SPINAL CORD TRANSPLANTS

IN A

RAT MODEL

OF

SPINAL CORD INJURY

by

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Abstract

Spinal cord injury is a devastating condition that results in permanent loss of motor and sensory function distal to the lesion. To date, there is no treatment that is able to restore function to a spinal cord injury patient. Neural transplantation is currently being studied as a potential and promising strategy for the treatment of spinal cord injury. Intraspinal transplants of rat fetal spinal cord (FSC) tissue have been shown to prevent axonal degeneration and support axonal regrowth following spinal cord injury in rodent models. However, to date there has been limited information regarding the survival and axonal regrowth of human FSC tissue transplants. In present study, we investigated the ability of human FSC tissue to survive grafting in the hemisection rat model of spinal cord injury. In order to promote axonal regrowth to the distal cord, we studied different grafting strategies that include a combination of bridge and double grafts rostral and caudal to the hemisection. In adult female, Wistar rats with spinal cord hemisections at the T9 level, a bridge graft of FSC cell suspension (E14) was placed into the transected space (bridge) and FSC cell suspensions were injected into the intact spinal cord rostrally and caudally to the lesion site (double graft). Control lesioned animals received bridge graft only, double grafts only or no graft. An additional unlesioned group received double grafts only. Animals were tested weekly on a motor grid task. At 6 to 7 weeks post grafting animals were injected with cholera toxin B (CTB) tracer in the motor cortex and sacrificed 72 hours later. Spinal cord sections were processed for serotonin (5-HT) immunohistochemistry to identify regenerating fibers. Rat FSC grafts were identified using cresyl violet stain and human FSC grafts were identified using human neuron specific enolase (NSE) immunohistochemistry. In grafted animals, human and rat FSC grafts survived and integration of the graft with the host spinal cord was observed. Intensely stained 5-HT positive axons were observed proximal to the lesion and entering the bridge graft, but not re-entering the distal host spinal cord. Significant improvement of locomotor function was observed in animals receiving double grafts only of human FSC. Improvement on the motor grid task was not observed in the other treatment groups. This study has demonstrated survival of human FSC grafts in the adult rat spinal cord which may have important implications in the possible application of neural transplantation for the treatment of spinal cord injury in humans in the future. Although a combination of transplant strategies such as bridge and double cell suspension grafting appears to facilitate at least some degree of axonal regeneration, no clear axonal regrowth to the distal spinal cord nor functional recovery was observed in these groups. However, increased motor function on the grid task was evident in the double graft only group suggesting that the bridge may be detrimental to graft-induced recovery. This observation merits further investigation.

List of Abbreviations

5-HT	serotonin
ABC kit	avidin/biotin complex kit
aFGF	acidic fibroblast growth factor
ANOVA	analysis of variance
AP	anteroposterior
B	bridge
BDNF	brain-derived neurotrophic factor
°C	degrees centigrade
CA	California
Ca^{2+}	calcium ion
CAM	cell adhesion molecules
cm	centimetres
CNS	central nervous system
CS/KS-PG	chondroitin sulfate /keratin sulfate proteoglycan
CST	corticospinal tract
CT	cytotactin / tenascin
CTB	cholera toxin subunit B
DAB	diaminobenzidine
DG	double graft
DMEM	Dulbecco's modified Eagle's medium
DNase	deoxyribonuclease
DV	dorsoventral
E14	embryonic day 14
ECM	extracellular matrix
FSC	fetal spinal cord
g	grams
GAP-43	growth associated protein - 43
H_2O_2	hydrogen peroxide
HX	hemisection
IgG	immunoglobulin G
IN-1	monoclonal antibody to NI35/250
i.p.	intraperitoneal
K^+	potassium ion
KCl	potassium chloride
kg	kilograms
KOH	potassium hydroxide
L	lateral
M	molar
MAG	myelin associated glycoprotein
mg	milligrams
$MgCl_2$	magnesium chloride
ml	milliliters
mM	millimolar

MN	Minnesota
mRNA	messenger ribonucleic acid
n	number of animals
NaH_2PO_4	sodium dihydrogen orthophosphate
N-CAM	neural cell adhesion molecule
NGF	nerve growth factor
NGS	normal goat serum
NHS	normal horse serum
NSE	neuron specific enolase
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
PB	phosphate buffer
PBS	phosphate buffered saline
PN	peripheral nerve
PNS	peripheral nervous system
s.c.	subcutaneous
SEM	standard error of the mean
T8	thoracic vertebra 8
T9	thoracic vertebra 9
T10	thoracic vertebra 10
T13	thoracic vertebra 13
WA	Washington
μl	microlitres
μm	micrometres

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1. *Introduction*

1.1 Historical highlights

Severe trauma to the spinal cord produces a number of adverse events which lead to neuronal death and axonal degeneration eventually resulting in functional loss characterized by the absence of sensory information and paralysis of muscles below the level of the lesion. Historically, there has been a pessimistic attitude towards the treatment and outcome of individuals suffering spinal cord injury. Nearly 5,000 years ago, an unknown Egyptian physician described the events following spinal cord trauma in the Edwin Smith Surgical Papyrus, and concluded that spinal cord injury was "an ailment not to be treated" (Guttman, 1976). This hopeless outlook was the prevailing belief in the medical community well into the twentieth century. Sir Ludwig Guttman (1976) summarized the views held toward spinal cord injured victims following the First World War: "Most of those men who managed to survive dragged out their lives as useless and hopeless cripples, unemployable and unwanted. ...[T]he view generally held was the sooner [the patient] died the better for all concerned." With the advent of the Second World War, an enormous number of spinal cord victims were anticipated and governments prepared for the overwhelming number of spinal cord cases by organizing spinal cord units as part of general military hospitals. The medical teams in these units were composed of doctors and nurses, as well as physiotherapists and occupational therapists. The primary objective was to "rescue these men and women from the human scrap-heap and to return most of them, in spite of their profound disability, to the community as useful and respected citizens." (Guttman, 1976). This concept of rehabilitation has continued as the basic premise of spinal cord injury treatment. Today, the treatment of secondary spinal cord injury complications, such as respiratory problems, renal failure, bladder infections, pressure sores, and physical and social rehabilitation, is the cornerstone of medical treatment for

spinal cord injury victims.

Although the life expectancy for spinal cord victims has improved significantly with time, few gains have been made in improving the neurological function lost at the time of injury. There has been no significant advance in the restoration of motor and sensory function affected by the spinal cord injury. In North America there are currently more than 250,000 individuals suffering from sensory and motor dysfunction as a result of trauma to the spinal cord, with 1,000 new cases reported annually in Canada alone (CORD, 1997). The majority of spinal cord injuries result from motor vehicle accidents, followed closely by falls, violence and recreational sports (NSCISC, 1998; CPA, 1997). The average lifetime cost for health care and living expenses can exceed $750,000 per individual (Zompa et al., 1997). This produces a huge financial burden on the patient, the health care system and society, which combined with the devastating physical and emotional consequences of spinal cord injury makes this condition one of the most important areas of biomedical research.

The devastating consequences of spinal cord injury has lead to the search for new strategies to repair the injured spinal cord. Neural transplantation is a promising approach that may result in restoration of motor and sensory function. The work described in this thesis explores the application of neural transplantation as a strategy for spinal cord repair.

1.2 Pathophysiology of spinal cord injury

The devastating neurological deficits observed following traumatic spinal cord injury are a consequence of massive cell death and damaged axonal pathways at the lesion site and in the area adjacent to the lesion. Experimental evidence suggests that two injury mechanisms are at play following spinal cord trauma: these are primary injury and secondary injury (Tator, 1991; Tator and

Fehlings, 1991). Primary injury to the spinal cord is due to direct tissue damage caused by the injury mechanics, such as compression, contusion or laceration (Tator and Fehlings, 1991; Zhang and Guth, 1997). Primary injury leads to further tissue injury that is caused by pathophysiological changes within the spinal cord elicited by the primary injury. The secondary damage has been attributed to edema, inflammation and vascular changes, including reduction of blood flow, loss of autoregulation and hemorrhage (Tator and Fehlings, 1991; Zhang and Guth, 1997), as well as electrolytic changes, such as increased intracellular Ca^{2+} and extracellular K^{+}. Biochemical changes also play an important role in secondary damage, including free radical production and arachidonic acid release (Tator and Fehlings, 1991). These secondary events lead to further neuronal death causing expansion of the lesion site followed by cavity formation and the development of a glial scar (Dusart and Schwab, 1994; Zhang et al., 1997).

1.3 Axonal reaction to injury

During embryonic development several proteins, such as growth associated protein-43 (GAP-43) and the cytoskeletal proteins actin and tubulin, are present in elevated amounts in the central nervous system (CNS) (Jacobson et al., 1986; Skene et al., 1986; Benowitz and Routtenberg, 1987; Skene, 1989). Heightened expression of these proteins is associated with the growth of newly developing axons. Specifically, GAP-43 appears to be important in the formation and modification of neuronal synapses (Benowitz and Routtenberg, 1987), while actin may play a role in growth cone motility (Smith, 1988). In the adult mammalian CNS these proteins are still present, but they are expressed at lower levels than during development (Jacobson et al., 1986; Curtis et al., 1993b).

Following axotomy, these growth associated molecules are re-expressed in elevated levels in the peripheral nervous system (PNS) and CNS (Tetzlaff et al., 1991; Strittmatter et al., 1992; Curtis et al., 1993a; Doster et al., 1991; Kobayashi et al., 1997). Sustained increases in GAP-43 are observed in fibers in the PNS which are capable of undergoing extensive regeneration (Skene and Willard, 1981; Benowitz and Routtenberg, 1987; Ide, 1996; Fu and Gordon, 1997), while only transient increases in GAP-43 and cytoskeletal proteins are observed in the CNS (Tetzlaff et al., 1991). The transient upregulation of growth associated proteins in the CNS is accompanied by sprouting of injured neurons (Doster et al., 1991; Tetzlaff et al., 1991). Similar to the short lived re-expression of the growth associated molecules this axonal sprouting is also temporary. Shortly following injury, sprouting axons in the dorsal columns of the adult rat can no longer be detected. The axons persist in this retracted state and in most cases form terminal swellings at the tip of the axons (Lampert and Cressman, 1964; Pallini et al., 1988; Schnell and Schwab, 1993).

1.4 Molecular environment for axonal regeneration

For many years it was generally accepted that axons of the PNS spontaneously regenerate following injury while damaged axons of the CNS do not have that capacity. However, the temporary sprouting response of axotomized CNS fibers indicate that these axons may possess the ability to regenerate, suggesting that the CNS environment may not be permissive for long distance axonal regeneration. Further evidence for this hypothesis was provided when investigators found that injured peripheral nerves (PN), which spontaneously regenerate in vivo, do not regenerate when placed into the CNS environment (Aguayo et al., 1979). In addition, when CNS tissue is placed in a PN environment axonal outgrowth from the transplanted tissue is observed

(Benfey and Aguayo, 1982; Richardson et al., 1984; So and Aguayo, 1985; Carbonetto et al., 1987). Two hypotheses have been put forward to explain the absence of regeneration in the adult mammalian CNS following injury.

One hypothesis is that in the adult mammalian CNS the absence or limited expression of growth promoting molecules prevents axons from regenerating following axotomy (Fraser et al., 1984; Thanos et al., 1984; Lander, 1989; Reichardt and Tomaselli, 1991). In addition to GAP-43, actin, tubulin and other molecules, including cell surface molecules, such as neural cell adhesion molecule (N-CAM), and components of the extracellular matrix (ECM), such as laminin, fibronectin and heparan sulfate proteoglycan, are involved in neural development and regeneration (Sanes, 1983; Lander, 1989; Reichardt and Tomaselli, 1991; Joosten, 1994; Joosten et al., 1996). During development cell adhesion molecules (CAM) and ECM components promote axonal growth and guidance (Fraser et al., 1984; Thanos et al., 1984; Reichardt and Tomaselli, 1991), and in the mature PNS, regeneration of injured axons is accompanied by the increased synthesis of these adhesion molecules (Fu and Gordon, 1997). The absence or limited expression of these molecules in the adult mammalian CNS may play a role in preventing axonal regeneration following injury. ECM components, laminin, fibronectin and heparan sulfate proteoglycan are absent in most mature CNS tissue (Carbonetto et al., 1987). However, high levels of laminin are found in animal species in which CNS tissue supports axonal regeneration, such as the goldfish optic nerve (Hopkins et al., 1985; Liesi, 1985) and in the embryonic rat spinal cord (Carbonetto et al., 1987). These studies support the notion that the adult mammalian CNS may not provide the environmental components necessary for axonal regrowth.

Neurotrophic factors have also been shown to play an important role in promoting axonal growth during development (Snider and Johnson, 1989). In particular, neurotrophin-3 (NT-3) is highly expressed during development of the

CNS, and decreases markedly as the CNS matures (Masonpierre et al., 1990). The exogenous application of NT-3 to damaged spinal cord axons increases the extent of axonal regeneration (Bregman et al., 1997b; Grill et al., 1997b). Recently, many investigators have examined the effects of additional neurotrophic factors on CNS regeneration. Several studies have demonstrated that the exogenous administration of neurotrophic factors, including brain derived neurotrophic factor (BDNF) (Bregman et al., 1997b; Novikova et al., 1997; Kobayashi et al., 1997), neurotrophin 4/5 (NT-4/5) (Kobayashi et al., 1997), and nerve growth factor (NGF) (Oudega and Hagg, 1996; Grill et al., 1997a), prevent atrophy and axonal retraction and promote axonal regeneration.

However, even in the presence of exogenously supplied growth promoting molecules, extensive axonal regeneration is not observed. More recently, a second hypothesis that the presence of neurite growth inhibiting factors may arrest axonal elongation following injury was put forward(Carbonetto et al., 1987; Crutcher, 1989; Savio and Schwab, 1989; Schwab et al., 1993). The theory of an inhibitory mechanism responsible for preventing CNS neurite outgrowth arose in the mid 1980s when Schwab and Thoenen (1985) found that CNS neurons regenerate in vitro into PN tissue but not into CNS tissue, such as optic nerve explants. The inhibitory effects of CNS tissue was confirmed by further studies (Carbonetto et al., 1987; Crutcher, 1989; Savio and Schwab, 1989; Bandtlow et al., 1993). An inhibitory component was isolated and found to be two proteins closely associated with CNS white matter, called NI 35/250 (Caroni and Schwab, 1988; Savio and Schwab, 1989). The experimental evidence to support the involvement of NI 35/250 in neurite growth inhibition was obtained from two separate experimental models, in one model using x-irradiation (Savio and Schwab, 1990), and in a second model a monoclonal antibody to NI 250, called IN-1 (Caroni and Schwab, 1988; Schnell and Schwab, 1993; Weibel et al, 1994; Bregman et al., 1995; Tatagiba et al., 1997). In the first experiment newborn rats

were x-irradiated to prevent oligodendrocyte development and myelin formation and then underwent transection of the corticospinal tract (CST). In x-irradiated, myelin-free animals axonal regeneration was observed 2-3 weeks following transection (Schnell and Schwab, 1990). Similar results were observed in the second experiment in which adult rats were treated with a monoclonal antibody to NI 35/250 (IN-1) (Rubin et al., 1994). Long distance axonal regeneration was observed in the optic nerve (Weibel et al 1994) and spinal cord of IN-1 treated animals (Caroni and Schwab, 1988; Schnell and Schwab, 1993; Bregman et al., 1995; Schwab and Brosamle, 1997). In addition, application of IN-1 to the damaged CNS resulted in enhanced plasticity of neurons within the brainstem and spinal cord (Schnell et al., 1994; Z'Graggen et al., 1998). Axonal regeneration in IN-1 treated rats was also associated with functional recovery as assessed by footprint analysis, grid testing and reflex testing (Bregman et al., 1995).

