Acidic Fibroblast Growth Factor's Association with Motor Neuronal Reaction to Injury

by

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TABLE OF CONTENTS

List of Tables

List of Figures

List of Figures Continued

List of Abbreviations

ACh	-	Acetylcholine
aFGF	-	Acidic Fibroblast Growth Factor
bFGF	-	Basic Fibroblast Growth Factor
CNS	-	Central Nervous System
CNTF	-	Ciliary Neurotrophic Factor
CNTFa	-	Ciliary Neurotrophic Factor alpha receptor component
DN		Dominant Negative
FGF	-	Fibroblast Growth Factor
HBGF	-	Heparin Binding Growth Factor
HSPG	-	Heparin Sulfated Proteoglycan
LIF	-	Leukemia Inhibitory Factor
MN	-	Motor Neuron
MEP	-	Motor End Plate
PNCI	-	Peripheral Nerve Crush Injury
PNS	-	Peripheral Nervous System
TK	-	Tyrosine Kinase

Acknowledgments

No research occurs in a vacuum. The quality and substance of research depends as much on external factors such as the intellectual environment, the current state of knowledge, and the challenge perceived as well as on internal factors such as life balance, curiosity, creativity and comfort with one's self. I have no more (and possibly quite less) drive or intellect than other graduate students. What I do have in abundance is balance through my family and friends. My escapes to sea and my medical studies have not detracted from my research activities, rather they have served to help me focus and keep an even keel when things in the lab were not at their best. Now on with my litany of thanks to the following people who contributed to the successful completion of my graduate education.

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Abstract

Acidic fibroblast growth factor has long been known to have potent *in-vitro* neurotrophic activity as well as non-neuronal stimulatory properties. The description of it's anatomical expression pattern in adult animals, where it is found in high concentrations within the cytoplasm of motor neuronal cell bodies and axons, has stimulated the search for aFGF's *in-vivo* role. When the factor's molecular properties have been examined, it is apparent that it is sequestered within cells, not being able to be released through the classic ER-Golgi export pathway. These clues have stimulated the development of the hypothesis that aFGF is a neuronally derived neurotrophic factor which is made available by the release of axoplasm at the time of axonal injury. It is this hypothesis which this thesis concentrates on.

The first step taken, was to describe the temporal and anatomical expression pattern of aFGF in developing rodents. This work demonstrated that aFGF is expressed in a slowly progressive manner within the same cellular populations it is found in the adult animal. The expression begins generally around P7 and increases to adult levels by P21/28.

Next, the peripheral nerve crush injury system was examined. This injury system having been determined to be the most reliable for experimental investigations in developing animals. These experiments demonstrated a striking resemblance to the time line seen in the aFGF expression work. Animals who were injured at young ages (when aFGF levels were low) had large amounts of neuronal cell death following the injuries. The older animals (at ages when aFGF expression was moderate or high) had limited neuronal cell death and adult animals had full survival and recovery.

Naturally these results only intensified the interest in aFGF's *in-vivo* role. As experimental methods to directly block aFGF activity following injury were unavailable, the converse approach was taken. Experimental injuries were performed in animals which normally suffer a moderate amount of post injury neuronal cell death, although in these experiments the endogenous amount of FGF was supplemented by either direct aFGF treatment or by using animals who overexpress the closely related family member bFGF. In both of these situations neuronal survivals were increased over the control animals.

These experiments support the hypothesis that aFGF is a neuronally derived neurotrophic factor which is made available at time of axonal lesion.

Chapter I: Introduction and Background

Introduction

The study of neuronal injury has been of interest to humans for centuries (Kandel, 1991). In modern times this research has been driven primarily by the immense psychological, emotional and dollar value cost which motor neuron injury places on both personal and societal resources (Harrison and Dijkers, 1991; Lundqvist *et al.*, 1991). My research interest has also been on nervous system injury. The traumatic loss of function and impact on quality of life which are seen following injury have driven me to attempt to understand the physical and biochemical interactions surrounding an injury event. It is my hope that the research presented here can in some small way help reduce the monetary and personal costs by providing a clearer view of motor neuronal injury response. The central theme of my research is the role of growth factors following motor neuronal injury.

This chapter introduces the aspects of motor neuronal cell biology which are relevant to the experimental work presented in later chapters. The motor neuronal cellular environment, developmental changes, reaction to injury are all considered. In addition two families of neurotrophic factors which are thought to play a role in post injury neuronal survival are introduced.

Motor Neurons

Environment

Primary motor neurons, (MNs), are the final common pathway for translation of cognitive planning / initiation / and direction of voluntary movement into the desired physical actions. In order to fulfill this unique role, motor neurons span three specialized cellular environments: The central nervous system, the peripheral nervous system and the motor end plate.

The MN cell bodies reside within the first of the specialized environments, that of the central nervous system (CNS). The CNS provides the motor neuron cell bodies with an environment which is biochemically isolated from the general circulation. Bathed in cerebral spinal fluid, with nutrients provided by the filtering action of the neural endothelium derived blood-brain barrier, these cell bodies are protected from many somatic insults (de Vries *et al.*, 1997; Risau, 1995). By virtue of the blood-brain barrier, the CNS is isolated biochemically from the systemic environment. Within the CNS, the MN cell bodies and initial projections are in direct contact (and directly influenced by) the other CNS cell types: Interneurons, projection neurons, other MNs, astrocytes, oligodendrocytes, and microglia.

The oligodendrocyte ensheathed axons project from the CNS into the second of the specialized environments, the peripheral nervous system (PNS), where the oligodendritic myelin sheath is replaced by that of Schwann cells. In the PNS the MN axons encounter two additional cell types; Schwann cells and endoneurial fibroblasts. These cells are found in the peripheral nerves which are made up from the MN axonal bundles. Endoneurial fibroblasts contribute to the establishment of the extracellular matrix found in peripheral nerves. In this process, the fibroblasts appear to provide signals to Schwann cells which further the development of the nerve (Bunge, 1993). Fibroblasts participate in the maintenance and post injury

remodeling of peripheral nerves (Roytta *et al.*, 1987). Recent investigations have shown that fibroblasts can also act as immunomodulators (Fries *et al.*, 1994) suggesting a mechanism for their role following injury. The functions of Schwann cells beyond the obvious role of myelination are many. They are sources for growth factors, provide physical protection of the axons, regulate local axonal structure, contribute to the extracellular matrix and promote regeneration of axons following injury (Bunge, 1993; Reynolds and Woolf, 1993). The Schwann cells provide the intimate covering for large axons, extending from the axon's exit from the CNS to it's arrival at the motor end plate.

The axons lose their Schwann cell sheath as they arrive at the third specialized environment, that of the motor end plate (MEP). The specialized structure of the MEP has been well described on the morphological, biochemical and functional levels (Bennett and Robinson, 1989; Carbonetto and Lindenbaum, 1995; Changeux *et al.*, 1990; Grinnell, 1995; Hall and Sanes, 1993). This close apposition of MN and muscle allows for two way interactions which go beyond the functional activity of turning ionic signals into chemical signals and then into physical movement. Some examples include muscle dependence on innervation to prevent atrophy (Fu and Gordon, 1995), promotion of neuronal survival with muscle extracts (Houenou *et al.*, 1991), rescue of motor neurons from developmental cell death when muscle activity is blocked (Harris and McCaig, 1984), as well as the influences of muscle activity on regeneration following MN injury (Little *et al.*, 1991).

The immediate local environments of the PNS and MEP are subject to somatic influences in special situations. The axonal bundles which make up the peripheral nerves are not normally invaded by immune cells, but following injury they are not exempt from local immune response. These responses include local inflammation, invasion by monocytes, as well as exposure to immune derived cytokines. (Fig. 1.1).

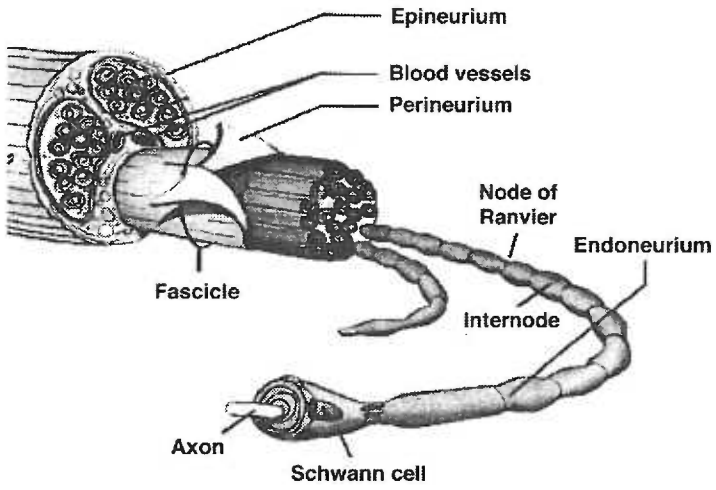


Figure 1.1
Diagram of the peripheral nerve anatomy. Note the relationship between individual axons, their associated Schwann cell covers, the encompassing perineurium, intrinsic vascular bundles and protective epineurium. Figure adapted from (Gartner and Hiatt, 1990).

