the positive effects of L1, not only for migration and differentiation, but also for survival of the

injected stem cells. Interestingly, the L1 expressing transgenic mice react less severely towards MPTP treatment when compared to their wild type control littermates. Finally, the importance of the HNK-1 carbohydrate expressed by neural recognition molecules enhances preferential regrowth of motor axons of the cut femoral nerve of adult mice into the correct nerve branch, identifying a molecular cue that determines an appropriate pathway choice. It is expected that these findings will be relevant in clinical settings of nervous system damage in humans.

## 20 CELL BODY TREATMENT STRATEGIES TO PROMOTE AXONAL REGENERATION AFTER ACUTE AND CHRONIC SPINAL CORD INJURY

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Regeneration of the injured spinal cord is hampered by an inhibitory environment associated with central nervous system myelin and the lesion site. In addition, the parental neurons in the brain fail to mount and maintain a vigorous growth response and many undergo massive atrophy or are believed to die. We have shown previously that the atrophy of rat rubrospinal neurons (RSN) after cervical axotomy could be fully reversed with the trophic factor BDNF applied to the vicinity of the red nucleus. This regimen was even successful when initiated as late as one year after injury. Importantly, we found no evidence for death of rubrospinal neurons. This treatment promoted the regeneration of the chronically injured neurons into peripheral nerve bridges which otherwise fails by 1–2 months after lesion. Unfortunately, the application of BDNF to the injured spinal cord (3 doses tested) was not successful when initiated at 2 months after injury and the number of rubrospinal neurons regenerating into PN bridges at that time was minimal. Immunohistochemistry for trkB demonstrated a marked decline along the proximal rubrospinal axon

stumps, consistent with the failure to respond to BDNF at the level of the cord. In contrast the RSN cell bodies still expressed trkB even a year after axotomy, albeit at low levels. We are currently exploring alternative delivery methods such as BDNF expressing viruses (using AAV and Lentivirus based vectors) which in our hands prevented some of the RSN atrophy in the acute setting. In addition, we are refining the cell body treatment regimen using cAMP, and observed in our preliminary series the sprouting of rubrospinal axons into and 1mm beyond the site of an acute crush of the lateral funiculus (C3).

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## 21 BALANCING INHIBITORY SIGNALS WITH GROWTH SIGNALS IN THE INJURED SPINAL CORD

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The failure of axonal growth in the injured central nervous system results from a lack of sufficient growth-stimulating factors, the absence of growth substrates in injury sites, and the presence of several inhibitors to growth. We will present evidence that the provision of growth-stimulating factors can tip the balance toward axon growth over inhibitory substrates in the injured spinal cord. Surprisingly, migrating Schwann cells are a source of both inhibitors (including NG2, a member of the chondroitin sulfate proteoglycan family of extracellular matrix molecules), and growth-stimulating factors (such as L1 and laminin) in the injured spinal cord. Overexpression of growth factors locally within sites of spinal cord injury by gene delivery increases axon growth, while paradoxically also increasing the expression of NG2 by Schwann cells. Thus, axon growth results from a balance of inhibition and stimulation,





and growth over an inhibitory milieu can be induced by delivery of sufficient quantities of growth stimulators.

## 22 THE INDUCTION OF NEURITE REGENERATION IN ADULT SPINAL CORD USING EQUINE INFECTIOUS ANAEMIA BASED LENTIVIRAL VECTORS

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Retinoic acid (RA) is a biologically active metabolite of vitamin A and is present in the nervous system in developing embryos and adult animals. In embryonal carcinoma cells RA induces neuronal differentiation and in many types of developing neurons RA stimulates the length and number of neurites. This suggests a role for RA in the nervous system. We have studied the retinoid receptor requirement for this neuronal effect and have shown that one specific receptor is activated, retinoic acid receptor b2 (RARb2). In embryonic mouse spinal cord which is stimulated to regenerate by RA, RARb2 is up-regulated, but in contrast in the adult mouse cord which does not respond to RA, RARb2 is not up-regulated. We have tested the importance of RARb2 in neurite outgrowth *in vitro* using equine infectious anaemia virus (EIAV)-based lentiviral vectors to transfect RARb2 into adult spinal cord. Neurite outgrowth was stimulated as a result. We have now begun a programme to ask whether EIAV vectors can be used *in vivo* to express high levels of RARb2 in the adult cord and dorsal root ganglia and to determine whether this can induce neurite outgrowth. We show that expression is possible *in vivo* and that the efficiency of transduction is much higher than can be achieved *in vitro*. Neurite regeneration was observed in cultured explants of transfected

cords and we will describe the results of anatomical and behavioural studies on such animals transfected *in vivo*. We have also begun a microarray analysis of the genes that are up- and down-regulated following transfection of spinal cord with RARb2.

## 23 FULLERENE NANOPARTICLES AS NEUROPROTECTANT AGENTS

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Nanotechnology is the focus of major research initiatives in the life sciences sector worldwide. It has been recognized that fullerenes and carbon nanotubes are one of the key building blocks for nanoscale materials. The fullerene molecule, a hollow sphere made up of 60 carbon atoms was discovered in 1985 as the third and unprecedented new form of elemental carbon in nature. It was dubbed buckminsterfullerene (or fullerene) because of its geodesic character. Fullerene compounds (fullerols), are ideal free-radical scavengers. The hallmark of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease or Lou Gehrig's disease is attack by oxygen radicals on neuronal tissue. Oxidative stress by oxygen radicals has been demonstrated to induce cellular instability by a cascade of events leading to programmed cell death. Abrogation of oxidative stress therefore remains a major focus of efforts for curtailing the devastating impact of neurodegenerative diseases. The excellent neuroprotectant efficacy of fullerenes reflects their ability to react with oxygen radical species such as superoxide radicals ( $O_2^{\cdot-}$ ) in addition to hydroxyl radicals ( $\cdot OH$ ) which attack lipids, proteins, DNA, and other macromolecules in the cell. When modified to allow water solubility and the ability to cross the blood/brain barrier, fullerols have been demonstrated to absorb multiple reactive oxygen radicals per fullerene molecule and to reduce the toxicity of free-radical assault on neuronal tissue. A Tris malonic acid fullerene derivative (C3 tris) has been shown to be a potent free-radical scavenger. It also inhibits the excitotoxic death of cultured cortical neurons induced by exposure to N-methyl-D-aspartate (NMDA),

amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), or oxygen-glucose deprivation. The C3-tris compound fully blocks even rapidly triggered, NMDA receptor-mediated toxicity. The C3-tris molecule also reduced apoptotic neuronal death induced by either serum deprivation or exposure to A1-42 protein. More importantly, the infusion of C3-tris compound in a transgenic mouse carrying

the human mutant (G93A) superoxide dismutase gene responsible for a form of familial amyotrophic lateral sclerosis (ALS), delayed both death and functional deterioration, in some cases by as much as 30%. Encouraging observations on the effect of fullerols in models of Parkinson's disease and occlusive stroke models have also been documented.