The inhibitory effects of CNS myelin were further demonstrated by the identification of another myelin-associated inhibitor of axonal outgrowth, called myelin associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Tang et al., 1997). Purified MAG prevents fiber outgrowth of CNS neurons in vitro, which is reversed when antibodies to MAG are administered (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Tang et al., 1997). However, myelin purified from MAG deficient mice still exerts inhibitory activity of neurite outgrowth in vitro (Li et al., 1996; Ng et al., 1996), suggesting that MAG may act in combination with other factors to inhibit axon regrowth following injury.

Injury to the CNS is usually followed by the formation of a glial scar (Dusart and Schwab, 1994). This response is characterized by hypertrophy and aggregation of astroglial cells at the lesion site to form a dense mass of scar tissue and has been interpreted as an attempt to maintain the integrity of the tissue adjacent to the injury site while limiting contact with the external

environment (Bovolenta et al., 1992). However, the glial scar may also act as a mechanical barrier to regenerating CNS axons (Reier and Houle, 1988; Houle, 1992). Furthermore, the glial scar may serve as a source of inhibitory growth factors for regenerating CNS axons. Cytotactin/tenascin (CT) and chondroitin sulfate/keratin sulfate proteoglycan (CS/KS-PG) are present in increased levels in the glial scar surrounding CNS lesions (Snow et al., 1990; McKeon et al., 1991). Tissue culture experiments have demonstrated that regenerating axons avoid areas where CS/KS-PG are present, suggesting that the presence of CT and CS/KS-PG in the glial scar may serve an inhibitory function following CNS injury (Snow et al., 1991; Snow and Letourneau, 1992). Recently reactive glia at the lesion site was demonstrated, in vivo, to be directly associated with failure of axon growth (Davies et al., 1997).

1.5 Peripheral nerve transplantation studies

The use of peripheral nerve tissue as a strategy to repair spinal cord injury was stimulated by the knowledge that peripheral nerves regenerate following injury, in contrast to CNS tissue which is less suitable for axonal regeneration. Early studies in the field of spinal cord injury transplanted segments of PNS tissue into spinal cord lesions in rats and other mammals (Sugar and Gerard, 1940; Barnard and Carpenter, 1950; Feigin et al., 1951; Kao, 1974; Richardson et al., 1980; Richardson et al., 1982; Richardson et al., 1984). One of the early spinal cord studies reported new nerve fibers growing into and traversing the length of transplanted sciatic nerve in the completely transected adult rat spinal cord (Sugar and Gerard, 1940). Following this early report several investigators attempted to duplicate the findings but failed (Barnard and Carpenter, 1950; Feigin et al., 1951). In contrast to the early study, they did not observe axonal regeneration into a peripheral nerve graft and speculated that in the Sugar and

Gerard investigation the spinal cord may not have been completely transected and that the remaining un-cut fibers were responsible for the improvement of function (Feigin et al., 1951). This is an important issue since the assessment of the degree of injury is crucial to transplantation experiments.

The field of PN transplantation in the spinal cord was left virtually untouched until the 1970s. At this time Kao and colleagues (1970) transplanted cerebral and cerebellar tissue into the transected adult dog spinal cord to investigate the effects of transplants on wound healing in the injured spinal cord. Following the observation that the transplants aided in the wound healing process, sciatic nerve and peripheral ganglion tissue were transplanted into the transected spinal cord of the adult dogs (Kao, 1974). Although Kao was once again interested in the effects of the transplants on scar formation, he found that the PN implants facilitated the regrowth of host axons. In 1977, Kao and colleagues confirmed their earlier findings using electron microscopy. They demonstrated that axons originating in the host spinal cord had entered and crossed sciatic nerve transplanted into the lesion site of a completely transected adult dog spinal cord (Kao et al., 1977a). This observation was confirmed by numerous studies in the early 1980s (Richardson et al., 1980; Richardson et al., 1982; Richardson et al., 1984). These studies demonstrated that grafts of peripheral neural tissue induce regeneration of injured axons in the adult rodent spinal cord. However, although CNS axons traversed the grafts to the distal end, the majority of axons did not re-enter the host cord. The few axons that did penetrate the host-graft interface were restricted to distances of less than one millimeter (Richardson et al., 1980; Richardson et al., 1982; Richardson et al., 1984). Other studies in the adult rat model used PN bridges to connect the spinal cord and the brain stem (David and Aguayo, 1981; David and Aguayo, 1985). In these studies injured spinal cord fibers were observed innervating the

PN bridges for distances up to 30 millimeters; however, they did not re-enter the CNS environment.

Despite limited results, research on PN transplantation is still on-going (Houle, 1991; Jaeger et al., 1993; Matsuyama et al., 1995; Cheng et al., 1996; Cheng et al., 1997; Ye and Houle, 1997, Asada et al., 1998). The main objective is to devise a strategy that permits axonal regeneration into the host spinal cord caudal to the lesion which leads to functional recovery. Recently Cheng and colleagues (1996) transplanted multiple intercostal nerves combined with the neurotrophic factor, acidic fibroblast growth factor (aFGF) into a completely transected adult rat spinal cord. The animals demonstrated improved motor function as assessed on several behavioral tests. In addition, anterogradely labeled corticospinal tract fibers were observed in the thoracic and lumbar spinal cord caudal to the lesion in treated animals. Retrograde tracing also revealed labeled neurons in brainstem nuclei and the motor cortex (Cheng et al., 1996). Additional studies have further investigated PN transplantation and neurotrophic factor treatment to promote spinal cord regeneration (Ye and Houle, 1997) and functional recovery (Cheng et al., 1997). However, the majority of research in recent years has focused on the use of fetal neural tissue transplantation which has been applied in other neurological disorders, such as Parkinson's disease and may be applicable to spinal cord injury.

1.6 Fetal Neural Tissue Transplantation Studies

Fetal neural tissue has been used in several transplant strategies for the spinal cord and is thought to act in several ways when transplanted into the adult host. Fetal tissue grafts can serve as a bridge for regenerating axons (Reier et al., 1986; Bregman, 1987; Jakeman and Reier, 1991; Diener and Bregman, 1998a) or as a site for relay of synapses (Privat et al., 1986; Jakeman et al.,

1989; Privat et al., 1989; Itoh and Tessler, 1990 a,b; Itoh et al., 1993; Houle et al., 1996). Mature neural tissue transplants have been shown to resemble areas of the normal spinal cord suggesting that fetal tissue may replace damaged neurons and restore spinal cord architecture (Reier et al., 1985; Reier et al., 1986; Jakeman et al., 1989). Fetal transplants have also been shown to be effective at reducing or preventing the formation of the glial scar barrier at the host-graft interface (Kruger et al., 1986; Bregman and Kunkel-Bagden, 1988; Tessler, 1991). In addition, fetal neural tissue has been shown to prevent atrophy and the subsequent death of axotomized neurons in the brainstem (Bregman and Reier, 1986; Bernstein-Goral and Bregman, 1997; Mori et al., 1997; Bregman et al., 1998; Diener and Bregman, 1998b) and lumbar enlargement (Diener and Bregman, 1994; Himes et al., 1994; Shibayama et al., 1998). These findings suggest that fetal tissue may rescue damaged neurons by releasing trophic factors.

a) Fetal transplants as a bridge

Several studies have demonstrated that both rat and human fetal spinal cord tissue can survive and differentiate when transplanted into an injured rat spinal cord (Bernstein et al., 1984; Pallini et al., 1989; Jakeman and Reier, 1991; Wictorin and Bjorkland, 1992; Theele et al., 1996; Giovanni et al., 1997; Zompa et al., 1997). As observed in peripheral nerve grafting, transplanted fetal tissue grafts have been shown to provide an environment suitable for regenerating axons. In the adult rat spinal cord injury model damaged spinal cord axons regenerate through fetal spinal cord (FSC) placed at the site of injury, although regrowth is limited to the transplant (Reier et al., 1986; Jakeman and Reier, 1991; Stokes and Reier, 1992). In the neonatal spinal cord injury model regenerating and late developing fibers grow across the FSC bridge and even re-enter the distal host spinal cord (Bregman, 1987; Bregman and Bernstein-Goral,

1991; Bernstein-Goral and Bregman, 1993; Bernstein-Goral et al., 1997; Bregman et al., 1997a,b). In the neonate animal model the FSC bridge facilitate axonal regeneration such that transplanted animals are almost indistinguishable from normal unlesioned animals on tests of motor function (Kunkel-Bagden and Bregman, 1990; Iwashita et al., 1994; Bregman et al., 1997b; Miya et al., 1997; Tessler et al., 1997; Diener and Bregman, 1998a).

b) Fetal transplants as a synaptic relay station

Fetal tissue may also allow the formation of functional connections within the graft, thereby serving as a synaptic relay station between the proximal and distal host spinal cord (Privat et al., 1986; Jakeman et al., 1989; Privat et al., 1989; Itoh and Tessler, 1990 a,b; Itoh et al., 1993; Houle et al., 1996). This has been supported by experiments on regenerating dorsal root axons (Itoh and Tessler, 1990 a, b). Using immunhistochemistry and anterograde tracing regrowing dorsal root axons were observed to form connections within the fetal transplant. These synapses were similar in distribution and structure to synapses made in lamina I of the dorsal horn in the normal intact spinal cord, suggesting that fetal transplants may support the formation of normal connections. Functional synapse formation by regenerating descending supraspinal neurons has also been demonstrated (Rajaofetra et al., 1992; Reier et al., 1992; Feraboli-Lohnherr et al., 1997). These regenerating fibers exhibited a pattern of reinnervated terminals in areas of the lumbar spinal cord, that are similar to the distribution of terminals in normal animals. The formation of functional synapses by regenerating neurons was further demonstrated by electrophysiological studies (Reier et al., 1992; Feraboli-Lohnherr et al., 1997)

c) Fetal transplants replace damaged neurons

There is also evidence that FSC transplants integrate with the host spinal cord and may replace neurons damaged by spinal cord injury (Reier et al., 1985; Reier et al., 1986; Jakeman et al., 1989). Differentiation of FSC transplants may be necessary to establish functional circuitry between the proximal and distal host spinal cord (Jakeman et al., 1989). Mature neurons in the transplant may serve as projection neurons to innervate areas of the host spinal cord or develop their own intrinsic circuitry (Jakeman et al., 1989). Differentiated FSC tissue grafts contain areas that are similar to regions of the normal adult spinal cord (Reier et al., 1985; Reier et al., 1986; Jakeman et al., 1989). Specifically, the development of substantia gelatinosa-like regions has been demonstrated, which is characterized by the presence of unmyelinated primary afferent fibers, small neurons and immunostaining for peptides found in the substantia gelatinosa of the normal adult spinal cord (Reier et al., 1985; Reier et al., 1986; Jakeman et al., 1989).

d) Fetal transplants as inhibitors of glial scar formation

Successful regeneration through a transplant and into the host spinal cord caudal to the lesion depends on the ability of regenerating axons to penetrate the host-graft interface. As previously described, injury to the spinal cord is followed by formation of a glial scar (Kao, 1977a,b; Dusart and Schwab, 1994), which is usually complete within two weeks of the injury (Kruger et al., 1986). The resulting dense network of astrocytic processes acts as a physical barrier (Reier and Houle, 1988; Houle, 1992) and may also be a source of inhibitory factors for regenerating axons (Snow et al., 1990; McKeon et al., 1991; Snow et al., 1991; Snow et al., 1992). The presence of a fetal CNS transplant in the brain has been shown to delay and in some regions inhibit the formation of the glial boundary (Kruger et al., 1986; Bregman and Kunkel-Bagden, 1988; Tessler, 1991). More

recently, it has been reported that FSC tissue grafts can modify an already existing glial scar and prevent the formation of a newly scarred tissue in chronic spinal cord lesion in the adult rat (Houle and Reier, 1988; Houle, 1992).

e) Fetal transplants rescue damaged neurons

Transplants of fetal tissue have also been shown to be effective in rescuing and preventing cell death of axotomized neurons. Axotomy leads to atrophy and eventual cell death of the majority of the neurons located in the red nucleus (Bregman and Reier, 1986; Bernstein-Goral and Bregman, 1997; Mori et al., 1997; Bregman et al., 1998; Diener and Bregman, 1998b) and Clarke's nucleus (Diener and Bregman, 1994; Himes et al., 1994; Shibayama et al., 1998). FSC transplants prevent the death of the majority of neurons that would have died in both the red nucleus (Bregman and Reier, 1986; Tetzlaff et al., 1994; Bernstein-Goral and Bregman, 1997; Mori et al., 1997; Bregman et al., 1998; Diener and Bregman, 1998b) and Clarke's nucleus (Diener and Bregman, 1994; Himes et al., 1994; Shibayama et al., 1998). The application of the neurotrophic factor, brain derived neurotrophic factor (BDNF) prevents the death of axotomized rubrospinal neurons (Diener and Bregman, 1994; Novikova et al., 1996; Kobayashi et al., 1997), while in both newborn and adult rats the neurotrophic factor, NT-3 rescues neurons in Clarke's nucleus (Diener and Bregman, 1994; Shibayama et al., 1998). A similar rescuing effect is also seen with neurotrophic factors applied to the spinal cord on axonal regrowth and restoration of functional recovery (Tetzlaff et al., 1994; Grill et al., 1997a,b), suggesting that fetal transplants may produce neurotrophic factors that rescue and prevent neuronal cell death.

Further support for this hypothesis comes from work investigating the effects of target versus non-target transplants on axonal regeneration (Bregman and Kunkel-Bagden, 1988; Himes et al., 1994). Transplants that are the normal

target tissue for descending axons were found to promote neuronal survival and axonal regeneration, whereas in most instances non-target tissue did not (Bregman and Kunkel-Bagden, 1988; Himes et al., 1994). These findings suggest that the fetal transplants that promote axonal regrowth provide trophic factors that rescue axotomized neurons (Bregman and Kunkel-Bagden, 1988; Himes et al., 1994). In fact, the target tissue transplants and the non-target transplant that promoted axonal outgrowth (neocortical tissue) contain high levels of mRNA for the neurotrophic factor, NT-3 (Maisonpierre et al., 1990; Himes et al., 1994). The non-target tissue investigated (fetal striatal tissue) contains very low levels of mRNA for NT-3, and subsequently were shown not to rescue axotomized neurons (Himes et al., 1994).

1.7 Clinical transplantation trials

Clinical application of intraspinal transplantation has been limited. In Moscow, investigators at the Brain Research Institute have transplanted human fetal neocortex tissue into forty-one chronically injured spinal cord patients over the past decade. Analysis indicated a very slight improvement in sensory function over two, three and sometimes five dermatomal levels. The results are difficult to interpret due to the inability to perform histological analyses on the grafted spinal cords. The primary conclusion is that the grafting procedure itself is safe, since mortality was nonexistent and patients did not report feelings of pain (Reier et al., 1994). More recently, a team of neurosurgeons in Florida transplanted human fetal spinal cord tissue into a patient suffering from syringomyelia, a disorder of the spinal cord characterized by the presence of fluid-filled cavities in the spinal cord. The main objective of this clinical trial is to determine if FSC tissue can survive for prolonged periods of time. These initial attempts to use neural transplantation to treat human spinal cord injury are

premature since most researchers believe that before neural transplantation for spinal cord injury can be employed in human clinical trials, as an approach for treating spinal cord injury, a clear understanding of the efficacy of neural transplantation is required using animal models.