Development

Motor neurons, in rodents, undergo their final mitosis between embryonic days 10 and 11, (E10-E11), (Phelps *et al.*, 1988). Rapidly following this event they extend their axonal process out into the PNS towards their target. Along the way, prior to arrival at their target, the MNs begin to express detectable levels of the transmitter synthesizing enzyme choline acetyltransferase (Chen and Chiu, 1992; Phelps *et al.*, 1991). The MN axons arrive at the target muscle between E13 and E17 during which time they begin to form polysynaptic connections with the target (Dennis *et al.*, 1981; Phelps *et al.*, 1991). Interestingly, this time of synapse formation overlaps with the normal developmental cell death which eliminates more that 50% of the starting MNs (Harris and McCaig, 1984). Naturally occurring MN cell death is followed by the normal elimination of polysynaptic connections at the MEP, which extends into the first postnatal weeks. Developmentally motor neurons appear to transition through several stages during which their response to injury differ (Sheard *et al.*, 1984).

Astrocytic migration begins around E16 when type 1 astrocytes migrate to their final positions and begin to contact both vascular structures and neurons (Miller *et al.*, 1985; Raff and Lillien, 1988). O2A cells, (oligodendrocyte precursors), begin to migrate towards their destinations as early as E15 (Miller *et al.*, 1985). Oligodendrocytes start their maturative steps at birth and continue to mature into the second week of life (Miller *et al.*, 1985). The immune cells of the CNS, the microglia, appear to be in place in the CNS as early as E13 (de Groot *et al.*, 1992; Perry *et al.*, 1985).

In the young PNS, Schwann cell precursors are proliferating and migrating along the established axons as early as E10. As they migrate these cells, in conjunction with fibroblasts, begin to lay down basal lamina as a step towards their

final differentiation (Bunge, 1993; Jessen and Mirsky, 1991). The process of differentiation of Schwann cells, including ensheathment of large and small axonal bundles forming the well known pattern of myelination, continues into the first postnatal week. Non-myelinating Schwann cells continue their proliferation and migration into the second month after birth (Jessen and Mirsky, 1991).

The target muscle formation begins with the fusion of myoblasts into early multinuclear myotubes, first seen at E14. The extension and striation of these young muscles continues through embryonic development and is essentially complete by birth. At the end plate, the first thickening of the basement membrane occurs concurrently with the detection of acetylcholine esterase and early acetylcholine receptor clusters around E15. The condensation of the end plate and the elimination of extra junctional ACh receptors extends from E18 till birth (Dennis *et al.*, 1981). All of these developmental events are outlined in graphical form in Figure 1.2.

Figure 1.2: Rodent Developmental Timeline

Central Nervous System
Motor Neurons

Final Mitosis
Axonal Elongation
Detection of Choline Acetyltransferase
Arrival at Target
Formation of Polysynaptic Connections
Neuronal Cell Death
Elimination of Polysynaptic Connections

Glia

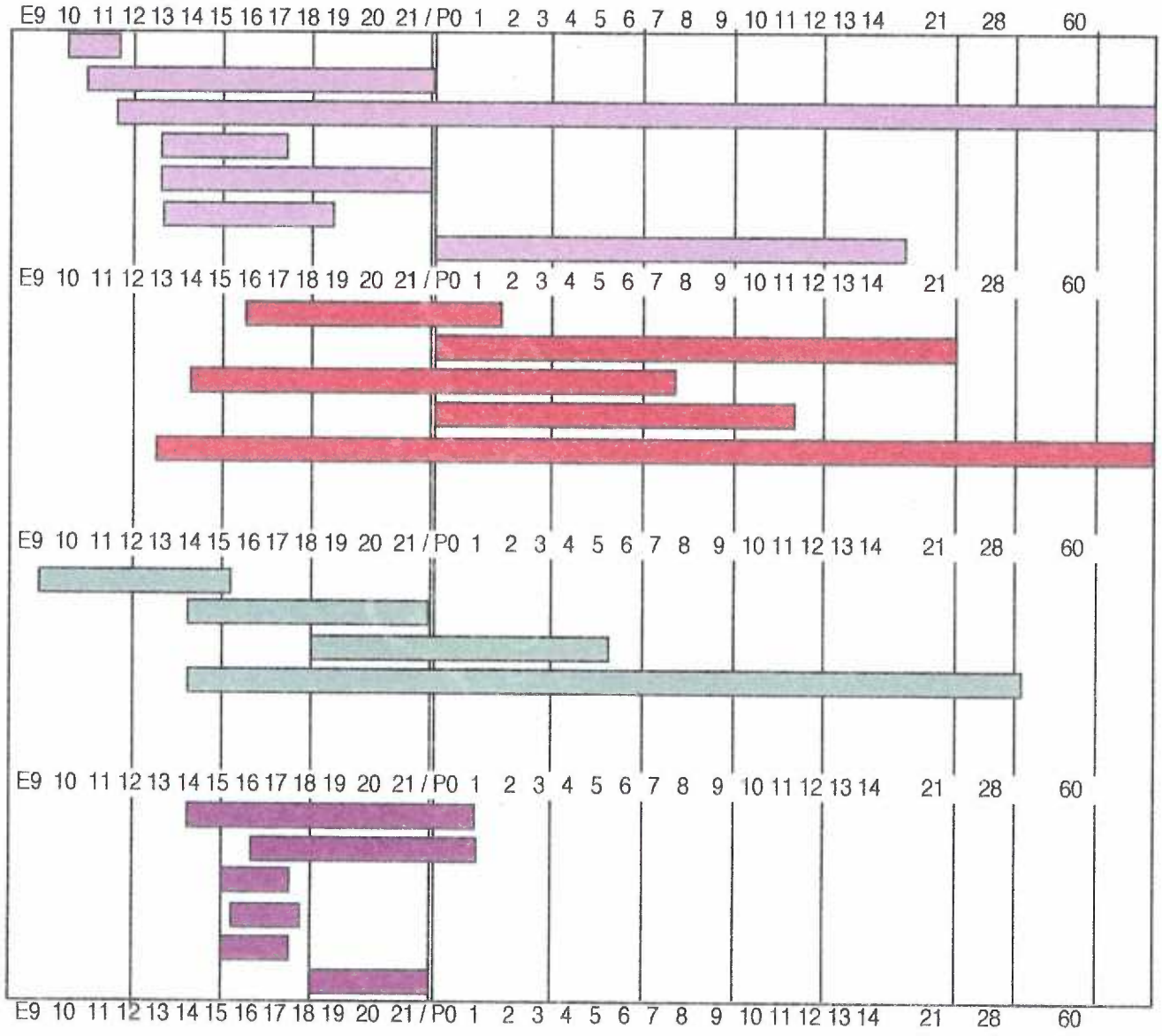
Migration / Mitosis ofType 1 Astrocyte
Maturation of Type 1 Astrocyte
Migration of O2A Cell
Differentiation of Oligiodendrocyte
Presence of Microglia

Peripherial Nervous System
Schwann Cells

Proliferation and migration of precursors
Differentiation & migration of myelinating SCs
Formation of Schwann Cell Myelin
Different. & migration of non-myelinating SCs

Motor End Plate
Muscle Cells

Fusion of Myoblast & Formation of Myotubes
Elongation & Striation of Muscle
Clustering of Acetylcholine Receptors
Appearance of MEP Basement Membrane
Appearance of Acetylcholine Esterase
Reduction in Extrajunctional Receptors



Experimental Motor Neuron Injury

Model systems

Central nervous system reaction to experimental injury of peripheral nerves has been under study for over a hundred years (Gudden, 1870). During this time a wide variety of injury protocols have been developed. Examples of these include: simple nerve transection (Schmalbruch, 1984), nerve transection with steps to prevent regeneration such as segment removal or deflection of the cut stumps (Kou *et al.*, 1995), nerve transection and target removal via limb amputation (Romanes, 1946), nerve avulsion (Torvik and Soreide, 1972), electrocautery (LaVelle and LaVelle, 1959), toxin injection (Streit and Kreutzberg, 1988), muscle paralysis (Greensmith and Vrbova, 1992), cold block (Wu *et al.*, 1993) and simple nerve crush (Romanes, 1946). Each of these investigations has aided our understanding of the nervous system's reaction to injury. Through these studies it has become clear that the central reaction to peripheral injury is influenced by various factors, the most important being: The injury protocol used (Torvik and Skjorten, 1971a; Torvik and Skjorten, 1971b); The species of animal being investigated (Torvik and Soreide, 1975); The age of the animal at the time of injury (Bueker and Meyers, 1951); The time after injury at which the CNS is examined (Kou *et al.*, 1995; Torvik and Skjorten, 1971a; Torvik and Skjorten, 1971b). It has been established by a number of investigators that the two most popular injury protocols, nerve transection and nerve crush, each stimulate their own specific injury response pattern when used in adult animals. Some examples of these differences include: Extensive neuronal loss seen in adult mice following transection injury as compared to full neuronal survival following crush injury (Crews and Wigston, 1990; Torvik and Skjorten, 1971a; Torvik and Skjorten, 1971b); Increased peri-neuronal microglial localization and bouton stripping following a transection injury as compared to crush injury

(Torvik and Skjorten, 1971a; Torvik and Skjorten, 1971b; Torvik and Soreide, 1975; Watson, 1974); Prolonged expression of mRNA for calcitonin gene-related peptide, cholecystokinin and galanin following a transection injury (Saika *et al.*, 1991); Long term increases in neurofilament mRNA following a crush injury and similar expression of actin, tubulin and GAP 43 mRNA expression following a transection injury (Tetzlaff *et al.*, 1991). These differences support the proposal that the adult transection protocol can be used as a model for central degenerative processes, and the adult crush protocol can be used as a model for regenerative processes (Torvik and Skjorten, 1971a; Torvik and Skjorten, 1971b; Torvik and Soreide, 1975).

Cellular model of injury

The differences in neuronal survival seen following these two injury models can be directly traced to the cellular consequences of the protocols. In the crush injury model the peripheral nerve sheath is crushed with fine forceps, creating a gap of 1-4 mm. This gap between axonal stumps is bridged by the epineurial covering of the nerve. Thus the axons which have been injured are presented with a restricted, directed area within which to regenerate. Immediately following the crush there is a small amount of axonal cytoplasm leakage rapidly followed by membrane sealing. Within a short time the proximal stump begins the formation of a growth cones, which will be delimited in their outgrowth by the epineurium. Meanwhile the distal stump undergoes traditional Wallerian degeneration which yields a loose Schwann cell tube directing the regrowing axons towards the target (Jessell, 1991) (Fig. 1.3). In adult mice, reinnervation and functional activity after facial nerve crush, as measured by blink and whisker movement, is restored within 14 days following the crush lesion (Kuzis, unpublished results).

For the transection protocol the cellular situation is substantially different. A true transection protocol includes steps to prevent regeneration from taking place, such as removal of a complete nerve segment (5-10mm). The axonal leakage and membrane sealing is undoubtedly the same following nerve transection, yet this is where the similarities end. As the gap created is of a large distance and lacks the epineurial boundary, the environment at the tip of the severed proximal stump provides little physical or molecular support for axonal growth cone activity. The absence of the epineurial boundaries makes the distant Schwann cell tube essentially unreachable. This causes the severed axons to attempt to regenerate in an inappropriate environment, yielding the classically observed disorganized neuroma. Over the ensuing weeks the neurons perish presumably from lack of target connection (Thomas, 1989; Umemiya *et al.*, 1993). Results from peripheral nerve transection protocols which do not include the step to insure stump separation must be evaluated with caution as a variable amount of axonal regeneration likely occurs.

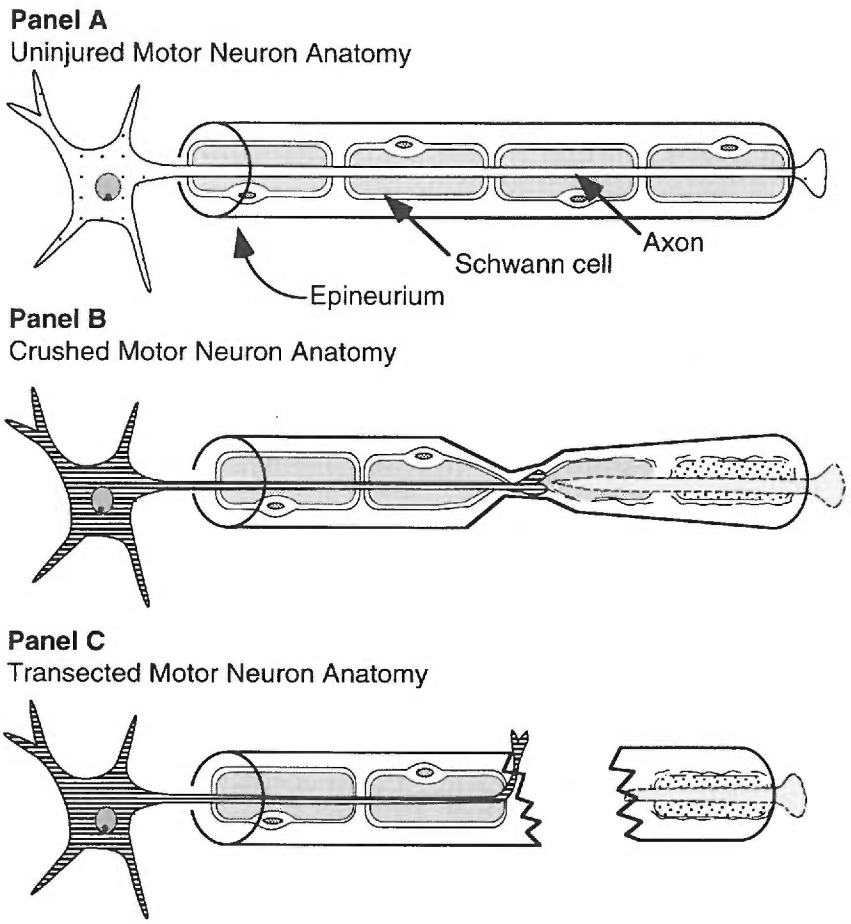


Figure 1.3
Cellular response to peripheral nerve injury.
Panel A - Uninjured motor neuron.
Panel B - Crush injury. Note division of axon, damage to Schwann cells and intact epineurium. Epineurium channels growth cone extension towards Schwann cell tube created via Wallerian degeneration.
Panel C - Transection injury. Note division of axon, damage to Schwann cells and disrupted epineurium. Lack of intact epineurium allows growth cone extension away from Schwann cell tube. Typically forming a neuroma and preventing reconnection with target muscle.

The overall difference between the adult animal transection and the crush protocols is that the transection model depends on the unsuccessful regeneration resulting from an experimentally created inappropriate environment while the crush model examines the successful regeneration inherent to the PNS. Therefore detailed examination of the crush model has the potential to yield insights into the

basic biological basis of peripheral nerve regeneration and MN survival which are not observed in the transection model.

It is clear from previous work that peripheral nerve crush injuries (PNCIs) in adults results in full motor neuronal survival and regeneration, which is in stark contrast to the significant retrograde neuronal cell death seen in animals injured at less than one week of age. (Ranging from 50% to >90% reduction) (Bueker and Meyers, 1951; Romanes, 1946; Schmalbruch, 1984; Soreide, 1981).

The cellular model of injury presented above provides adequate explanation for the neuronal regeneration and survival differences observed between the adult transection and crush injury models. These cellular differences alone are unable to account for the age related differences in neuronal survival seen within the crush injury system. In order to account for the neuronal survival differences between neonatal and adult animals we must consider the neurotrophic factors available within the motor neuron environment.

Neurotrophic Influences

General Considerations

Even in adult animals, the maintenance of target connections is important to the ultimate survival of motor neurons. It is likely that this target derived support is in part due to trophic factors produced by the muscle (Crews and Wigston, 1990; Umemiya *et al.*, 1993). The experimental separation of motor neurons from their target is common to the transection and crush injuries. The difference, as presented above, is that the crush injury provides a pathway for regeneration allowing ultimate reconnection to the target. The complex cellular environment of the motor neuron offers many possibilities for neurotrophic support during the period which a crushed axon is striving to reconnect to it's target. For motor neurons, such as those of the facial nucleus, potentially important factors at the site of injury are derived

from the ensheathing Schwann cells (ciliary neurotrophic factor, CNTF) and from the motor neurons themselves (acidic fibroblast growth factor, aFGF). Additional factors whose role following axonal injury remain to be described include: Basic fibroblast growth factor (bFGF) from astrocytes surrounding the motor neuron cell body; Leukemia inhibitory factor (LIF) from periaxonal fibroblasts and immune cells; Glial cell-line derived neurotrophic factor (GDNF) from Schwann cells; and muscle derived neurotrophins.

This selection of potentially available trophic factors has led to the development of a post injury recovery model which relies on neurotrophic contributions from several factors as opposed to a single "lesion factor". Evidence for this concerted injury response model, where factors derived from various cells involved in an injury each contribute to promote neuronal survival and repair, is slowly accumulating (Nishi, 1994). *In-vivo* support for this model comes from the abilities of GDNF, CNTF and brain derived neurotrophic factor (BDNF) to rescue independently facial motor neurons which would have died following an injury in young animals (Henderson *et al.*, 1994a; Henderson *et al.*, 1994b; Sendtner *et al.*, 1992; Sendtner *et al.*, 1990), as well as the synergistic activity of CNTF and BDNF in halting motor neuron degeneration (Mitsumoto *et al.*, 1994). Evidence for a FGF contribution comes from the ability of FGFs to act synergistically with CNTF in promoting motor neuronal sprouting (Gurney *et al.*, 1992). (Fig. 1.4).

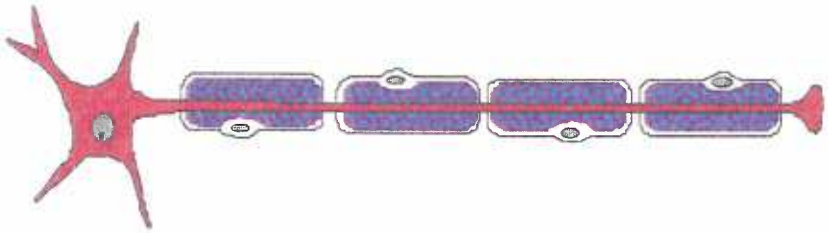


Figure 1.4
The primary neurotrophic factors in the motor neuronal environment.
CNTF within Schwann cells, aFGF within motor neurons.