1.8 Project objectives and hypothesis

a) Establish a model of spinal cord injury

The first objective of this project is to establish a reliable rat model of spinal cord injury in our laboratory. This part of the project should allow us to have a standardized animal model in which to test transplant strategies designed to promote axonal regeneration and functional recovery. We have chosen to explore two models: complete spinal cord transection model and the hemisection model. These models were chosen because they produce a clear disruption of spinal cord axons resulting in a distinct sensorimotor deficit that could be tested and quantified and are also suitable for neural transplantation experiments.

b) Double grafting as a strategy for spinal cord repair

Current treatment for spinal cord injured patient focuses on preventing secondary damage, rather than restoring spinal cord circuitry and functional loss resulting from the primary injury. Recent studies have shown that fetal transplants may promote axonal regeneration, suggesting that neural transplantation has the potential to reconstruct spinal cord circuitry and possibly restore sensory and motor function. Although fetal transplants have been shown to promote some degree of neuronal survival and increase axonal regrowth, circuit reconstruction and functional recovery is limited. We propose a novel

technique of double grafting fetal tissue into the adult rat spinal cord to enhance the extent of axonal regrowth, circuit reconstruction and functional restoration.

The double grafting technique has been used previously in animal models of Parkinson's disease to increase the degree of axonal regrowth over long distances by guiding regenerating axons to appropriate targets (Mendez et al., 1996; Mendez and Hong, 1997; Mehta et al., 1998). The success of the double grafting technique in circuit reconstruction and functional recovery in the Parkinson's disease model supports the idea that double grafts in spinal cord injury may promote axonal regeneration by guiding fibers into the distal host spinal cord and eventually restore function. **We hypothesize that the double grafting technique will promote axonal regeneration and improve hindlimb motor function in a rodent model of spinal cord injury.**

Using either the complete transection or the hemisection model of spinal cord injury, lesioned animals will receive bridge grafts and/or double grafts of rat or human FSC cell suspension. The use of human FSC tissue will be very helpful in identifying transplants in the rat spinal cord and may provide important information in regards to the ability of human FSC cells to survive transplantation and restore spinal cord circuitry and functional loss. Functional improvement will be assessed using a grid locomotor test. Following the observation period, graft survival and axonal regeneration in the spinal cord will be analysed using immunohistochemical and tracing studies.

2. Pilot Lesion Studies

To determine the best spinal cord injury model for the proposed transplantation experiments we conducted pilot studies to test the complete transection and hemisection lesion models.

2.1 Complete transection model

The complete transection model of spinal cord injury was explored to determine its suitability for neural transplant experiments. Animals with complete transection of the spinal cord at the level of T9, exhibited complete paralysis of their lower limbs and loss of normal bladder function. These animals also demonstrated significant weight loss, in most instances greater than 10% of normal body weight, and required extensive postoperative care. The mortality rate for completely transected animals was high (50%) due primarily to autotomy (self-mutilation) and urinary tract infections. It was clear that the complete transection model was not a good model due to the high mortality and morbidity, which made this model impractical for transplantation experiments.

2.2 Hemisection model

Hemisection of the adult rat spinal cord was performed at the level of T9 vertebra. The hemisection produced paralysis of the ipsilateral hindlimb. Animals with hemisection exhibited a significant improvement in morbidity and mortality (1.8%) compared to animals with complete transection (50.3%). These animals experienced loss of bladder function which returned to normal within ten days following surgery. Hemisected animals gained weight during the observation period, and it was clear that this was an optimal model for transplant experiments. Therefore, the hemisection model of spinal cord injury was used in all experiments.

3. Materials and Methods

3.1 Study Design

This study was designed to determine the effects of rat and human FSC tissue transplantation on the thoracic hemisected adult rat spinal cord injury model. A total of fifty-seven female Wistar rats (Charles River, St. Constant, Quebec) weighing 200-225 grams were used in this study. Animals were housed two per cage under standardized conditions, on a twelve hour light/dark cycle with free access to food and water. All animal procedures were in accordance with the guidelines of the Canadian Council on Animal Care and the University Council on Laboratory Animals.

The animals were randomly divided into five study groups (Table 1): 1) double graft only (DG); 2) hemisection only (HX); 3) hemisection + double graft (HX+DG); 4) hemisection + bridge (HX+B); and 5) hemisection + bridge + double graft (HX+B+DG). The timeline of experimental procedures are summarized in Figure 1 and a diagram of the experimental design is depicted in Figure 2.

3.2 Hemisections

Animals were anaesthetized with a mixture (2.0 ml/kg) of 25% ketamine hydrochloride (Ketalean, MTC Pharmaceuticals, Cambridge, Ontario), 6% xylazine (Rompun, Miles Canada, Etobicoke, Ontario), 2.5% acepromazine maleate (Wyeth-Ayerst Canada, Montreal, Quebec) in 0.9% saline. Their backs were shaved and skin swabbed with Betadine surgical scrub (Purdue Frederick Inc.; 7.5% Povidone-iodine). Under a dissecting microscope the dorsal surface of the vertebral column was exposed and a laminectomy performed to expose the spinal cord between vertebrae T8 and T10. The location of vertebrae T8 to T10 can be determined by using the lowest rib as a reference for T13. Vertebrae

Table 1: Number of animals in each experimental group.

Treatment	Rat FSC	Human FSC
DG	4	4
HX	6	6
HX + DG	4	4
HX + B	8	8
HX + B + DG	10	9

DG: double grafts only; HX: hemisection only; HX+DG: hemisection with double grafts; HX+B: hemisection with bridge; HX+B+DG: hemisection with bridge and double grafts.

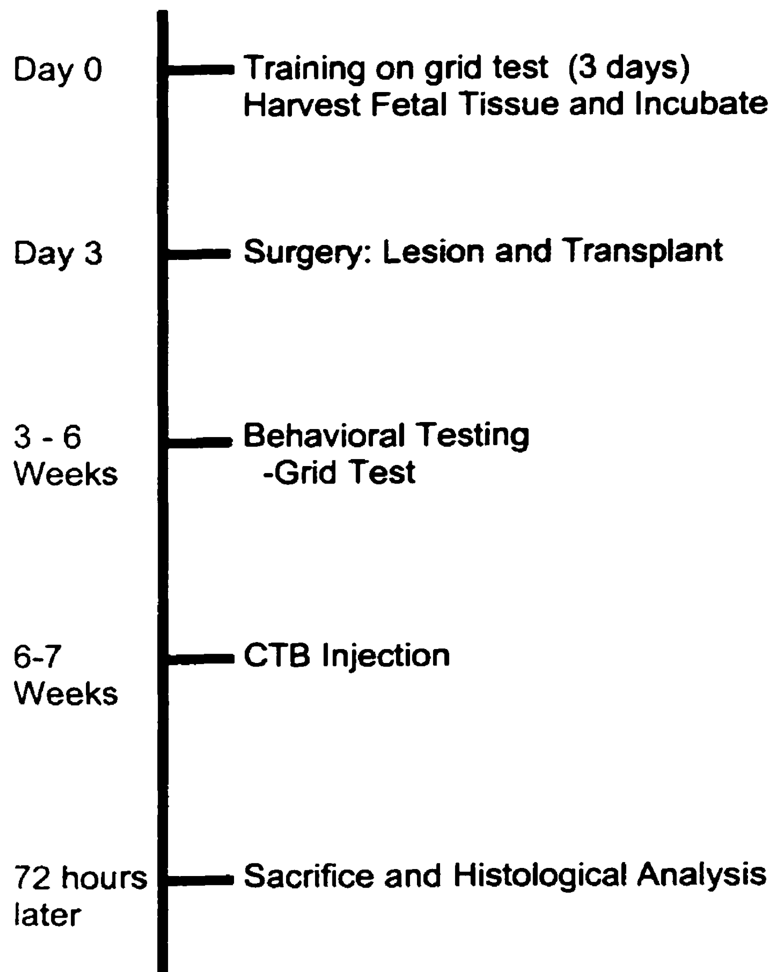


Figure 1: Timeline of experimental procedures.

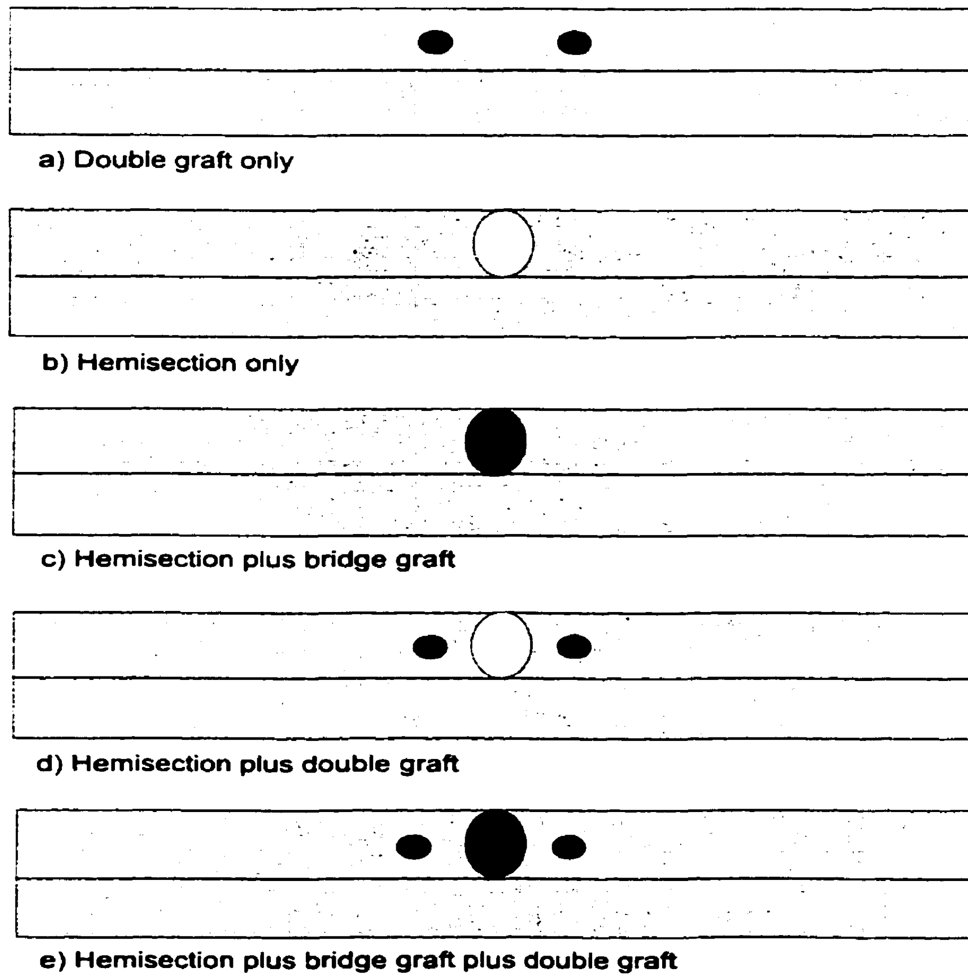


Figure 2: Diagram of experimental groups showing hemisections and transplantation in the adult rat spinal cord at the level of T9. Shaded areas indicate grafts and non-shaded areas indicate lesions without transplants.

T9 can be further confirmed by the caudal slant of the spinous process while the spinous processes of adjacent vertebrae are almost perpendicular to the vertebral column. Blood vessels in the area of T9 were cauterized using a sterile low temperature cautery (Solan; Jacksonville, FL), to minimize hemorrhage during surgery. The dura was opened and hemostasis of the spinal cord surface was controlled with saline soaked gelfoams.

Hemisection was performed on the left half of the spinal cord using a number 12 blade (Aesculap; Tuttlingen, Germany). The posterior median sulcus of the spinal cord, in which a vein is usually encountered, and the center of the adjacent spinous processes were used to identify the midline of the spinal cord. To ensure that all fibers were severed the blade was drawn through the lesion site several times. Animals receiving hemisection only and no further treatment had the lesion site covered with fibrin glue (Tisseel; Immuno), to prevent connective tissue ingrowth and other debris from entering the lesion site. The overlying muscle and skin was sutured together in layers using polyglactin 3.0 sutures (Ethicon; Sommerville, New Jersey). Immediately following lesioning the remaining animals received FSC transplants (see section 3.6).

3.3 Preparation of FSC cell suspension

a) Rat FSC cell suspension

The rat FSC tissue used for transplantation was obtained from timed pregnant rats. At embryonic day 13-14 (E13/14) the dams were anesthetized with an injection of sodium pentobarbital (65 mg/kg i.p.). Fetuses were removed under sterile conditions and placed into a Petri dish containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco). The spinal cords were dissected free of the vertebral canal and the meningeal membranes were then removed. An

attempt was made to dissect only the thoracic spinal cord. Intact spinal cords were stored for 72 hours at 4°C in hibernation media (30 mM KCl, 5.0 mM glucose, 0.24 mM $MgCl_2$, 10.95 mM NaH_2PO_4, 5.0 mM Na_2HPO_4, 20 mM lactic acid, 32.18 mM KOH, and 164.7 mM sorbitol, pH 7.4), to mimic the conditions of harvesting human spinal tissue (see below).

Following the hibernation period FSC cell suspensions were prepared by the following method. The tissue was incubated in 0.1% trypsin/0.05% DNase/DMEM (Trypsin: Worthington; DNase: Sigma DN-25) at 37°C for 20 minutes, then rinsed four times in 0.05% DNase/DMEM. The tissue was then mechanically dissociated using a 1ml pipette and then a 200ul Eppendorf pipettor until a uniform single cell suspension was obtained. The tissue was centrifuged at 1000 rotations per min for five minutes and the supernatant discarded.

b) Human FSC cell suspension

Human FSC was obtained from tissue used by The Clinical Neural Transplantation Program at the QEII Health Sciences Center. Tissue was obtained from routine elective abortion procedures under sterile conditions and under strict ethical guidelines currently in use in at the QEII Health Sciences Center. The age of the tissue corresponded to gestational age 8-9 weeks. Under this program, a delay of 72 hours between procurement of tissue and transplantation is required for microbiological screening for pathogens. Only tissue that was pathogen free was used. Cell suspensions were prepared in a similar manner as described for rat FSC.

3.4 Immunosuppression

Animals receiving transplants of human FSC were immunosuppressed with cyclosporin A (Sandoz; Dorval, Quebec; 10 mg/kg i.p., daily) beginning

twenty-four hours prior to surgery and continued for the duration of the study, to suppress the immune system and prevent rejection of the human transplants (Wictorin and Bjorkland, 1992).

3.5 Trypan blue dye exclusion method for cell viability and cell concentration

Cell viability of the prepared cell suspension was determined using the trypan blue dye exclusion method. Under brightfield illumination, cells with an intact cell membrane exclude the dye and therefore appear clear; dying or dead cells incorporate the trypan blue and thus appear blue.

With the aid of a hemocytometer final cell concentration was determined to be approximately 5.0×10^5 cells/μl for rat FSC cell suspensions. For human FSC cell suspension final cell concentration was 2.8×10^5 cells/μl. In all experiments cell viability was >98%.