Fibroblast Growth Factor Family

FGF Family Members

One family of neurotrophic factors which have a potential role following motor neuron injury is the Fibroblast Growth Factor family, also known as heparin binding growth factors (HBGFs). This family consists of at least fifteen structurally related proteins that are characterized by their selective binding to the sulfated glycosaminoglycan heparin and by their overlapping mitogenic or neurotrophic actions on a variety mesodermal, ectodermal and neuroectodermal cell types (Burgess and Maciag, 1989; Eckenstein, 1994).

Acidic and basic FGF were the first two members of the FGF family to be isolated and characterized. For review see (Burgess and Maciag, 1989). The two factors share about 55% amino acid sequence homology, and both can act as mitogens for nonneuronal cells, including astrocytes (Pettmann *et al.*, 1985), Schwann cells (Davis and Stroobant, 1990), and oligodendrocytes (Eccleston and Silberberg, 1985), and as neurotrophic factors for a large variety of peripheral (Eckenstein *et al.*, 1990; Schubert *et al.*, 1987; Unsicker *et al.*, 1987) and central neuronal populations (Engele and Bohn, 1991; Morrison *et al.*, 1986; Walicke *et al.*, 1986). The concentrations of these factors required to stimulate *in-vitro* half-maximal effects are typically less than one nanogram per milliliter of culture medium (Eckenstein *et al.*, 1991). One major distinction between the two factors is that aFGF requires the addition of exogenous heparin in order to be fully active in *in-vitro* assays, while bFGF is active in the absence of heparin in similar assays (Burgess and Maciag, 1989; Eckenstein *et al.*, 1991). Moreover, in contrast to several other members of the FGF family, both aFGF and bFGF lack an amino terminal hydrophobic signal sequence, which is required for the sorting of proteins into the secretory pathway (Walter and Lingappa, 1986). Thus it is currently unclear

to what extent and by what mechanism aFGF and bFGF are released from intact cells.

In the adult CNS, aFGF and bFGF represent the bulk of mitogenic activity present (Eckenstein *et al.*, 1991; Thomas, 1987a; Thomas, 1987b). Early studies reported conflicting data with widespread and overlapping expression patterns for the two factors (Grothe and Unsicker, 1992; Pettmann *et al.*, 1986; Wilcox and Unnerstall, 1991). Undoubtably, the close primary structural homology between the two factors contributed to the difficulty in developing tools with consistent properties. Subsequent studies found that aFGF and bFGF are localized in distinct cell populations within the adult rat CNS: aFGF being present in specific subcortical neuronal populations, including primary motor neurons (Elde *et al.*, 1991; Schnurch and Risau, 1991; Stock *et al.*, 1992); bFGF in astrocytes and in a population of hippocampal pyramidal neurons (Emoto *et al.*, 1989; Woodward *et al.*, 1992).

FGF Receptors

The biological effects of the FGFs are mediated by a family of structurally homologous transmembrane receptors arising from four separate genes, (FGFRs). For review see (Johnson and Williams, 1993). Three members of the high affinity FGF receptor family, (FGFR-1 (Flg), FGFR-2 (Bek), and FGFR-3), are known to be expressed throughout the CNS. FGFR-1 and FGFR-2 are most commonly expressed in CNS (Werner *et al.*, 1992). *In-situ* hybridization studies in the adult CNS have localized FGFR-1 mRNA expression mainly to neuronal populations, including motor neurons (Wanaka *et al.*, 1990), while FGFR-2 mRNA seems to be expressed predominantly by non-neuronal cells (Asai *et al.*, 1993). Both FGFR-1 and FGFR-2 exist in multiple forms, generated by differential splicing of the

respective RNAs. The splice variants of both receptor types most common in CNS appear to be able to bind and mediate the effects of both aFGF and bFGF. For review see (Jaye *et al.*, 1992; Johnson and Williams, 1993). In addition to these high affinity receptors, cell surface heparan proteoglycans are necessary co-factors for the biological activity of FGFs, presumably by binding and presenting FGFs to the high affinity transmembrane receptors (Klagsbrun and Baird, 1991; Rapraeger *et al.*, 1991). *In-vitro* the addition of soluble heparin increases the biological activity of FGFs (Eckenstein *et al.*, 1991; Klagsbrun and Baird, 1991).

Ciliary Neurotrophic Factor and Leukemia Inhibitory Factor

Factor Description

The other trophic factors thought to have a role in post PNCI remodeling includes CNTF and LIF. These two factors are independent gene products with limited primary amino acid homology (Stockli *et al.*, 1991) but predicted structural homology (Bazan, 1991) which allows them to share a transmembrane receptor complex. Of particular interest to this thesis is the anatomical localization of both of these factors to the periaxonal area, CNTF appearing within Schwann cell cytoplasm and LIF produced by fibroblasts and local immune cells found near lesion sites. As a complete review of these factors and their interactions with motor neurons is presented in chapter five only a brief overview of these factors is presented here.

CNTF temporal and anatomical localization

Within the specialized environment of the MN, CNTF is localized to only a few cell types. In the central nervous system, CNTF messenger RNA and protein have been localized to a widely distributed subpopulation of astrocytes and to oligodendrocyte-like cells in the spinal cord (Dobrea *et al.*, 1992; Stockli *et al.*, 1991). In the peripheral nervous system some, but not all, myelinating Schwann cells have been shown to produce CNTF in high concentrations (Dobrea *et al.*, 1992; Friedman *et al.*, 1992; Gupta *et al.*, 1992; Rende *et al.*, 1992). Immunohistochemistry has shown CNTF reaction product located in the cytoplasm of these cells, presumably sequestered there as the mature protein lacks the traditional signal sequence required for efficient secretion (Rende *et al.*, 1992; Stockli *et al.*, 1989).

In rodents, CNTF mRNA is detected by northern blot in extracts from E11 head

(Ip *et al.*, 1993b), whereas CNTF protein is first detected at postnatal day four. The levels of mRNA and protein increase to reach adult levels by postnatal day 21 (Dobrea *et al.*, 1992; Sendtner *et al.*, 1991; Stockli *et al.*, 1989). In addition, low levels of CNTF mRNA has been found in adult muscle by northern blot. (Ip *et al.*, 1993b).

LIF anatomical localization

Within the specialized environments of the MN, LIF is found in only a few cell types. *In-vivo* expression of LIF is found in skeletal muscle extracts, activated monocytes and injured peripheral nerves (Anegon *et al.*, 1990; Curtis *et al.*, 1994; McManaman *et al.*, 1990). The observation that LIF is required for normal immune cell production is consistent with its expression within immune cells (Escary *et al.*, 1993). *In-vitro*, LIF has been found in astrocyte and fibroblast cultures suggesting that cells within these cultures are capable of expressing LIF under certain circumstances (Wesselingh *et al.*, 1990). The observation that LIF knockout mice have no detectable motor abnormality suggests that LIF is less important in MN development and maintenance (Escary *et al.*, 1993; Stewart *et al.*, 1992).

Localization of CNTF/LIF overlapping receptor complexes

The signaling ability of CNTF and LIF are determined by the distribution of their shared receptor complex. The receptor complex is assembled from the common gp130 and LIFb subunits. The addition of the CNTF alpha subunit allows CNTF activation of the receptor complex and thus limits the possibilities for CNTF signaling (Ip *et al.*, 1993a; Ip and Yancopoulos, 1992). The current thought is that LIF requires only gp130 and LIFb components of the receptor complex resulting in a distribution of responsive cells which completely overlaps with those defined as

CNTF responsive (Gearing *et al.*, 1994; Ip and Yancopoulos, 1992). With respect to motor neurons, *in-situ* hybridization detection of the specific CNTF alpha (CNTFa) receptor component has identified CNTFa message in motor neurons from early development into maturity (Ip *et al.*, 1993a). In addition northern blots of adult tissue demonstrate the CNTFa component in muscle and sciatic nerve mRNA (Ip *et al.*, 1993a). It appears that Schwann cells, target muscle, and motor neurons themselves have the receptor complex, thereby allowing them to respond to CNTF / LIF.

Molecular model of injury

The cellular model of PNCI, as described previously, relies on the retention of overlying epineurium to guide growth cone elongation. The molecular model I propose for neurotrophic support following PNCI is similar. Upon breach of the plasma membrane, axonal and Schwann cell cytoplasm is released in limited quantities within the injury zone. The released cytoplasm contains concentrated aFGF and CNTF. This free factor is bound by prexisting receptors located both on axonal segments and on Schwann cell membranes. Receptor activation promotes neuronal survival via a cascade which is incompletely understood. It can by hypothesized that receptors exist on both neuronal axons and Schwann cells so on a molecular basis both aFGF and CNTF may act in autocrine and paracrine manners. (Fig. 1.5).