3.6 Transplantation

Transplants of FSC cell suspension were implanted during the same surgical session, immediately following lesioning. Animals receiving transplants were divided into four groups: a) DG only, b) HX + DG, c) HX + B, and d) HX + B + DG (Figure 2). The DG only group received double grafts of FSC cell suspension in the unlesioned spinal cord. The cell suspension was injected into the intact spinal cord at the level of T9 as described below. Hemisections of the spinal cord were performed on the remaining animals. In all grafted animals, slow hardening fibrin glue (Tisseel; Immuno) was injected over the lesion site to protect and seal the surgical area. This was also done in hemisected animals not receiving transplants. The fibrin glue ensured that injected cell suspensions

remained in the lesion cavity and also prevented connective tissue and other debris from entering the lesion site. In animals receiving bridge grafts, FSC cell suspension of 1 x 10^6 cells (rat FSC: 2.0 μl; human FSC: 4.0 μl) was injected into the hemisected area using a Hamilton syringe connected to a glass microcapillary (70 μm tip diameter) over a period of five minutes to ensure adequate dispersal of cells throughout the cavity. In the bridge grafted animals that received double grafts, a glass microcapillary connected to a Hamilton syringe was used to implant grafts into the intact cord 1-2 mm rostral and caudal to the hemisection at a depth of approximately 1 mm from the dorsal surface of the spinal cord. Cell suspensions containing 2.5 x 10^5 cells (rat FSC: 0.5 μl; 1.0 μl) were injected over a period of five minutes. The final group of animals, received double grafts without the bridge graft placed in the hemisection.

3.7 Postoperative Care

Post-operatively all animals were placed on a heating pad turned to a low setting to keep them warm while recovering from the anesthesia. Animals were given 5 ml s.c. saline, and Bupremorphine (Reckitt & Colman Products; Hull England; 0.025 mg/kg s.c.) in 0.9% saline as an analgesic. Antibiotic therapy, 10% enrofloxacin in sterile water (Baytril, Bayer Inc., Etobicoke, Ontario; 5.0 mg/kg s.c.) daily for seven days, was routinely used post-operatively to prevent urinary tract infection. Animals were provided with soft bedding and hay and were housed singly until fully recovered (usually five to seven days) and did not display aggressive behavior. Animals were then housed in pairs. Environmental stimuli, such as wooden blocks and tubes were also provided to distract animals and reduce autotomy. Softened food was prepared daily and placed on the floor of the cage within reach of the rats. Animals were weighed daily for the first two weeks and then twice weekly thereafter as weight loss was not a problem once

animals had recovered from the surgery. Bladders were expressed twice a day until bladder reflexes returned, within seven days.

3.8 Functional Testing

Functional testing consisted of grid locomotion. This motor task has been standardized and validated (Kunkel-Bagden et al., 1993). Prior to surgery animals were trained on the grid test daily for three days (five trials per day). Motor function testing was performed in all groups beginning at postoperative week three and continued once a week until postoperative week six. All functional testing was analyzed by an observer who was blinded to both the experimental group and the chronological order of the testing.

In the grid locomotion test, animals were required to walk across a three foot long grid runway containing 2.5 x 2.5 cm holes. On each testing day, five trials were attempted for each animal. Footfalls were defined as failure to grasp a rung resulting in dropping of the foot below the plane of the grid. Each session was videotaped from a distance of 1.7 meters of the lateral aspect of the runway, which allowed a clear view of the step cycle of the animals. The number of footfalls made by the right and left hindlimb and the total number of steps taken to cross the grid for each hindlimb were quantified using slow motion analysis of videotaped sessions. Data was presented as the number of footfalls divided by the number of steps required to cross the platform. The average was calculated from the five trials for each test day. Unlesioned animals were used as controls of performance in this test and compared to the experimental group.

3.9 Anterograde Labeling

Six weeks post transplantation, animals were injected with the tracer, cholera toxin subunit B (CTB; List Biological Laboratories, Campbell CA) in the sensorimotor cortex to anterogradely label descending corticospinal axons. Animals were anaesthetized with a mixture (2.0 ml/kg) of 25% ketamine hydrochloride (Ketalean, MTC Pharmaceuticals, Cambridge, Ontario), 6% xylazine (Rompun, Miles Canada, Etobicoke, Ontario), 2.5% acepromazine maleate (Wyeth-Ayerst Canada, Montreal, Quebec) in 0.9% saline. The skin overlying the cranium was shaved and cleaned with Betadine surgical scrub (Purdue Frederick Inc.;7.5% Povidone-iodine). Under a dissecting microscope the skin was incised. A small hole was drilled with a high speed drill and a Hamilton syringe connected to a glass microcapillary (70 μm diameter) was used to inject 2.5 μl total of 1% CTB solution bilaterally into each of six stereotactically defined sites through layer V of the motor cortex (Paxinos and Watson, 1986). Coordinates were AP + 0.8, L + 2.0 and - 2.0, DV - 1.9; AP + 1.8, L + 2.2 and - 2.2, DV - 1.9; AP + 2.8, L + 2.7 and - 2.7, DV -1.9; tooth bar -3.3; coordinates from bregma and the dorsal surface of the brain. The solution was injected over two minutes and the needle was withdrawn after five minutes. Animals were sacrificed 72 hours following CTB injection allowing sufficient time for transport of the tracer to the spinal cord. Brains and spinal cords were removed and processed for CTB immunohistochemistry as described in section 3.10 and 3.12.

3.10 Tissue Preparation

At six to seven weeks post transplantation, animals were anaesthetized with an overdose of anaesthetic (25% ketamine hydrochloride; Ketalean, MTC Pharmaceuticals, Cambridge, Ontario; 6% xylazine; Rompun, Miles Canada, Etobicoke, Ontario; 2.5% acepromazine maleate; Wyeth-Ayerst Canada,

Montreal, Quebec; in 0.9% saline) and perfused transcardially with 150 ml of 0.1M ice cold phosphate buffer, pH 7.4, followed by 300-400 ml of 4% paraformaldehyde. Brain and spinal cord segments containing the grafts were dissected and post-fixed for an additional 24 hours by immersion in 4% paraformaldehyde at 4°C followed by cryoprotection overnight in 30% sucrose in 0.1M phosphate buffer at 4°C. Brains were then cut coronally in 40 µm sections on a freezing microtome at -37°C and stored in Millonig's solution (16.88 mg/ml $NaH_2PO_4H_2O$, 3.86 mg/ml NaOH, 0.006% NaAzide in distilled water). To cut straight sections on the microtome, spinal cord segments were embedded in hardened gelatin. First, the spinal cord was incubated in 10% gelatin at 37°C for three hours. For each spinal cord a plastic container was prepared for embedding by hardening a layer of 10% gelatin on the bottom of the container by placing the gelatin and container at -20°C for 5 minutes. The spinal cord was removed from the 10% gelatin immersion an arranged longitudinally in the embedding container with the dorsal surface facing up. 10% gelatin was slowly added until the spinal cord was completely covered. Care was taken to ensure that the spinal cord remained flat in the embedding container. Subsequently the embedding container was placed at -20°C for 15 minutes to allow the 10% gelatin to harden, then placed in 4% paraformaldehyde at 4°C. The following day the embedded spinal cord was transferred to 30% sucrose at 4°C and left overnight. Spinal cords were cut longitudinally in 40 µm sections on a freezing microtome at -37°C stored in Millonig's solution at 4°C.

3.11 Cresyl Violet Stain

Cresyl violet staining was performed to identify the lesion sites and transplant. Sections to be stained for cresyl violet were mounted on gelatin-covered slides and air dried. The following day slides were placed in 0.1M PB for

five minutes, dipped quickly in distilled water and four times each in 50% then 70% ethanol. Slides were placed for five minutes in 95% ethanol containing approximately 10 drops of glacial acetic acid/200 ml of ethanol. Slides were dipped four times each in 70% and 50% ethanol then distilled water before placement in 0.1% cresyl violet acetate in distilled water. After eight minutes in cresyl violet slides were dipped twice in distilled water and four times each in 50% and 70% ethanol then placed in 95% ethanol with glacial acetic acid for one to two minutes until sections were slightly darker than desired. Slides were further passed through a series of alcohol immersions, including twice in both 95% and 100% ethanol for five minutes each. Slides were immersed twice in xylene and coverslipped directly from the second xylene immersion.

3.12 Immunocytochemistry

Spinal cord sections were processed for serotonin, CTB, and human neuron specific enolase (NSE) immunocytochemistry. Serotonin [5-hydroxytryptamine (5-HT)] was used to identify descending brainstem fibers and CTB was used to identify descending corticospinal fibers labeled in the somatosensory cortex. In animals receiving transplants of human FSC, sections were also processed for NSE to identify transplanted human FSC. Specificity of each immunocytochemical procedure was established by omission of the primary antibody in control sections.

a) Serotonin

Free-floating sections were rinsed three times, for five minutes each time, in 0.1M phosphate buffered saline (PBS), and quenched for thirty minutes in 0.1M PBS containing 10% methanol and 3% hydrogen peroxide. Sections were rinsed three times, for five minutes each time, in 0.1M PBS and incubated for

sixty minutes in a blocking solution consisting of 0.3% Triton in 0.1M PBS and 5% normal goat serum (NGS). Spinal cord tissue was incubated overnight at 4°C in primary antibody [polyclonal rabbit anti-5-HT (Incstar, Stillwater, MN); 1:50000] with 0.3% Triton X-100 in 0.1M PBS and 5% NGS. Sections were washed three times, for five minutes each time, in 0.1 M PBS and incubated for sixty minutes at room temperature with the secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories; Burlingame, CA; 1:500 dilution) in 0.1 M PBS and 5% NGS. Spinal cord tissue was rinsed three times, for five minutes each time, in 0.1 M PBS and incubated for sixty minutes with avidin-biotinylated peroxidase complex (Vector Elite ABC Kit, Vector Laboratories, Burlingame, CA) diluted 1:500 (A and B each) in 0.1 M PBS. Sections were washed three times, for five minutes each time, in 0.1 M PBS and reaction product was visualized with diaminobenzidine (DAB; 0.5 mg/ml in 0.1M phosphate buffer, 6.6 μl/20 ml of 30% H_2O_2).

b) Cholera toxin B

Sections to be stained for antibody against CTB were rinsed three times, for five minutes each time, in 0.1M PBS and incubated for 30 minutes in 10% methanol and 3% hydrogen peroxide in 0.1M PBS. Tissue was rinsed three times, for five minutes each time, and incubated at room temperature in 0.1M PBS containing 5% normal rabbit serum (NRS) and 0.3% Triton X-100. Sections were transferred to primary antibody (polyclonal goat anti-CTB; List Biological Laboratories Inc.; 1:40 000 dilution). Following 48 hours incubation at 4°C in primary antibody, sections were rinsed three times, for five minutes each time, in 0.1 M PBS and transferred to a solution containing the secondary antibody (biotinylated rabbit anti-goat IgG; 1:500 dilution), 5% NGS in 0.1M PBS for sixty minutes at room temperature. Sections were washed three times, for five minutes each time, in 0.1M PBS and incubated for sixty minutes at room

temperature with avidin-biotinylated peroxidase complex (Vector Elite ABC Kit, Vector Laboratories, Burlingame, CA) diluted 1:500 in 0.1 M PBS. Spinal cord tissue was rinsed three times, for five minutes each time, in 0.1M PBS and reaction product was visualized with 0.05% DAB solution in 0.1M phosphate buffer containing D(+) glucose (0.25 g/ml distilled water; 160 µl/20 ml DAB solution), ammonium chloride (0.2 g/ml distilled water; 40 µl/20 ml DAB solution), glucose oxidase (3 mg/ml distilled water; 20 µl/20 ml DAB solution), and ammonium nickel sulfate (100 mg/20 ml DAB solution).

c) Human neuron specific enolase

For NSE staining, sections from spinal cords of animal transplanted with human FSC were rinsed three times, for five minutes each time, in 0.1M PBS and quenched for thirty minutes in 10% methanol and 3% hydrogen peroxide in 0.1M PBS. Spinal cord tissue was rinsed three times, for five minutes each time, and incubated at room temperature with 5% normal horse serum (NHS) and 0.3% Triton X-100 in 0.1M PBS. Sections were washed three times, for five minutes each time, and incubated overnight at 4°C in primary antibody (monoclonal mouse antibody; Novacastra, Newcastle upon Tyne, United Kingdom; 1:100 dilution), 0.3% Triton X-100, 5% NGS in 0.1M PBS. Sections were rinsed three times, for five minutes each time, and incubated for sixty minutes at room temperature in secondary antibody (rat adsorbed biotinylated horse anti-mouse IgG; Vector Laboratories, Burlingame CA; 1:200 dilution) with 0.3% Triton X-100 and 5% NHS in 0.1M PBS. Spinal cord tissue was rinsed three times, for five minutes each time, in 0.1 M PBS and incubated for sixty minutes with avidin-biotinylated peroxidase complex (Vector Elite ABC Kit, Vector Laboratories, Burlingame, CA) diluted 1:500 in 0.1 M PBS. Sections were rinsed three times, for five minutes each time, and reacted with DAB; 0.5 mg/ml in 0.1M phosphate buffer, 6.6 µl/20 ml of 30% H_2O_2) to reveal the reaction product.

Following DAB reaction sections processed for immunohistochemical detection of 5-HT, CTB and NSE were rinsed five times, for five minutes each time, in 0.1M PB and mounted onto gelatin coated glass slides, air-dried and dehydrated through a series of alcohol and xylene immersions. Mounted sections were then covered with Permount and coverslipped. Sections were examined microscopically for graft survival and extent of lesion. Immunolabeled sections were examined to determine the phenotype and extent of fiber penetration into and through grafts.

3.13 Histological Analysis

Mounted longitudinal sections were examined under light microscopy to determine the extent of the lesion, transplant survival, the presence of 5-HT fibers and retraction of CST fibers labeled by CTB. A computerized image software system (Optimus) was used for histological analysis (Optimas Corporation, Bothell, Washington). All images were captured using Adobe Photoshop software.

a) Lesion assessment

Lesions were identified in longitudinal sections as obvious disruptions in the normal architecture of the spinal cord. Lesions were easily identified in cresyl violet and 5-HT stained sections. A lesion was determined to be exactly midway if a hemisection reached but did not extend past the midline to the contralateral spinal cord segment.

b) Identification of transplanted tissue

Transplanted rat FSC tissue was identified in cresyl violet stained sections. Grafts were determined to have survived if neurons were identified in

the grafted tissue. Transplanted human fetal spinal cord tissue was identified using antibody to NSE which specifically recognizes human tissue.

c) Regeneration of serotonergic fibers

Mounted longitudinal sections were examined to assess regeneration of 5-HT positive fibers. The pattern and distribution of immunolabeling was described in the spinal cord proximal and distal to the lesion as well as in the grafts.

d) Corticospinal tract retraction

Corticospinal tract retraction was quantified by measuring the distance from the lesion site or host-graft interface to the first terminal swelling of a retracting axon. Terminal swellings were identified as intensely stained CTB filled axon tips as described and illustrated in Pallini et al. (1988). The retraction distance was calculated using a computerized program specifically designed for this task. The retraction distance was analyzed twice by the primary investigator and once by a blinded investigator to assess inter and intra observer variability.

3.14 Statistical Analysis

Differences in quantitative variables between groups and within groups were tested by ANOVA. *Post hoc* differences were determined by Tukey's *post hoc* test. A students t-test was used to determine significant differences within and between observers for CST retraction, and to determine significant differences between right and left hindlimb for performance on the grid test.