Under this model direct neurotrophic support is available via the autocrine action of aFGF binding to HSPGs and FGFRs on the axonal membrane as well as by the paracrine action of CNTF released from Schwann cells binding to CNTF receptor complex on axonal membranes. Indirect neurotrophic support would be available by Schwann cell stimulation. This stimulation would again occur via

Study Goals

The working hypothesis for this thesis is that aFGF has a role within the concerted injury response presumed to occur following motor neuronal injury. Several lines of evidence contribute to development of this hypothesis. We have observed high levels of aFGF expression in motor neuronal populations in adulthood (Stock *et al.*, 1992). The observed aFGF has been localized to the cytoplasmic space adjacent to the membrane of both neuronal cell bodies and axons (Elde *et al.*, 1991; Stock *et al.*, 1992). When mitogenic activity attributable to aFGF is extracted from tissues, the effective concentrations exceed 200 ng/mg soluble protein (Eckenstein *et al.*, 1991; Stock *et al.*, 1992). While the amount of aFGF extractable from neural tissue is in the hundreds of nanograms per milligram of soluble protein, the concentrations required for half maximal stimulation *in-vitro* are typically less than 1 ng/ml of culture medium. The potent neurotrophic and non-neuronal mitogenic activities of aFGF are transduced through tyrosine kinase receptors suggesting that release from the cell is necessary for action. Yet the fact that aFGF lacks an amino terminal hydrophobic signal sequence indicates that it is not released through the traditional ER-Golgi secretion pathway. These observations suggest that aFGF's role may be fulfilled after being released at the site of an injury, by breach of the plasma membrane.

From the data presented above we know that acidic fibroblast growth factor is a potent neurotrophic agent which is found in high concentrations, in an anatomically appropriate location to play a role following motor neuronal injury. With this in mind the specific hypothesis I set out to test can be stated as:

Motor neuron derived acidic fibroblast growth factor acts to support motor neuronal survival following peripheral nerve crush injury.

The first prediction of my hypothesis is that the decreased neuronal survival following PNCI seen in young animals should be proportional to the detectable levels of aFGF found at the time of PNCI. This prediction builds upon the molecular model above by considering that injury induced cytoplasmic leakage is independent of age at PNCI, but concentration of aFGF within the released cytoplasm is dependent on age at time of injury. This hypothesis also predicts that increased availability of aFGF (or FGFR stimulation) in a relatively FGF poor environment (such as in neonates) should increase neuronal survival following injury. Finally, the hypothesis predicts that if FGFR activation is blocked in a relatively FGF rich environment (such as in adults) neuronal survival following injury should be reduced.

The body of this document describes my efforts to test the above predictions for this hypothesis. The question of developmental expression pattern of aFGF is addressed in Chapter 2. The description of a developmentally regulated injury system as well as the effects of increasing FGF availability are presented in Chapter 3. Unfortunately tools for direct blocking of aFGF activity were not available so this prediction remains untested. Chapter 4 provides a discussion of the potentially synergistic lesion factors, CNTF and LIF. Finally, Chapter 5 documents my additional experiments and observations related to testing the stated hypothesis. In Chapter 6 I summarize and discuss the results and how they relate to my hypothesis. I close Chapter 6 by proposing additional experiments to refine my original hypothesis.

Chapter 2:

Developmental expression of acidic and basic fibroblast growth factor in rat

As our laboratory had previously investigated the localization of aFGF in adult rats, (Stock *et al.*, 1992), and collaborated on the adult localization of bFGF, (Woodward *et al.*, 1992), the next step was to examine the expression of these growth factors during development, as well as the regional expression of the predominant receptor forms (FGFR-1 & FGFR-2). This work can be found in publication as: (Kuzis *et al.*, 1995) and is reproduced here in it's final form.

"Developmental time course of acidic and basic fibroblast growth factors expression in distinct cellular populations of the rat central nervous system"

Abstract

Acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) are expressed in high levels in adult central nervous system (CNS). Here we report the time course of developmental appearance and distribution of these factors and of two FGF receptors, FGFR-1 and FGFR-2, in the CNS of rats ranging in age from embryonic day 16 to adult. Immunohistochemical analysis showed that sensory neurons in the midbrain were the first cells to contain detectable aFGF immunoreactivity at embryonic day 18. The next cell group to contain aFGF were motor neurons, which were found to be aFGF-positive at the day of birth. A number of other subcortical neuronal populations were observed to contain aFGF immunoreactivity after postnatal day 7. Adult levels and distribution patterns of aFGF were reached in all CNS areas by postnatal day 28. Basic FGF immunoreactivity was observed at postnatal day 0 in neurons in the CA2 subfield of hippocampus. Astrocytes contained detectable bFGF immunoreactivity starting at postnatal day 7. Adult levels and patterns of distribution of bFGF were reached in all CNS areas by postnatal day 28. These immunohistochemical observations were confirmed using bioassay and western blot techniques. FGFR-1 and FGFR-2 mRNA were expressed in significant levels in all CNS areas at all time points analyzed. The observation that aFGF and bFGF appear in specific and distinct cellular populations at relatively late developmental times suggests that these FGFs may be involved in specific mechanisms of CNS maturation, maintenance and repair.

Introduction

The family of fibroblast growth factors (FGFs, also known as heparin binding growth factors or HBGFs) consists of at least nine structurally related proteins that are characterized by their selective binding to the sulfated glycosaminoglycan heparin and by their overlapping mitogenic or neurotrophic actions on a variety mesodermal, ectodermal and neuroectodermal cell types (Burgess and Maciag, 1989; Eckenstein, 1994). The biological effects of the FGFs are mediated by a family of transmembrane receptors (currently consisting of at least four structurally homologous, but different genes), which contain intracellular tyrosine kinase domains. Two members of the high affinity FGF receptor family, FGFR-1 (Flg) and FGFR-2 (Bek), are known to be expressed in the CNS. Both FGFR-1 and FGFR-2 exist in multiple forms, generated by differential splicing of the respective RNAs. The splice variants of both receptor types most common in CNS appear to be able to bind and mediate the effects of both aFGF and bFGF, for review see (Jaye *et al.*, 1992; Johnson and Williams, 1993). In addition, cell surface heparan proteoglycans are necessary co-factors for the biological activity of FGFs, presumably by binding and presenting FGFs to the high affinity transmembrane receptors (Klagsbrun and Baird, 1991; Rapraeger *et al.*, 1991).

Acidic and basic FGF were the first two members of the FGF family to be isolated and characterized (for review see (Burgess and Maciag, 1989)). The two factors share about 55 % amino acid sequence homology, and both can act as mitogens for nonneuronal cells, including astrocytes (Pettmann *et al.*, 1985), Schwann cells (Davis and Stroobant, 1990), and oligodendrocytes (Eccleston and Silberberg, 1985), and as neurotrophic factors for a large variety of peripheral (Eckenstein *et al.*, 1990; Schubert *et al.*, 1987; Unsicker *et al.*, 1987) and central (Engele and Bohn, 1991; Morrison *et al.*, 1986; Walicke *et al.*, 1986) neuronal

populations. One major distinction between the two factors is that aFGF often requires the addition of exogenous heparin in order to be fully active in *in-vitro* assays, while bFGF is active in the absence of heparin in similar assays (Burgess and Maciag, 1989; Eckenstein *et al.*, 1991). Moreover, in contrast to several other members of the FGF family, both aFGF and bFGF lack an amino terminal hydrophobic signal sequence, which is required for the sorting of proteins into the secretory pathway (Walter and Lingappa, 1986). Thus it is currently unclear to what extent and by what mechanism aFGF and bFGF are released from intact cells.

In the adult CNS, aFGF and bFGF represent the bulk of mitogenic activity present (Eckenstein *et al.*, 1991; Thomas, 1987). Earlier studies found that aFGF and bFGF are localized in distinct cell populations within the adult rat CNS: aFGF being present in specific subcortical neuronal populations (Elde *et al.*, 1991; Schnurch and Risau, 1991; Stock *et al.*, 1992), and bFGF in astrocytes and in a population of hippocampal pyramidal neurons (Emoto *et al.*, 1989; Woodward *et al.*, 1992). A series of conflicting studies, however, reported different and more widespread expression patterns for the two factors (Grothe and Unsicker, 1992; Pettmann *et al.*, 1986; Wilcox and Unnerstall, 1991). *in-situ* hybridization studies have localized FGFR-1 mRNA expression mainly to neuronal populations (Heuer *et al.*, 1990; Wanaka *et al.*, 1990), while FGFR-2 mRNA and FGFR-3 mRNA seem to be predominantly expressed by non-neuronal cells (Asai *et al.*, 1993; Peters *et al.*, 1993).

The present study investigates, at the cellular level, the developmental appearance and distribution of aFGF and bFGF in rat CNS, and correlates, using northern blot techniques, FGFR-1 and FGFR-2 mRNA levels in the same tissue. While aFGF and bFGF distribution is mainly investigated by immunohistochemistry, western blot and bioassay methods are used for confirmation, which is important considering the conflicting reports in the literature (see above).

Methods

Antisera

The rabbit polyclonal anti-aFGF serum used here for immunohistochemistry has been characterized previously (Stock *et al.*, 1992). This rabbit antiserum, however, showed poor sensitivity for localization of aFGF on western blots. Therefore additional antisera to aFGF were prepared and characterized for the present study, using methods identical to those described earlier (Stock *et al.*, 1992). Several antisera prepared in mouse strongly stained recombinant aFGF while they did not detect an excess of bFGF on western blots.

The mouse monoclonal anti-bFGF ascites fluid used for both immunohistochemistry and western blots (#3886, a gift from Dr. C. Hart, Zymogenetics) has been characterized in detail earlier (Eckenstein *et al.*, 1991; Woodward *et al.*, 1992).

Immunohistochemistry

Long-Evans rats of different ages, embryonic days E16, E18, postnatal days P0, P7, P14, P21, P28 and adult (between P90 to P120) were used for this study. Timed pregnant rats were obtained from Simonsen. At least three animals were analyzed at each developmental stage. P14 and older animals were anesthetized and perfused through the heart with 50 ml of PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.2), followed by 300 ml of fixative (10% (v/v) formalin, 0.4% glutaraldehyde, 100 mM sodium phosphate, pH 7.2 for aFGF immunohistochemistry, or 5% (v/v) formalin, 100 mM sodium phosphate, pH 7.2 for bFGF immunohistochemistry). Tissues were dissected immediately after perfusion, postfixed in the same fixative at room temperature for either 30 min (bFGF) or 90

min (aFGF). Younger animals were sacrificed by asphyxiation with CO_2, and tissues were quickly dissected and immersion fixed at room temperature for two hours (bFGF) or overnight (aFGF) in the fixative described above. Tissues were infiltrated with 15% sucrose in 100 mM sodium phosphate, pH 7.2, followed by 30% sucrose in 100 mM sodium phosphate, pH 7.2. Coronal 50 µm thick frozen sections were cut from the olfactory bulb to the cervical spinal cord and processed for immunohistochemical staining of FGFs. As a control measure marked sections from adult animals, where the FGF staining pattern is known, were included in each batch of developing tissue which was processed.

For aFGF immunohistochemistry, equally spaced sections were incubated for 30 min in 30.0 % (v/v) ethanol and 5.0 % (v/v) hydrogen peroxide in 65 mM Tris, pH 7.3, to inactivate endogenous peroxidases, followed by two five minute washes in TBS (100 mM Tris, 150 mM NaCl, pH 8.2). The sections were then incubated for 90 minutes in blocking solution (100 mM sodium phosphate, 10.0 % (v/v) horse serum, 0.5 % (v/v) Triton X-100, 0.2 % (w/v) sodium azide), followed by overnight incubation (minimum of 12 hrs) in rabbit antiserum to aFGF or rabbit pre-immune serum (both diluted 1:1000 in blocking solution). Sections were washed three times for ten minutes each in TBS, incubated for one hour in goat anti-rabbit antiserum (Sternberger-Meyer) diluted 1:250 in blocking solution containing 5% (v/v) rat serum), washed as described above, incubated for one hour in rabbit peroxidase-anti-peroxidase (Sternberger-Meyer) diluted 1:100 in blocking solution containing 5% (v/v) rat serum and no sodium azide, washed and reacted with 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% (v/v) hydrogen peroxide in PBS. The reaction was stopped by washing twice for five minutes each with PBS. The stained sections were mounted on gelatin coated glass slides and coverslipped according to standard procedures.

For bFGF immunohistochemistry, tissue sections were incubated for 60 minutes in blocking solution, followed by overnight incubation in mouse monoclonal antibody to bFGF (ascites fluid # 3886) or mouse pre-immune serum (both diluted 1:2000 in blocking solution), washed three times for ten minutes each with PBS, incubated for one hour in biotinylated goat anti-mouse (Vector) diluted 1:100 in blocking solution containing 5% (v/v) rat serum), washed, incubated for one hour in avidin--peroxidase complex (Vectastain ABC from Vector) according to the manufacturer's specifications, washed, and reacted with DAB as described above.

For anatomical identification of stained cells in P7 through adult sections, the atlases by (Paxinos and Watson, 1986) and by (Swanson, 1992), were used. For tissues of younger ages (E16-P0) the atlas by (Paxinos *et al.*, 1991), was used.

Preparation of tissue extracts

Long-Evans rats of the above ages were sacrificed by asphyxiation with CO_2, the CNS was quickly removed and immediately dissected into the following areas: forebrain (including cerebral cortex and hippocampus), midbrain/hindbrain, cerebellum and spinal cord. Dissected tissues were immediately frozen on dry ice and stored at -70 ˚C for up to three weeks. For analysis of FGF-levels, tissue from at least three animals was combined, soluble extracts were prepared and protein concentration determined as described earlier (Eckenstein *et al.*, 1991).

Western-blot analysis.

FGFs were enriched from extract supernatants by heparin affinity chromatography. Heparin-Affigel (Biorad) columns (400 µl bed volume per 500 mg tissue wet weight) were packed, washed with 2 bed volumes of 20 mM Tris, 2 M NaCl, pH 8.0, and equilibrated with 3 bed volumes of 20 mM Tris, pH 8.0. Supernatants of tissue extracts were applied onto the heparin affigel columns (care

was taken to apply equal amounts of protein per column volume) and the flow through was collected and re-applied to the column a second time. Under these conditions more than 80% of FGF-activity bound to the column, as determined by bioassay. The columns were washed, with 2 bed volumes of 20 mM Tris, pH 8.0, and eluted with 2 bed volumes of hot (60 ^{o}C) SDS buffer (1%(w/v) sodium dodecylsulfate, 1% (v/v) mercaptoethanol in 20 mM Tris, pH 8.0). Eluates were concentrated by lyophilization and reconstituted with H_2O to 10% of the starting supernatant volume. Twenty microliters of each eluate was loaded onto a single lane of polyacrylamide gel (16%) and electrophoresed in the presence of SDS under standard conditions. Separate lanes were loaded with 10 ng recombinant human aFGF and 2 ng recombinant bovine bFGF as standards. Proteins from these gels were electroblotted onto 0.2 µm nitrocellulose membranes using standard conditions. The membranes were allowed to air dry, then they were incubated overnight in blocking solution at room temperature. Detection of aFGF and bFGF immunoreactivity was performed using methods detailed earlier (Eckenstein *et al.*, 1991).

Assay for mitogenic activity.

The mitogenic effect of extracts was tested using a serum free ^{3}H-thymidine incorporation assay, modified slightly from a previously detailed method (Shipley, 1986). Briefly, 0.5 ml fresh MCDB 402 medium containing diluted supernatant (prepared by centrifugation at 15,000 g from tissue extracts) was added to confluent, serum-starved AKR-2B cells, followed 22 hr later by the addition of 1.0µCi/ml ^{3}H-thymidine. Cells were incubated in isotope for 1 hr after which ^{3}H-thymidine incorporation into cold 10 % trichloracetic acid insoluble material was determined. The amount of mitogenic activity present in tissue supernatants was

calculated as described previously (Eckenstein *et al.*, 1991). In this assay aFGF is only active in the presence of heparin, whereas bFGF is active also in the absence of heparin. Thus, dose response curves were determined with and without heparin. The ratio of extract activity measured in the presence and absence was then used to estimate the level of aFGF in the extracts as described earlier in detail (Eckenstein *et al.*, 1991). Three different bioassay experiments were performed for each age and tissue series.

Northern Blot Analysis

Total RNA was extracted from tissues by a one step phenol guanidinium method (Chomczynski and Sacchi, 1987) using TriReagent (Molecular Research Center). Fifteen micrograms of total RNA was loaded onto a lane of an agarose gel (1%), electrophoresed in the presence of formaldehyde and transferred onto Gene Screen Plus (Dupont-NEN) using standard conditions. Equal loading of lanes and efficiency of transfer were determined by staining the blots with methylene blue prior to further processing. ^{32}P-dCTP labelled DNA probes for FGFR-1 and FGFR-2 were prepared by standard methods from plasmid inserts encoding the extracellular domains of the two receptors (a gift of Dr. Craig Dionne, see (Dionne *et al.*, 1990)) using a random prime kit (Dupont-NEN). Blots were incubated at 62˚C for 72 hrs in 10^6 dpm/ml of probe in high efficiency hybridization buffer (from Molecular Research Center), washed at 65˚C in 150 mM NaCl, 15 mM sodium citrate pH 7.0, containing 1% (w/v) SDS, and labelled bands were detected by standard film autoradiographic methods.

Electronic preparation of figures

Figures representing immunohistochemical results were prepared by scanning 35 mm photographic slides and acquiring the digitized image with a Macintosh computer (Apple). Figures illustrating western and northern blots were similarly acquired using a flatbed scanner (under reflective illumination for western blots and trans-illumination for northern blots). Adobe Photoshop software package was used to adjust the brightness and contrast of figures to similar levels (care was taken that these adjustments did not alter the information represented by the figures), which were then printed on a dye-sublimation printer (Supermac).

Results

An initial survey established that aFGF and bFGF became detectable around the day of birth and increased substantially over the next three to four weeks. A detailed analysis was then performed to encompass ages from embryonic days 16 and 18 (E16 and E18), postnatal days 0, 7, 14, 21, 28 (P0, P7, P14, P21, 28) to adult (animals between 90 to 120 days old).