4. Results

4.1 Animal Model

Animals with hemisection exhibited significant improvement in morbidity and mortality (1.8%) compared to animals with complete transection in which the mortality was 50.3%. Although hemisected animals experienced loss of bladder function it returned to normal within ten days following surgery. All hemisected animals gained weight during the survival period of six to seven weeks. Only two animals with hemisection were sacrificed due to postoperative complications: one animal had extensive autotomy of the hindlimb and the other had a lower limb infection that did not respond to antibiotic therapy

In hemisected animals the rostral and caudal spinal cord stumps were closely apposed. There was little evidence of extensive damage, such as hemorrhage and lesion cavities.

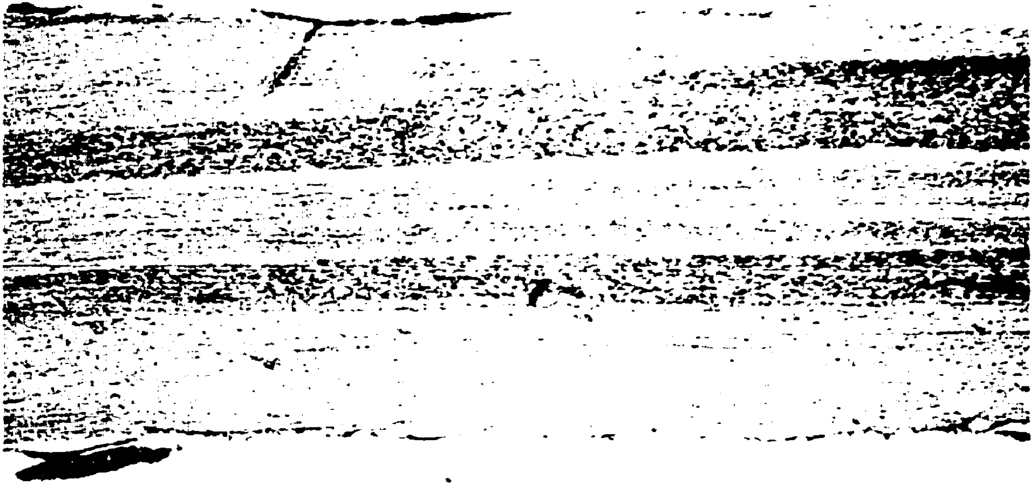
4.2 Histological observations

a) Lesion Assessment

The extent of the lesion and degree of hemisection was assessed under microscopic examination. Hemisection was defined as a lesion that reached but did not extend past the midline. Lesions were identified in longitudinal sections as obvious disruptions in the normal architecture of the spinal cord (Figure 3a). Normal architecture of the spinal cord rostral and caudal to the lesion displayed a distinct white and grey matter arrangement which was not observed at the site of the lesion or transplanted area (Figure 3b). The extent of the lesion for each animal was assessed by analysing serial sections for each animal.

Figure 3: Horizontal longitudinal sections of the rat thoracic spinal cord stained with cresyl violet: a) normal cord and b) cord with a hemisection only (arrow). Note the disruption of the normal architecture of the cord in (b). scale bar = 250 μm.

a



b

Figure 3

Under-hemisections were observed when the lesion did not extend to the midline and over-hemisection were observed when the lesion extended past the midline. The majority of animals (86%) were over-hemisected with lesions ranging from slight interruption of the contralateral white matter to almost complete interruption of contralateral grey matter (Table 2).

b) Identification of Transplants

i) Bridge grafts

In serial sections of the spinal cord from animals receiving bridge transplants there was some variability in the extent to which the transplants filled the lesion site. In several sections transplants almost entirely filled the lesion site with some areas appearing continuous with the host spinal cord (Figure 4a), while in other sections large cavities could be seen within the transplant and adjacent host spinal cord (Figure 4b). In human FSC bridge grafts the bridges were easily distinguished from the host by NSE staining (Figure 4c). These tissue bridges were most commonly seen on the lateral aspect of the cord and sometimes observed in the center of the lesion.

ii) Identification of rat FSC transplants

Transplanted rat FSC was identified as tissue in the grafted site that did not demonstrate the characteristic grey and white matter of the normal spinal cord. Survival was confirmed by the identification of neurons within the transplanted tissue. In animals receiving cell suspension grafts injected into the host spinal cord rostral and caudal to the lesion (double grafts). The grafts were oval in shape and contained more densely packed neurons and glial cells than the host spinal cord (Figure 5a). Numerous densely packed glial cells were seen at the margin of the grafts in the proximal and distal host spinal cord and at the

Table 2: Description of degree of hemisection in lesioned animals.

	Rat FSC	Human FSC	Hemisection
Under Hemisection	1	0	0
Hemisection	4	1	0
Over Hemisection	17	20	6
Total	22	21	6

Figure 4: Horizontal longitudinal sections of the rat thoracic spinal cord: (a) cresyl violet stain of a hemisected animal with a bridge graft of rat fetal spinal cord (asterisk) filling the space of the hemisection (arrow). The lesion does not extend to the midline and is classified as an under-hemisection; (b) cresyl violet stained with a bridge graft of rat fetal spinal cord (asterisk) spanning the space of the hemisection (arrow) with cavities. In this animal the lesion extends beyond the midline and would be classified as an over-hemisection; and (c) immunostained for neuron specific enolase (NSE) with grafts of human fetal spinal cord (asterisk) filling the space of the hemisection (arrow).
scale bars = 250 μm.

Figure 4

Figure 5: Horizontal longitudinal sections the rat thoracic spinal cord: (a) cresyl violet stain of a hemisected animal with double grafts of rat fetal spinal cord (asterisks) in the intact cord rostral and caudal to the hemisection (arrow). The grafts are outlined by a dense border of glial cells; (b) cresyl violet stain of high power view rat fetal spinal cord graft in (a), indicating the graft-host interface identified by the density of glial cells (arrow) at the border of the graft and host adult rat spinal cord; and (c) higher power view of a graft of rat fetal spinal cord containing both glia and neurons. Neurons are distinguished from glia by the increased size and the presence of a nucleus (arrows). The presence of neurons within the graft indicates graft survival.
scale bars = 250 μm.

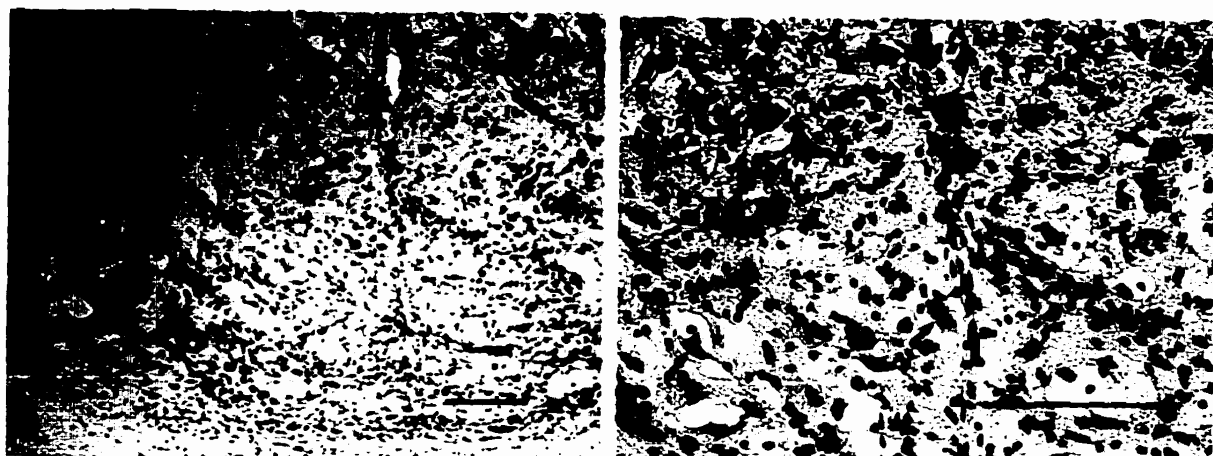


Figure 5

margin of the lesion site. Neurons were easily identifiable within the graft by the large size and shape compared to surrounding glial cells (Figure 5c) and were not as densely stained as glial cells. Table 3 shows the number of animals per experimental group with surviving transplants of rat FSC.

iii) Identification of human FSC transplants

Transplants of human fetal spinal cord cell suspension were easily identified using antibody to NSE. Table 3 shows the number of animals per experimental group with surviving transplants of human FSC. In some of the animals with positive NSE staining only small areas of the transplants were observed, while in other animals transplanted tissue was observed filling the entire lesion site (bridge grafts) and as distinct oval grafts in the intact host spinal cord (double grafts) (Figure 6a). NSE immunohistochemical staining could be seen extending into the lesion site as well as rostrally and caudally in the intact spinal cord (Figure 6b and 6c).

c) Identification of serotonergic fibers

A robust density of 5-HT immunostaining was observed in the spinal cord rostral to the lesion in all experimental animals. In some animals, 5-HT immunostaining was also seen caudal to the lesion. Although difficult to quantify, it appeared that the degree of 5-HT immunostaining was greater on the contralateral side compared to the ipsilateral side to the lesion and the caudal host spinal cord was minimally stained compared to the rostral spinal cord (Figure7). 5-HT positive fibers could also be seen crossing the midline from the grey matter contralateral to the lesion to the grey matter ipsilateral to the lesion. This was especially prominent in the grey matter surrounding the central canal. In some animals there were several sections that appeared to have intact 5-HT

Table 3: Table showing the number of animals with surviving transplants using cresyl violet stain and human neuron specific enolase staining.

	Rat FSC	**Human FSC**
DG only	4/4	4/4
HX+DG	4/4	2/4
HX+B	5/8	4/8
HX+B+DG	7/10	6/9
Total	20/26	16/25

DG: double grafts only; HX: hemisection only; HX+DG: hemisection with double grafts; HX+B: hemisection with bridge; HX+B+DG: hemisection with bridge and double grafts

Figure 6: Horizontal longitudinal sections of the rat thoracic spinal cord: (a) human neuron specific enolase immunostain of a hemisected animal with double grafts of human fetal spinal cord tissue rostral and caudal to the hemisection (asterisk). Using human neuron specific enolase staining the grafts of human fetal spinal cord tissue can be easily identified within the host rat spinal cord; (b) high power view of the distal graft illustrating immunostaining extending distally along the host cord (arrow) and (c) high power view of the proximal graft of human fetal spinal cord with immunostaining extending proximally along the host cord (arrows). scale bars = 250 μm.

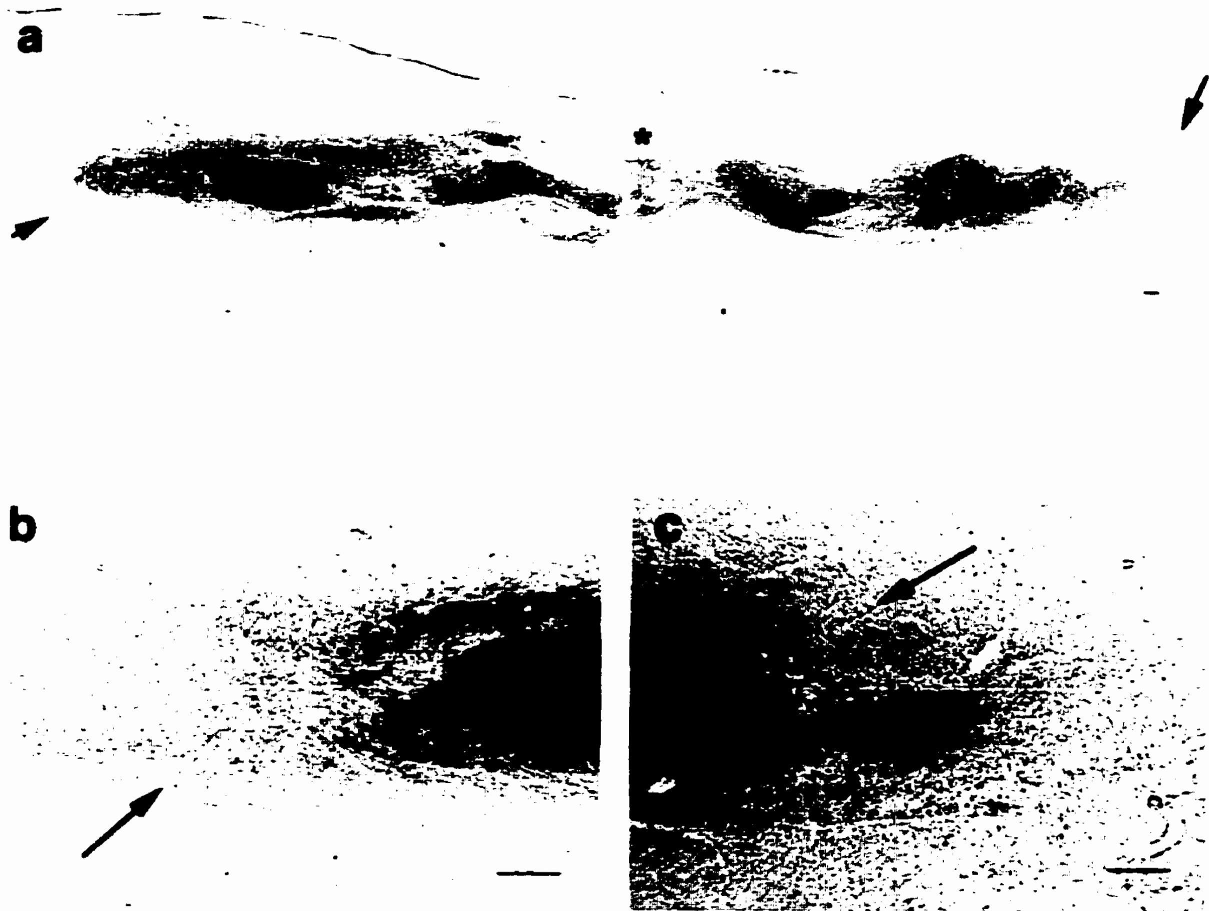


Figure 6

Figure 7: Horizontal longitudinal sections of the rat thoracic spinal cord immunostained for 5-HT: (a) low power view of normal cord; (b) higher power view of (a) demonstrating the presence of 5-HT positive fibers within the grey matter; (c) low power view of a hemisected (arrow) cord with a bridge graft of human fetal spinal cord tissue. Note the presence of 5-HT positive fibers in the grey matter proximal (right side) extending up to the lesion and the loss of immunostaining distally (left side) to the hemisection; and (d) higher power view of (c). Serotonergic positive fibers can be observed clustering at the proximal host-graft interface. Scale bars = 250 µm.

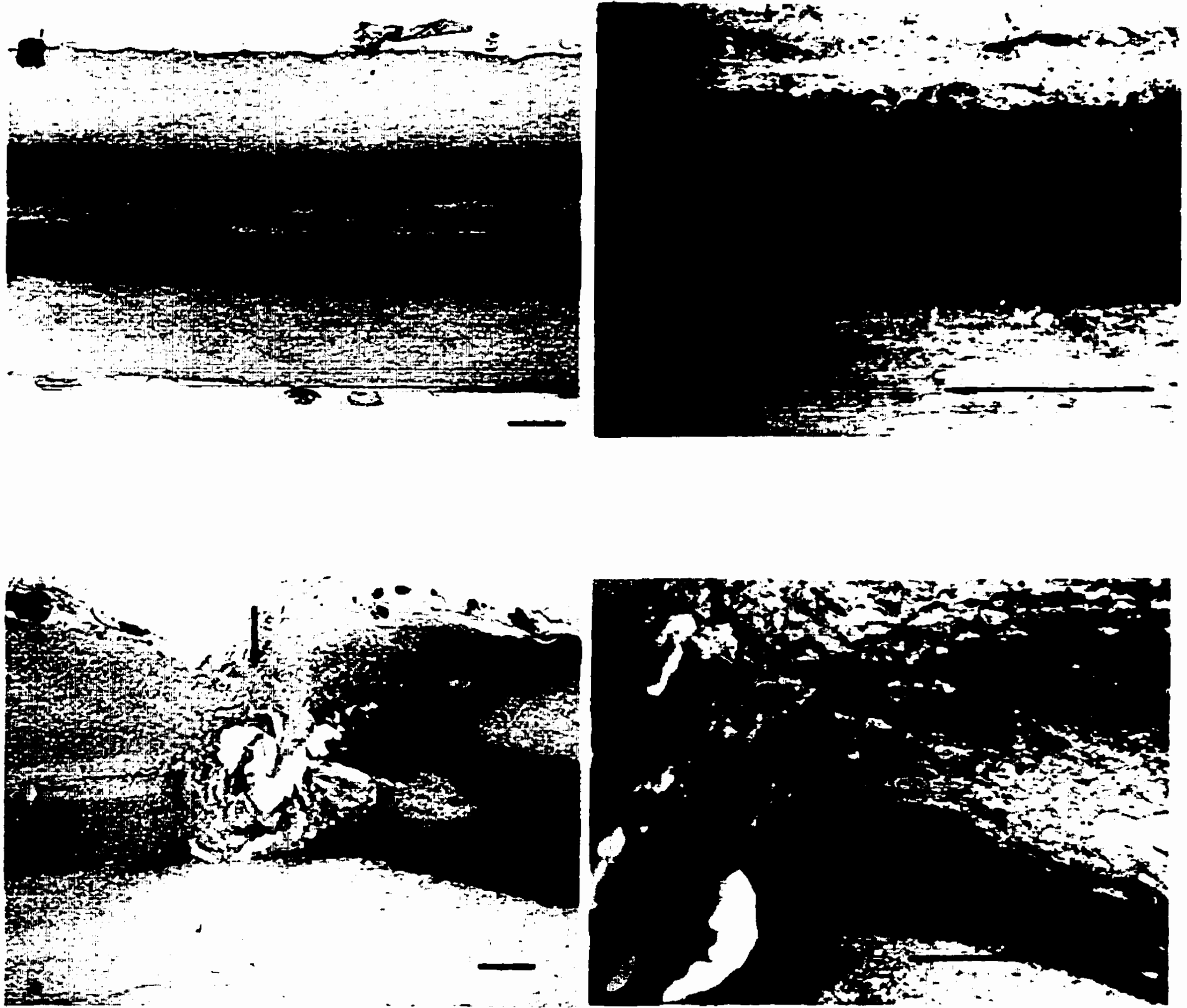


Figure 7

fibers at the lateral edge of the lesion, suggesting that these fibers may not have been severed during the surgery.

i) Hemisection only

In animals receiving a lesion only and no transplant (n=6) 5-HT positive fibers were observed clustering at the rostral host-graft interface. No 5-HT positive fibers were seen crossing the lesion site nor entering the caudal host spinal cord (Figure 7b).

ii) Double Graft Only

Eight unlesioned animals received injections of FSC (rat FSC n=4, human FSC n=4) into the intact spinal cord. Double grafts appeared as two distinct grafts located in the grey matter of the spinal cord. The grey matter rostral and caudal to the double grafts contained a normal serotonergic distribution of fibers. The grafts were oval in shape and did not appear to affect the grey matter rostral and caudal to the grafts (Figure 8a). Numerous serotonergic positive fibers were observed traversing through the grafts, however they were not as dense as the adjacent host spinal cord. Serotonergic fibers were observed extending around the grafts.

iii) Hemisection plus double grafts

Eight lesioned animals received double grafts of FSC (rat FSC n=4, human FSC n=4) into the spinal cord rostral and caudal to the lesion. The grafts were oval in shape and located in the grey matter. In the animals with clearly surviving double grafts the stumps of the rostral and caudal spinal cords were clearly apposed. In these animals there were very few cavities observed at the lesion site (Figure 8b). 5-HT immunolabeling was observed in both the rostral

Figure 8: Horizontal longitudinal sections of the rat thoracic spinal cord immunostained for 5-HT: (a) unlesioned adult rat spinal cord with double grafts (asterisk) of rat fetal spinal cord tissue. In this animal the double grafts have grown together and appear as one graft; (b) hemisected adult rat spinal cord with double grafts of rat fetal spinal cord rostral and caudal to the hemisection (arrow). scale bars = 250 μm.

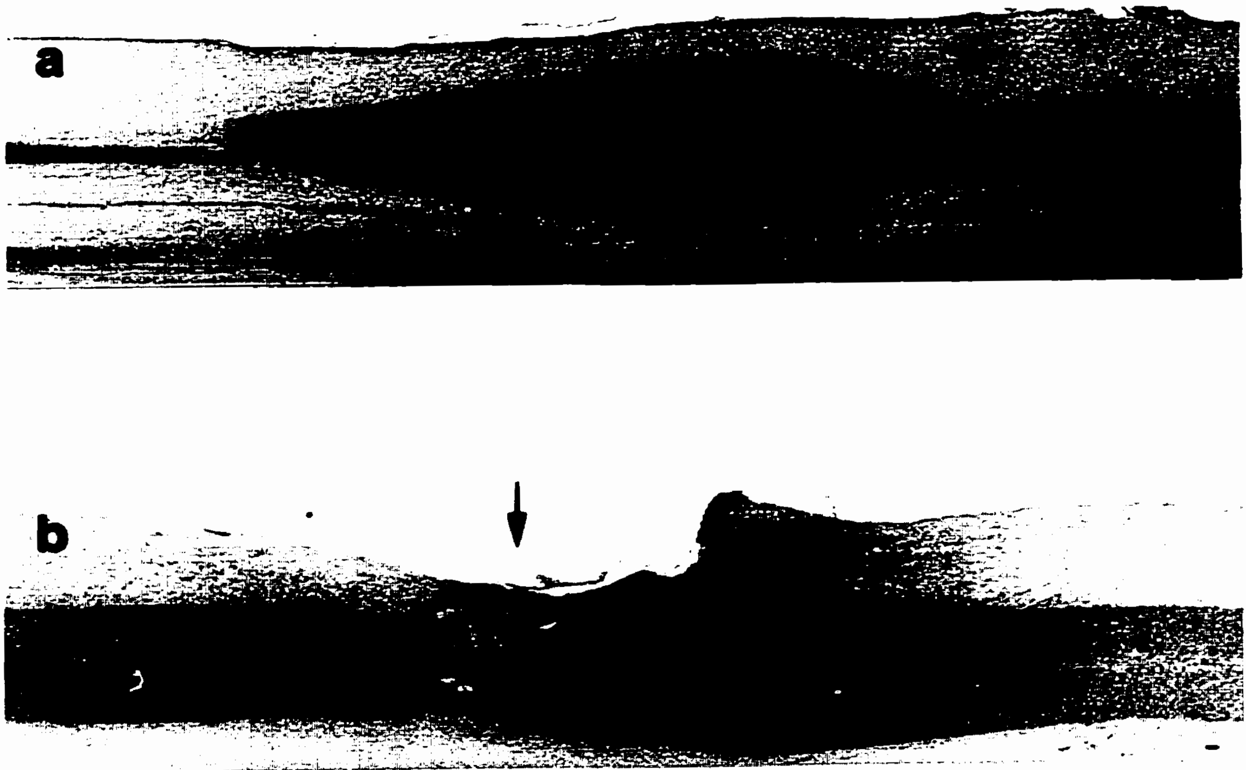


Figure 8

and caudal graft. In no instances were 5-HT positive fibers observed crossing the lesion site.

iv) Hemisection plus bridge grafts

Sixteen animals received lesion and bridge grafts of FSC cell suspension (rat FSC n=8, human FSC n=8) in the gap produced by the lesion. In all animals 5-HT positive fibers were observed in the rostral spinal cord extending and clustering at the host-graft interface (Figure 9a). In almost all animals the majority of fibers stopped abruptly at the host-lesion interface, however a few 5-HT positive fibers were observed extending into the bridge graft. These fibers did not traverse the entire length of the bridge and fibers were not observed re-entering the distal host spinal cord (Figure 9b).

v) Hemisection plus bridge plus double grafts

Nineteen lesioned (rat FSC n=10, human FSC n=9) animals received bridge grafts of FSC cell suspensions and double grafts of FSC cell suspension injected into the spinal cord rostral and caudal to the lesion (Figure 10a). 5-HT immunolabeling was observed in the bridge (Figure 10b), however, no fibers were observed crossing the host-graft interface into the distal spinal cord (Figure 10c). In most of these animals there were extensive areas of cavitation at the lesion site, which were usually associated with numerous red blood cells.

d) Identification of corticospinal tract fibers

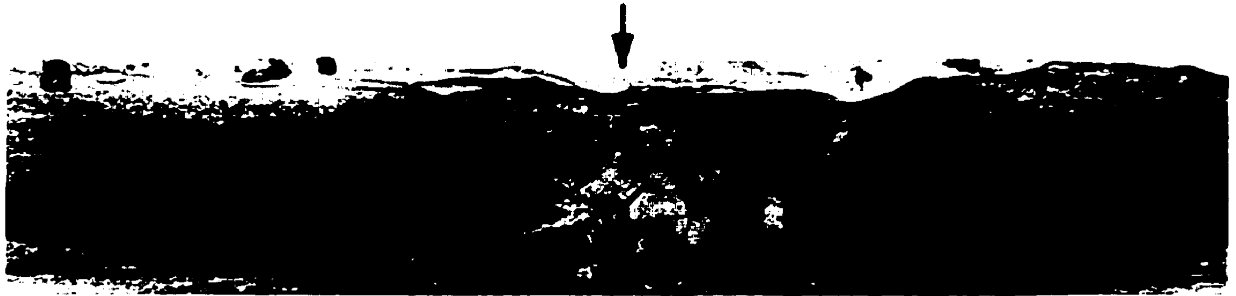
i) Site of CTB injection

In animals receiving injections of CTB, cells in the sensorimotor cortex were positive for CTB immunostaining (Figure 11a). Staining was also observed in several basal ganglia structures that are involved in descending motor

Figure 9: Horizontal longitudinal sections of the rat thoracic spinal cord immunostained for 5-HT: (a) hemisected adult rat spinal cord with bridge graft of rat fetal spinal cord tissue (asterisk) in the space spanning the hemisection and (b) high power view of (a) illustrating the dense cluster of immunostained fibers at the graft-host interface (arrow). Fibers are not observed entering the rat fetal spinal cord graft. scale bars = 250 μm.

Figure 9

Figure 10: Horizontal longitudinal sections of the rat thoracic spinal cord immunostained for 5-HT: (a) hemisected adult rat spinal cord with a grafts (asterisks) of rat fetal spinal cord tissue in the lesion site and in the intact cord rostral and caudal to the hemisection (arrow); (b) high power view of (a) showing immunostained fibers (arrow) entering the bridge graft from the proximal side of the lesion. Fibers were not observed spanning the entire length of the transplant to the distal host spinal cord; and (c) a lack of immunostained fibers extending from the bridge graft into the distal cord (arrow). scale bars = 250 µm.



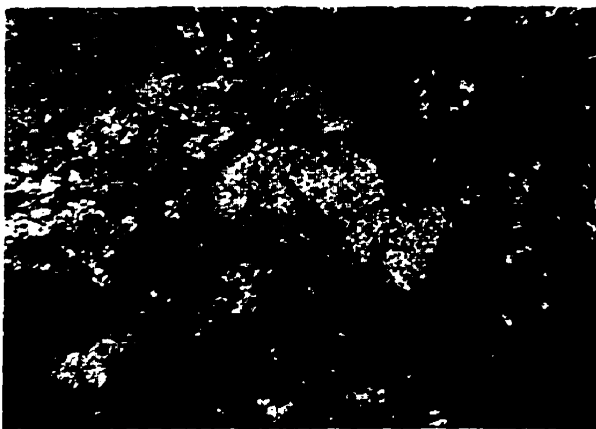


Figure 10

pathways, primarily the thalamic nuclei. In most brain sections there was evidence of leakage of the CTB into the cerebrospinal fluid illustrated by intense CTB staining on the surface of the brain around the injection site.

ii) Identification and measurement of CST retraction

In all animals CST fibers could be identified in the thoracic spinal cord in the grey and white matter (Figure 11d). CTB labeled fibers were not seen caudal to the lesion site. The terminal swellings of the retracting axons were identified and the distance measured to the lesion site by two observers (Figure 11c). There was not a significant difference between the recorded retraction distances between ($p>0.05$ in ANOVA) and within observers ($p>0.05$ in ANOVA) for rat FSC and human FSC respectively.

The average CST retraction and SEM for each experimental group is presented in Figure 12. There was not a significant difference between hemisection only animals and transplanted groups.

4.3 Functional Testing

i) Grid Test

All animals were capable of traversing the grid. Animals used the same motor pattern as observed in overground locomotion. The hindlimb and the opposite forelimb were in contact with the rungs of the grid simultaneously (Figure 13). Normal control rats and double graft only (unlesioned) rats crossed the grid making few errors (Figure 13a). For those animals the number of errors did not exceed one error for each crossing. In contrast, lesioned animals were

Figure 11: CTB immunostained coronal section of (a) rat cortex illustrating immunostaining within the motor cortex around the site of injection (arrow); (b) horizontal longitudinal section of the hemisected rat spinal cord with double grafts of rat fetal spinal cord. CTB has leaked out of the injection site and has been transported to the spinal cord through the cerebrospinal fluid, resulting in non-specific staining of the lesion site and the double grafts rostral and caudal to the lesion; (c) horizontal longitudinal section of the hemisected rat spinal cord with bridge graft of rat fetal spinal cord tissue, showing CTB stained CST fibers proximal to the lesion; and (d) high power view of (c) illustrating the retraction bulbs of the CST fibers. Scale bars = 250 μm.