Immunohistochemical localization of aFGF

The neuronal populations containing aFGF immunoreactivity in the developing CNS were identical to those identified earlier in the adult (Stock *et al.*, 1992) and no cell populations exhibited transient aFGF immunoreactivity. The cellular distribution of aFGF immunoreactivity was cytoplasmic in appearance, filling the cell body, dendrites and axons of labelled cells. Acidic FGF immunoreactivity became detectable in specific neuronal populations at significantly different developmental times. These times ranged from P 0 to P14, and it was possible to group populations as being aFGF-positive at "early" (P 0), "intermediate" (P 7) or "late" (P 14) time points. In general, the intensity of aFGF staining was weak to moderate in all neuronal populations when first detected and increased gradually over the next 7 to 14 days to reach the level of intensity observed in the adult.

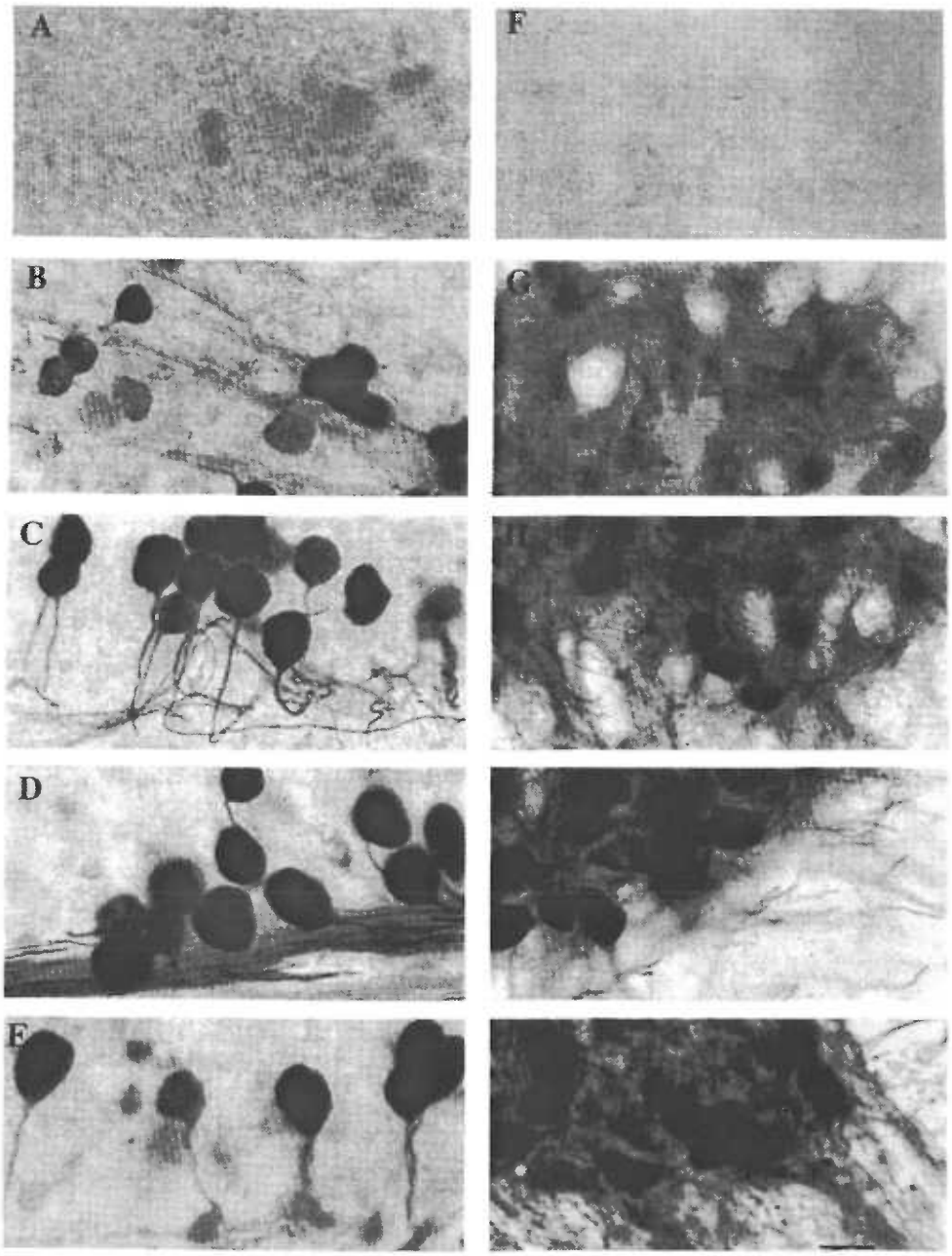


Figure 2.1: Immunohistochemical localization of aFGF in mesencephalic sensory (A-E) and oculomotor neurons (F-J) at different developmental ages (A and F: E 18; B and G: P 0; C and H: P 7; D and I: P 14; E and J: P 21). The mesencephalic sensory neurons are the first to become aFGF positive, followed closely thereafter by motor neurons. These two neuronal populations also reach close to adult levels of aFGF-staining intensity earlier than most other aFGF positive populations (P 7 for sensory and P14 for motor neurons). Note the impressive growth in size of the mesencephalic sensory neurons over the developmental time span studied and that the immunoreactivity fills the entire cytoplasm of labelled cells, including in dendrites and axons (bar in panel J = 25 μm).

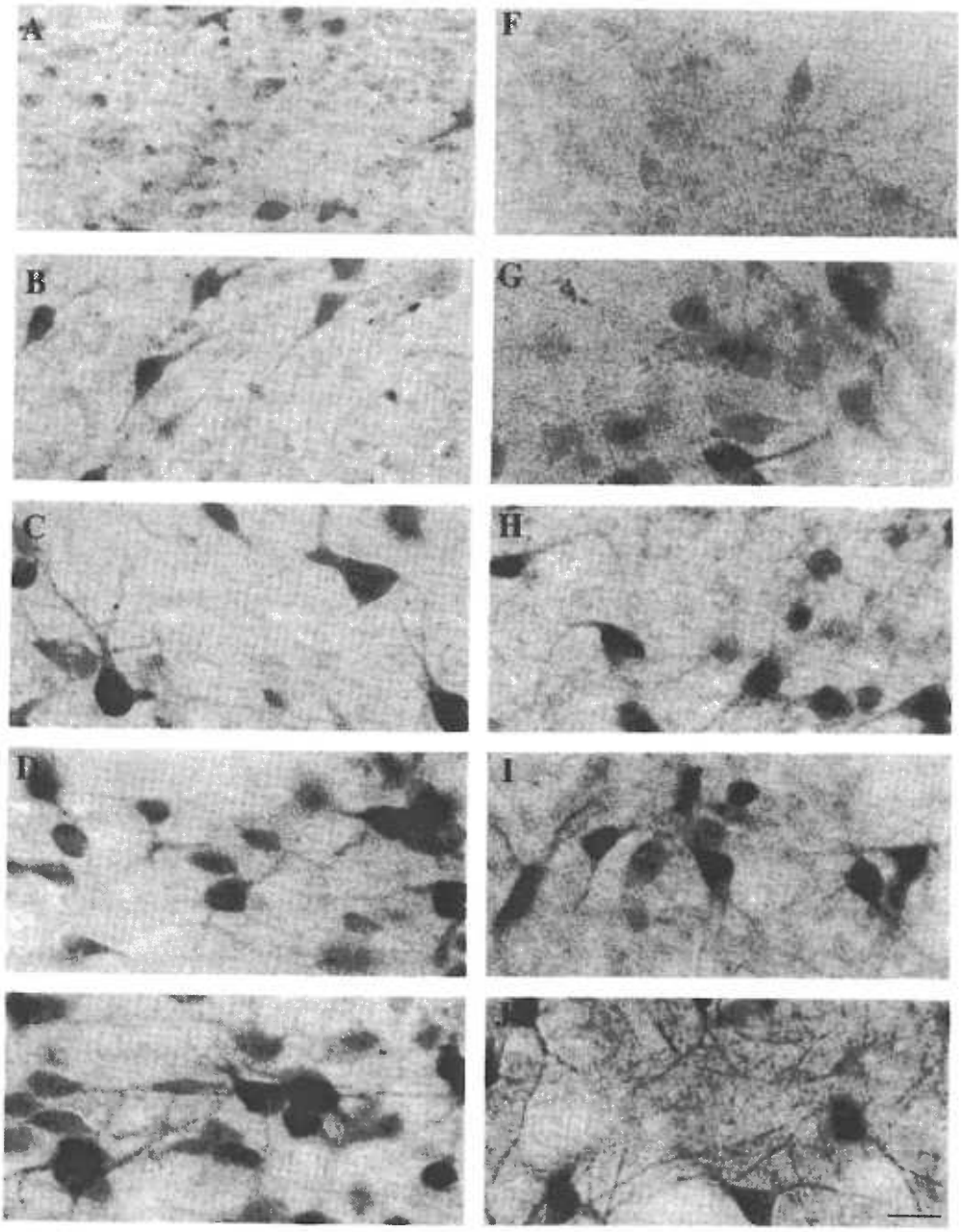


Figure 2.2: (A-E) Immunohistochemical localization of aFGF in neurons in the diagonal band of Broca at different developmental ages (A : P 7; B: P 14; C : P 21; D: P 28; E: adult). These cells are among the neurons that become aFGF positive at intermediate developmental time points (P 7) and reach close to adult levels of aFGF staining intensity around P 14. (F-J) Immunohistochemical localization of aFGF in neurons of the substantia nigra at different developmental ages (F: P 7; G: P 14; H: P 21; I: P 28; J: adult). These cells are among the neurons that become aFGF positive at late developmental time points (P 14) and reach close to adult aFGF-staining intensity around P 28.
(bar in panel J = 25 μm).