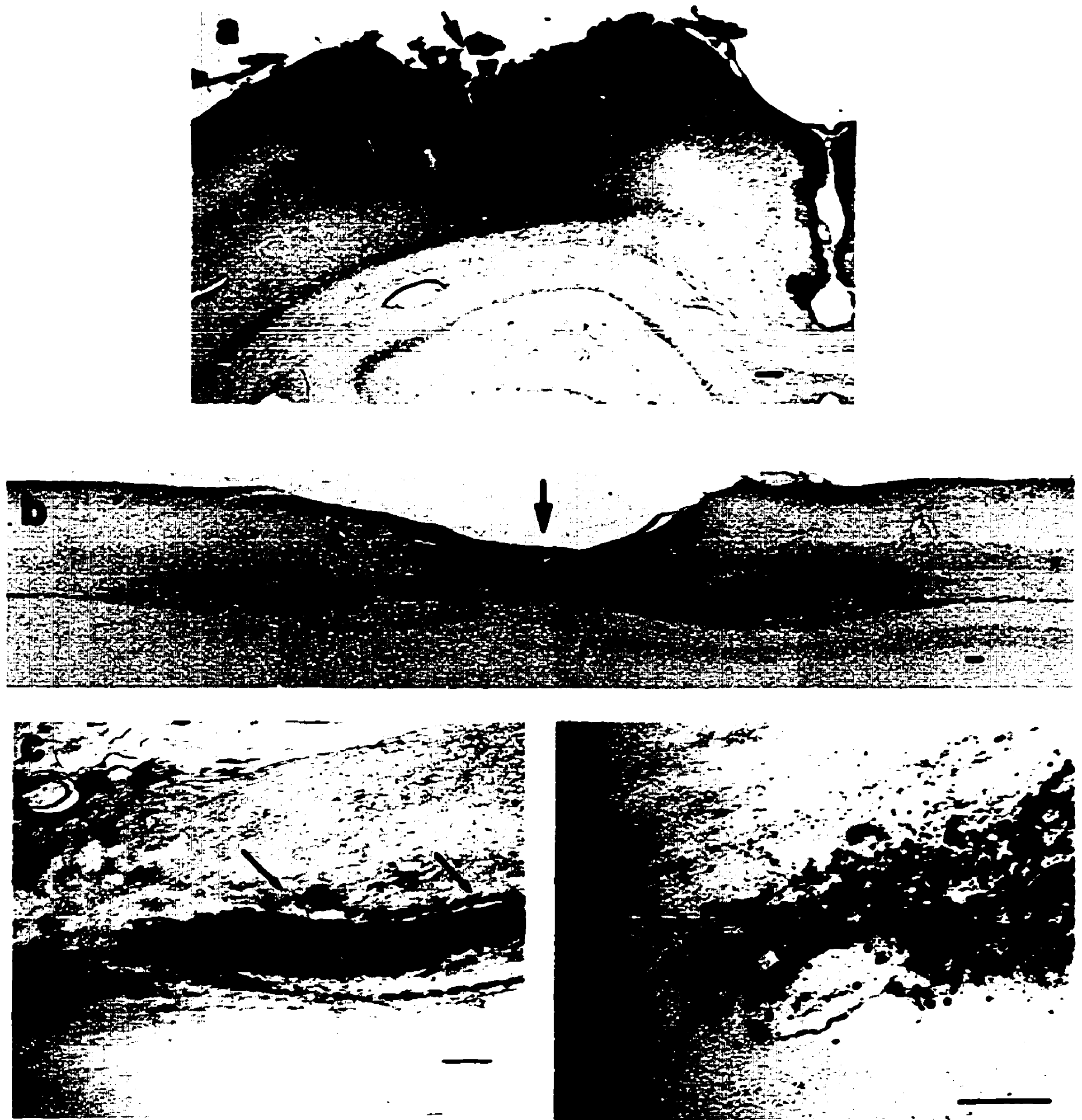
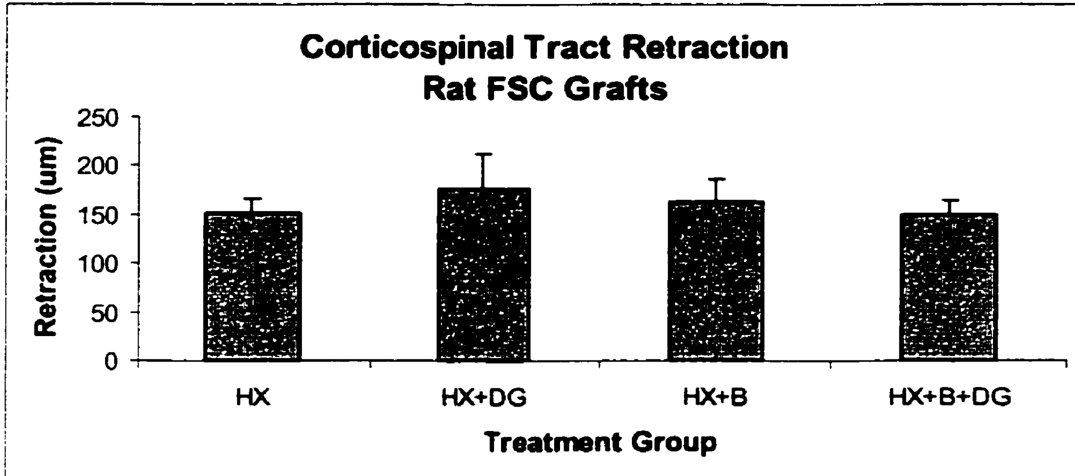


Figure 11

A



B

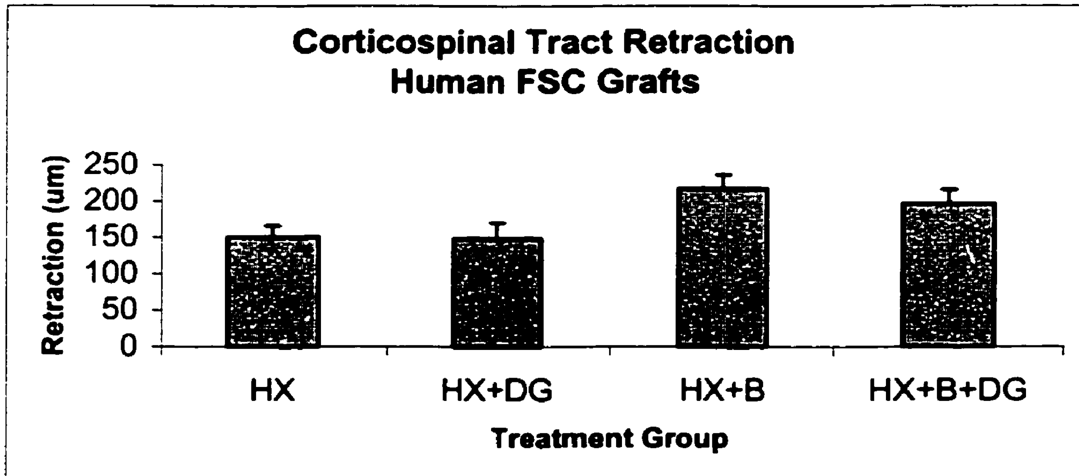


Figure 12: Retraction of corticospinal tract fibers from the site of lesion six to seven weeks following surgery. There was no significant difference between HX only animals and transplanted groups (HX+DG, HX+B, HX+B+DG) ***A,*** Animals transplanted with rat FSC tissue. ***B,*** Animals transplanted with human FSC tissue. Each bar represents mean ± SEM, n=2-6, with the exception of both rat and human FSC HX+DG where n=1.

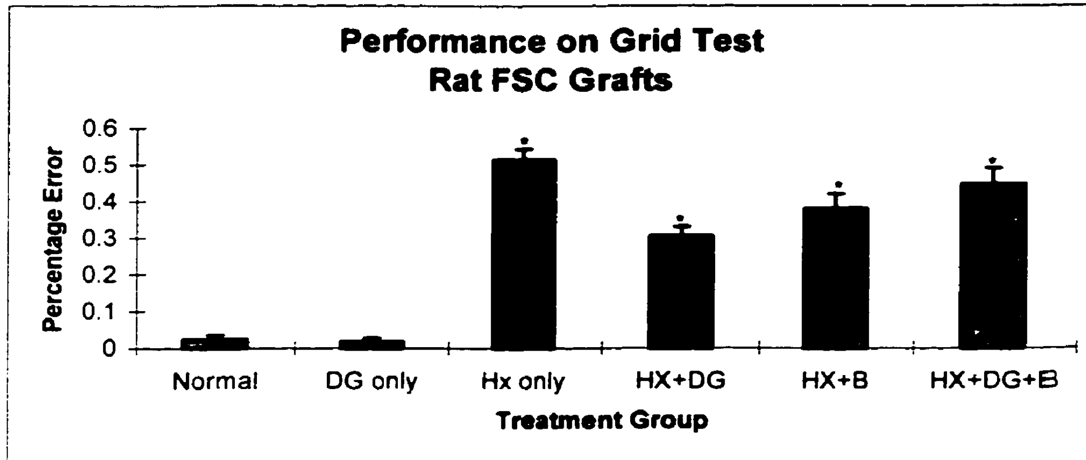
Figure 13: Photographs of animals performing the grid test to assess functional recovery. (a) unlesioned animals crossing the grid. These animals grasp the rungs as they traverse the grid, making few errors; (b) lesioned animal dragging its hindlimb across the grid. During the first two weeks following surgery motor function of the hindlimb was significantly impaired and in some cases paralysed. This was followed by a certain degree of natural recovery, and therefore motor testing did not begin until two weeks following surgery. Animal with continued paralysis during the entire four weeks of motor testing were not used in the analysis; and (c) lesioned animal with a footfall through the rung of the grid. Hemisection of the spinal cord resulted in numerous errors when crossing the grid.

Figure 13

only able to cross the grid either dragging the hindlimb (Figure 13b) or with numerous footfalls (Figure 13c). Hemisected animals made footfall errors 51.4% of the time, while normal animals made errors 2.3% of the time. There was no significant difference on the grid test among each of the four weekly sessions within each group. Data for each treatment group for the four week test period was combined. Figure 14 shows the average percentage error and standard error of the mean (SEM) for each treatment group for the four weeks. Percentage error scores were also recorded for the right hindlimb, ipsilateral to the lesion. Using the student's t-test it was determined that there was a significant difference between footfalls made by the right and left hindlimb.

Percentage error scores for normal, HX only and transplant groups are presented in Figure 14. For animals receiving transplants of rat FSC there was no significant difference among any of the groups. For animals receiving transplants of human FSC there was not a significant difference between the HX, HX+B and HX+B+DG treatment groups ($p<0.05$). However there was a significant difference between the HX+DG group and the HX and HX+B+DG treatment groups ($p<0.05$). For both rat and human FSC groups there was not a significant difference between the unlesioned normal and the unlesioned double grafted animals ($p>0.05$). However for both rat and human FSC grafted animals there was a significant difference between the unlesioned and lesioned animals ($p<0.05$).

A



B

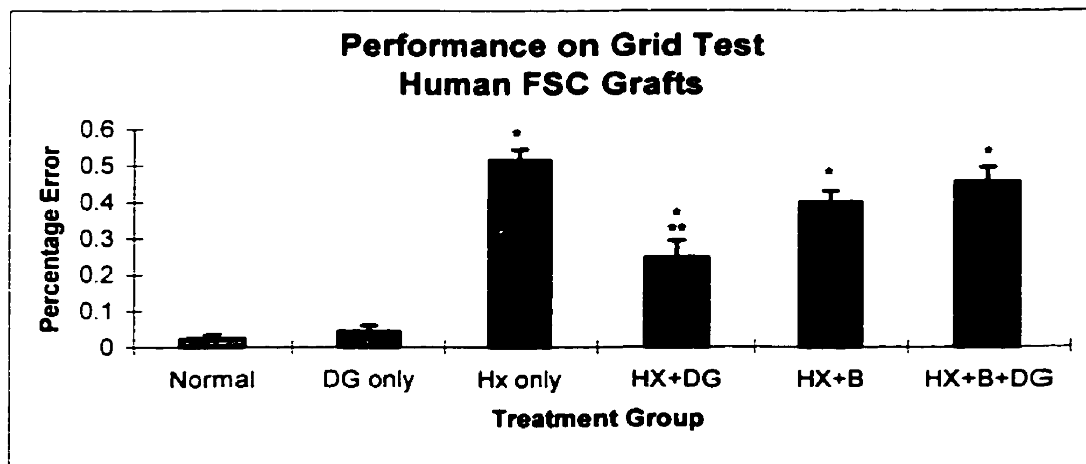


Figure 14: Effect of thoracic hemisection and transplantation on footfall error scores (percent error) on the grid test. ***A,*** Animals transplanted with rat FSC tissue. All lesioned animals showed significant deficits on the grid test. (* $p < 0.05$ compared to normal and DG only). There was a significant difference between HX+DG and HX only and HX+B+DG (** $p < 0.05$ compared to HX only and HX+B+DG). ***B,*** Animals transplanted with human FSC tissue. All lesioned animals showed significant deficits on the grid test. (* $p < 0.05$ compared to normal and DG only) There was no significant difference between HX only and transplanted groups (HX+DG, HX+B, HX+B+DG). Each bar represents the mean ± SD, n=2-6, with the exception of the HX+DG group grafted with human FSC where n=1.

5. Discussion

Grafts of rat FSC tissue have been shown to survive and differentiate when transplanted into the injured adult rat spinal cord (Reier et al., 1986). Regeneration of damaged spinal cord axons is also promoted when FSC tissue is placed at the site of the lesion (Reier et al., 1986; Bregman, 1987; Jakeman and Reier, 1991; Diener and Bregman, 1998a). These observations suggest that FSC grafts may be used as a feasible strategy for repair of the injured adult spinal cord in humans in the future. In the present study we have demonstrated survival of rat and human FSC grafts when transplanted in the hemisected adult rat spinal cord. Although these grafts appear to promote some degree of regeneration of 5-HT fibers no clear effects on functional recovery of the hemisected rats as assessed by the grid test were observed.

A major problem in using neural transplantation as a strategy for spinal cord injury repair has been the lack of reinnervation of the spinal cord distal to the lesion. To overcome this problem of axonal regrowth we have used a novel strategy of double grafts in which FSC tissue was injected into the intact cord above and below the lesion. The double grafting technique has been used successfully in animal models of Parkinson's disease to reconstruct the dopaminergic nigrostriatal pathway (Mendez et al., 1996; Mendez and Hong, 1997; Mehta et al., 1998). In these studies, simultaneous grafts of fetal ventral mesencephalon grafts placed in the striatum and substantia nigra lead to long distance axonal growth of axons from the nigra to the striatum. The double grafts promoted improved functional recovery and a greater reinnervation of the striatum by dopaminergic fibers when compared with animals receiving single grafts in the striatum or substantia nigra alone. These studies suggest that the double graft strategy provided guiding and trophic factors which stimulated long distance axonal regrowth from the nigra to the striatum. We hypothesized that a similar strategy in the spinal cord may also lead to increased axonal growth and functional recovery.

5.1 Animal Model

The first objective of this project was to establish a reliable model of spinal cord injury in the laboratory. We originally investigated the complete transection as a model of spinal cord injury. The advantage of the complete transection model is that the surgery is easily replicated and ensures total transection of fibers at the level of T9. Following complete spinal cord transection, 5-HT content below the level of the lesion is usually completely abolished (Hadjiconstantinou et al., 1984; Bregman, 1987). This observation is very helpful since in a completely transected adult rat spinal cord, any 5-HT positive fibers observed within the transplant and below the site of the injury are assumed to be regenerating fibers originating from the proximal host spinal cord. However, this model had major disadvantages. Postoperative care of completely transected animals was extensive and time consuming. Lesioned animals required manual emptying of the bladder two to three times daily, for the first ten to fourteen days, which decreased to once a day afterwards. Animals also required daily baths and cage changes to prevent skin breakdown and infection. Several animals developed pressure sores from the extended periods of lying on one side of their lower body. Mortality in completely transected animals was extremely high, greater than 50.3%, primarily from autotomy and urinary tract infections. Despite intensive efforts to decrease these complications using enhanced environments, to prevent autotomy, and prompt antibiotic therapy to decrease the incidence of urinary tract infection, mortality remained high. It was clear that the mortality rate in the thoracic complete transection paradigm made this model impractical and not well suited for this study.

In contrast, the thoracic hemisection animal model had a relatively low mortality rate of 1.8%. The animals recovered from the surgery rapidly and the majority of the animals were active by the end of the first week. Bladder function returned to normal, requiring no manual emptying of the bladder beyond five

days following surgery. Another advantage of this model is that the contralateral intact side can serve as a structural and functional control for the lesioned side. In this model there is paralysis of the ipsilateral hindlimb, therefore functional assessment can be easily made by comparing the affected limb with the contralateral intact limb. The major disadvantage is that the hemisection is difficult to replicate and may not transect all fibers on the ipsilateral side. Nonetheless, we found the thoracic hemisection model a reproducible and reliable model in which to study the structural and functional effects of the double grafting strategy of neural transplantation in the adult spinal cord.

5.2 Identification of transplants

Numerous studies have reported long term survival of rat FSC following transplantation into the adult rat spinal cord (Reier et al., 1984). Optimal survival of FSC tissue has been attributed to several factors, including the age of the donor. Spinal cord tissue harvested from E11-E15 fetuses has shown excellent survival following transplantation (Patel and Bernstein, 1983; Bernstein et al., 1984; Reier et al., 1984). Contact of the graft with the vascular surface of the host spinal cord also promotes graft survival. Low survival of transplanted FSC tissue, was observed in animals exhibiting extensive damage to host spinal cord tissue and blood vessels (Reier et al., 1984).

Rat FSC tissue grafts were identified by the presence of tissue in the lesion site that did not have the characteristic grey and white matter distribution of the intact host spinal cord. In addition, cresyl violet stain revealed large neurons, presumably motoneurons, within the grafted tissue. Sections meeting these criteria were deemed to have surviving transplants. In 76% of animals receiving transplants the grafts were easily identified. Grafts that did not survive occurred in animals that had extensive lesions and damage to the blood supply

of the host spinal cord. These results correlate well with previous studies that suggest decreased graft survival associated with extensive damage to the host spinal cord tissue and blood vessels during surgery (Reier et al., 1986).

The use of human FSC made graft identification easy. The antibody, human neuron specific enolase, stains specifically human neural tissue and not rat tissue. Human FSC grafts were found to survive in 64% of animals receiving transplants. In most animals the human FSC grafts were well defined and integrated to the host spinal cord, in several instances fibers could be seen extending into the host spinal cord tissue.

The observation that the human FSC grafts survive and integrate into the adult immunosuppressed rat is the principle finding of this study. This novel observation is important because it shows the ability of human spinal cord tissue to maintain it's viability after the trauma of harvesting, dissociation and transplantation. These results also have implications in the field of human neural transplantation. It is well known that human fetal cells survive and promote functional recovery in Parkinson's patients (Lindvall, 1998; Kordower et al., 1997; Lindvall, 1997; Wenning et al., 1997; Kordower et al., 1996; Lindvall et al., 1994). Several clinical trials are currently underway to determine if neural transplants of human fetal dopaminergic neurons are clinically effective (Lindvall, 1998; Kordower et al., 1997). Initial attempts have been made to transplant fetal tissue in spinal cord injury in humans (Reier et al., 1994), however there is a lack of animal data to indicate that this strategy is feasible. In the present study we have shown that human fetal spinal cord tissue can survive and integrate into the adult spinal cord of an immunosuppressed rat. These results indicate that potential strategies using human fetal grafts to treat spinal cord injury patients are feasible since human fetal spinal cord cell suspensions are viable after grafting.

5.3 Identification of serotonergic fibers

5-HT immunocytochemistry is useful for identifying descending pathways. There is a dense and highly organized descending projection of 5-HT neurons to the spinal cord. 5-HT fibers in the adult rat spinal cord originate in the brainstem, in the medullary raphe nuclei, and descend to the spinal cord through the ventral and dorsolateral funiculus. The fibers terminate at all levels of the spinal cord, predominantly in layers 1 and 2 of the dorsal horn, the intermediolateral column, surrounding the central canal (layer 10) and the motor nuclei of the ventral horn (Paxinos, 1995).

a) Hemisection only animals

Following hemisection 5-HT immunoreactive fibers dramatically decrease distal to the injury. Damaged spinal cord axons cluster at the host-lesion interface and fail to regenerate across the site of injury. The failure of CNS neurons to spontaneously regenerate has been attributed to many factors, including the presence of a glial scar at the injury site creates a physical barrier (Reier and Houle, 1988; Houle, 1992) and also the general non-permissive nature of the CNS environment (Fraser et al., 1984; Thanos et al., 1984; Caroni and Schwab, 1988; Lander, 1989; Schnell and Schwab, 1993; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Weibel et al, 1994; Tang et al., 1997). Controlling these adverse factors may be crucial in promoting regeneration of injured axons and may be important for the development of neural transplants for spinal cord injury.

b) Bridge grafts in hemisected animals

Although placement of a FSC graft into the site of the lesion has been shown to permit regeneration of descending fibers across the site of injury (Reier et al., 1986; Bregman, 1987; Jakeman and Reier, 1991; Itoh et al., 1993; Houle

et al., 1996; Diener and Bregman, 1998a), axonal regrowth terminates at the distal host-graft interface before re-entering the distal spinal cord (Bregman 1987; Bregman et al., 1989; Bregman et al., 1997b). In the current study, we have shown similar results. The presence of a FSC tissue bridge at the injury site promoted regeneration of 5-HT positive fibers into the bridge graft. However, consistent with previous studies, regenerating axons did not grow beyond the permissive FSC environment into the distal host spinal cord. Several possible mechanisms have been proposed to explain this phenomenon.

It is possible that the FSC bridge graft provided a physical support structure, free of neurite growth inhibiting factors, that facilitated axonal regeneration. Neuronal replacement at the bridge site has also been observed (Reier et al., 1985; Reier et al., 1986; Jakeman et al., 1989). It is also likely that bridge grafts participate in restoration of spinal cord circuitry by serving as a site for relay of synapses for host axons (Privat et al., 1986; Jakeman et al., 1989; Privat et al., 1989; Itoh and Tessler, 1990 a,b; Itoh et al., 1993; Houle et al. 1996). In addition, the FSC bridge may provide trophic support (Bregman and Reier, 1986; Bernstein-Goral and Bregman, 1997; Mori et al., 1997; Bregman et al., 1998; Diener and Bregman, 1998b) and modify the glial response at the site of the injury decreasing scar formation and permitting axons to cross the graft-host interface (Kruger et al., 1986; Bregman and Kunkel-Bagden, 1988; Tessler, 1991). However, regenerating axons in the bridge grafts do not re-enter the distal host spinal cord. It is possible that although FSC transplants reduce the degree of glial scarring, there remains areas of gliosis at the lesion site (Kruger et al., 1986; Bregman and Kunkel-Bagden, 1988; Tessler, 1991). This mechanical barrier may prevent axons from crossing the graft-host interface into the distal spinal cord. Another important factor in limiting axonal regeneration is the non-permissive nature of the spinal cord environment, specifically the presence of myelin (Caroni and Schwab, 1988; Savio and Schwab, 1989; Weibel et al, 1994)

and the absence of growth promoting molecules such as ECM components, laminin, fibronectin, heparan sulfate proteoglycan (Carbonetto et al., 1987) and neurotrophic factors (Masonpierre et al., 1990). It is conceivable that axons located in the proximal spinal cord enter the bridge which may have a permissive environment for growth and cannot find the double graft signals to continue to grow to the distal non-permissive cord.

c) Bridge grafts combined with double grafts in hemisected animals

An essential part of this project was to investigate the ability of double grafts to promote regeneration of descending axons across the bridge through the distal graft-host interface. It was hypothesized that double grafts of FSC into the host spinal cord may provide the additional trophic support needed to guide the regenerating axons to traverse the lesion area and re-establish connections with normal target areas in the distal spinal cord. The premise in this group of animals was that the proximal graft may rescue degenerating axons and promote their growth towards the lesion site which contains a bridge graft. The distal graft would in turn promote axonal growth to the distal spinal cord.

In several animals receiving bridge grafts plus double grafts of FSC tissue 5-HT positive fibers were observed in the bridge graft. However, similar to the bridge graft only group, 5-HT positive fibers were not observed re-entering the distal host spinal cord. These results suggest that the presence of the distal graft did not change the non-permissive environment of the distal cord and did not increase 5-HT fiber regeneration to the distal spinal cord.

d) Double grafts in hemisected animals

In the group receiving double grafts only without a bridge graft, no 5-HT fibers were observed crossing the lesion site. However, there were some 5-HT fibers present in the distal cord. It is possible that the distal graft may have

promoted sprouting of 5-HT fibers of the distal cord or may have encouraged axons to cross the midline proximal to the lesion and enter the distal cord. These concepts will have to be tested using tracing experiments, but may indicate that the distal graft is important for guiding proximal fibers to the distal cord.

5.4 Identification of corticospinal tract fibers

The descending corticospinal tract (CST) plays an important role in regulating motor function (Paxinos, 1995). Following spinal cord injury, axotomized CST fibers undergo permanent retrograde degeneration (Schwab and Brosamle, 1997; Tetzlaff et al., 1994; Pallini et al., 1989; Pallini et al., 1988), in the completely transected animal averaging 45.6 um/day (Pallini et al., 1988). FSC tissue transplants alone have been shown to have no effect on decreasing the extent of CST retraction (Pallini et al., 1989). However, FSC combined with IN-1, an antibody to the neurite inhibitor myelin, promote CST fiber regeneration through the lesion site and into the distal host spinal cord (Schnell and Schwab, 1993). Administration of IN-1 (Schwab and Brosamle, 1997; Schnell et al., 1994) and neurotrophic factor (Grill et al., 1997a,b) without FSC transplants have also been shown to increase CST regeneration. Transplantation studies involving alternative tissue, such as olfactory ensheathing cells (Li et al., 1997), and peripheral nerve grafts (Cheng et al., 1996) have also reported CST regeneration through the site of injury.

In the current study there was no significant difference in CST retraction between experimental groups and control lesioned animals. However, in no sections were CST fibers observed degenerating as far rostrally as previously reported (Pallini et al., 1988; Pallini et al., 1989).

A possible explanation for these results may be attributed to technical problems with the CTB injection. In the adult rat, CST neurons are located

mainly in the primary motor cortex and in the forelimb and hindlimb areas of the primary sensory cortex, near the dorsal surface of the rat brain. Injecting CTB into these areas will anterogradely label CST fibers, however CTB not absorbed can leak into the subarachnoid space. In this way the tracer can be carried in the cerebral spinal fluid throughout the CNS. If the CTB is transported to the site of the lesion, it may be picked up by fibers at the lesion site and even by the transplant. In brain sections, motoneurons were labeled, however there was also intense CTB positive staining at the dorsal surface of the brain, suggesting that CTB leaked out of the injection site and stained non-specifically the surface of the brain. Further, in most sections the lateral edge of the spinal cord was also stained with CTB at all levels, although not as intensely as the CST fibers. These technical problems make interpretation of the results difficult.

5.5 Functional Recovery

Functional impairment in the control hemisected rats correlates well with previous studies (Bregman et al., 1997b; Zompa et al., 1997). Permanent impairment in limb placement accuracy on the grid walk motor test in the hemisected adult rat has been validated previously (Bregman et al., 1997b; Zompa et al., 1997). Control of movement has been attributed to several descending supraspinal influences. For instance, the CST mediates voluntary movement through connections with interneurons and by directly synapsing on motoneurons (Liang et al., 1991; Paxinos, 1995). The consequence of specific CST lesions on forelimb use has been well examined. Lesions of this pathway impaired forelimb reaching, including lifting, aiming, pronation and supination of the forelimb, but did not significantly impair grasping (Z'Graggen et al., 1998; Grill et al., 1997b; Whishaw et al., 1993). Although the specific effects of pyramidotomy on hindlimb use have not been examined extensively, general

overground locomotion is not affected (Z'Graggen et al., 1998; Grill et al., 1997b; Fehlings and Tator, 1995; Whishaw et al., 1993). In addition to the CST, several other supraspinal pathways have been implicated in the control of movement, including the rubrospinal, vestibulospinal and reticulospinal pathway (Whishaw et al., 1998; Grill et al., 1997b, Whishaw et al., 1993, Whishaw et al., 1990). Specifically the vestibulospinal and reticulospinal fibers control balance and postural adjustments during movement (Whishaw et al., 1998; Paxinos, 1995; Holstege and Kuypers, 1987; Jones and Yang, 1985).

Hemisection of the thoracic spinal cord theoretically severs all supraspinal input to the ipsilateral lumbar spinal cord. However, within a week following surgery, all animals are active and using the ipsilateral hindlimb, but this limb is still significantly impaired when compared to normal controls or the contralateral hindlimb. Although it was impossible to distinguish exact hemisections to the midline of the spinal cord, in most animals extensive over-hemisections had been performed. In these animals the use of the ispilateral hindlimb was significantly impaired compared to animals with incomplete hemisections. These observations suggest that there were residual descending fibers or there was crossing over of fibers from the contralateral side of the spinal cord, contributing to the control of movement of the ipsilateral hindlimb. Z'Graggen et al. (1998) have suggested that a lesion may induce reorganization of the intrinsic motor system of the spinal cord and facilitate the release of chemotrophic and neurotrophic factors that induce recruitment of collateral axons from the contralateral spinal cord. In addition, mechanisms, such as the activation of previously inactive synapses (Wall, 1987), increased sensitivity of neurons, upregulation of receptors (Bregman, 1994), and sprouting of intact fibers (Murray and Goldberger, 1974, Zhang et al., 1995) have been proposed to account for the partial recovery recovery of ipsilateral limb function.

In the current study the grid walk test of motor function was used to assess improvement in limb placement accuracy. Previous studies have not reported functional improvement on the grid walk test following transplants of FSC tissue into the injured adult rat spinal cord (Bregman et al., 1997a,b; Zompa et al., 1997, Pallini et al., 1989). Similarly, in this study statistically significant improvement in grid walk test scores was not observed in any of the treatment groups receiving rat FSC when compared to control lesion only animals. In animals receiving human FSC grafts there was not a significant difference in grid walk test scores between controls and the treatment groups: HX+B, HX+B+DG, however, there was a significant difference between the HX and HX+B+DG groups and the HX + DG group. Although this observation requires further investigation it correlates with the notion that the distal grafts alone may increase 5-HT fiber regeneration around the lesion site into the distal cord. Further studies are necessary to elucidate if this hypothesis is correct. Another question to answer is the degree of regeneration of descending fibers necessary for recovery of affected limbs. Bregman and colleagues (1997a) suggested that improvement of hindlimb precision requires complete regeneration of descending pathways and possibly restoration of ascending sensory pathways. Although the difference in grid walk scores was not significant, the average footfall error scores for transplanted groups were slightly lower than for the control lesion only animals. This suggests that there may be at least partial regeneration of descending pathways. Another possibility to explain this observation is that some regenerating fibers synapse on donor neurons and influence spinal cord circuitry distal to the lesion. It is also possible that the presence of FSC at the site of injury has induced functional reorganization of the spinal cord circuitry distal to the lesion that is independent of descending input. It is important to note that although the double grafting strategy did not lead to clear improvement in motor scores, the scores were not worsened. This observation is important since

placing a proximal graft into the non-injured spinal cord has potential to worsen the degree of trauma caused by the hemisection.

5.6 Summary

In summary, this study has established the hemisection model of spinal cord injury as a useful model for spinal cord injury in neural transplantation experiments. The demonstration of survival of human FSC tissue grafts is the main observation of the study. The use of human tissue in the rat model of spinal cord neural transplantation has allowed clear differentiation of grafts from rat tissue and has shown the feasibility of human graft survival. This observation has potential implications in clinical neural transplantation for spinal cord injury in the future. Although the double graft strategy did not clearly show advantages in relation to bridge grafts, it has shown that it is not detrimental to spinal cord function and merits further study.

Neural transplantation for spinal cord injury is in its infancy. Our results indicate that the ability of FSC transplants to compensate for the loss of descending input is at this time still limited. It is possible that neural transplantation may need to be combined with other enhancing strategies, such as neurotrophic factors or antibodies to neurite inhibiting factors, to be more effective. However, it is clear that regeneration and reconstruction strategies for spinal cord injury have great potential and provide a novel and exciting approach for the future treatment of this devastating condition.

6. References

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