Neurons that became aFGF-positive at early times included primary sensory neurons in the mesencephalic nucleus of the trigeminal nerve (Figure 2.1, A-E), primary motor neurons (Figure 2.1, F-J), and large neurons in the reticular formation. In a few instances very faint labelling of mesencephalic sensory neurons was observed at E 18. The mesencephalic sensory neurons were the most intensely stained population at all the time points analyzed, reaching adult levels of staining as early as P 7. Motor neurons and reticular neurons also became intensely aFGF-positive at P 14 and P 21, respectively.

Populations that became aFGF-positive at intermediate times (P7) included neurons the diagonal band of Broca (Figure 2.2, A-E), in the hypothalamus and the thalamus. At P 14 these three groups exhibited aFGF staining of moderate intensity similar to that observed in adult animals. Hypothalamic and thalamic aFGF-positive neurons consisted of a number of different neuronal populations, defined by their specific locations, as described in detail in an earlier publication (Stock *et al.*, 1992). The timing of the appearance of aFGF immunoreactivity was similar in all these groups.

Populations that became aFGF-positive at late times (P14) included neurons in the deep cerebellar nuclei, in the raphe nuclei, the red nucleus, the substantia nigra (Figure 2.2, F-J), the nucleus basalis, the globus pallidus, the caudate and the ventral pallidum. Most of these populations showed moderate, adult-like levels of aFGF staining by P 21 to P 28. An exception were large aFGF-positive neurons in the caudate, which continued to mature and stained intensely in the adult. Only a very small number of stained neurons were observed in the cerebral cortex and the hippocampus. These neurons also became aFGF-positive around P 14.

aFGF immunoreactivity was also observed in dendrites, fiber tracts and terminal fields. Most prominently labelled were motor neuron axons, the axons of mesencephalic sensory neurons (Figure 2.1), and terminals in central areas

innervated by peripheral sensory neurons. The timing of aFGF appearance in axons and terminals paralleled the appearance of the factor in the corresponding cell bodies.

A general posterior to anterior gradient in the appearance and levels of aFGF immunoreactivity was observed, with immunoreactivity appearing earlier in the hindbrain and midbrain than in the forebrain. In addition, the neuronal populations that became aFGF-positive at an early time point also were more intensely labelled in the adult when compared to neurons in which aFGF became detectable later in development. Exceptions to these general rules, however, were also observed. For example the labelled neurons in the caudate became aFGF-positive late, yet still were intensely stained in adulthood. In addition, not all aFGF-positive neurons in the mid- and hindbrain became aFGF-positive at an early time. Figure 2.1 shows, for example, that small neurons in the vicinity of mesencephalic sensory neurons became aFGF-positive only at P 14. The pattern of developmental distribution of aFGF immunoreactivity is summarized in Table 1. It is important to note that only the most prominent aFGF-positive neuronal cell groups are identified in this table, and that several other populations, mainly in the mid- and hindbrain, also exhibit aFGF immunoreactivity.

Immunohistochemical localization of bFGF

In good agreement with earlier work (Woodward *et al.*, 1992), bFGF immunoreactivity was found predominantly in astrocytes and in neurons in the CA2 subfield of hippocampus, and no populations showing transient developmental expression of bFGF immunoreactivity were observed. The cellular distribution of immunoreactivity in labelled cells was mainly nuclear, with perinuclear labelling being observed in some cells. This nuclear localization of some forms of bFGF is in good agreement with earlier studies (Bugler *et al.*, 1991; Florkiewicz and Sommer, 1989; Renko *et al.*, 1990; Woodward *et al.*, 1992).

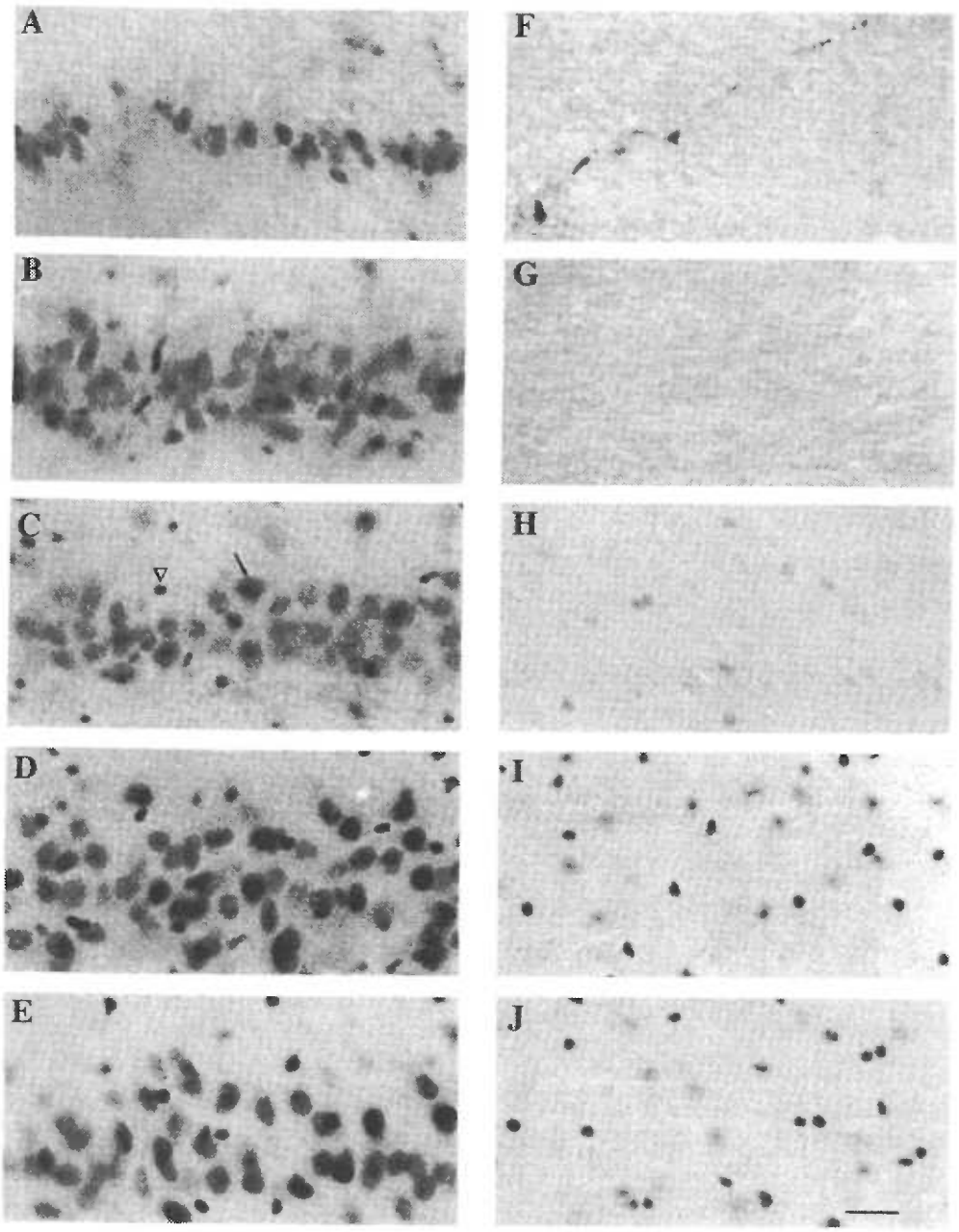


Figure 2.3: Immunohistochemical localization of bFGF in area CA2 of hippocampus (A-E) and cerebral cortex (F-J) at different developmental ages (A and F: P 0; B and G: P 7; C and H: P 14; D and I: P 21; E and J: P 28). Note that the staining is most intense in the nucleus of labelled cells. Small labelled nuclei (open arrowhead in panel C) represent astrocytes, which are present throughout the CNS, and large labelled nuclei, which are present in area CA2, represent neurons (arrow in panel C), labelled structures in panel F are red blood cells, which show non-specific staining. Hippocampal CA2 neurons are bFGF-positive earlier (at P 0) than hippocampal astrocytes, which are faintly stained at P 7 and astrocytes in cerebral cortex become bFGF positive roughly one week after hippocampal astrocytes (bar in panel J = 25 μm). CA2 neurons reach adult bFGF-staining intensity around P 7, hippocampal astrocytes around P 14 and cortical astrocytes around P 21.

Pyramidal cells in area CA2 of hippocampus were the first cells to contain detectable amounts of bFGF immunoreactivity at P 0, when they showed weak but clear bFGF staining. At P 7 the staining of these neurons was of moderate intensity, similar to the intensity of staining observed in adult CA2 neurons. Astrocytes in hippocampus and some subcortical areas became bFGF-positive by P 7, while astrocytes in cerebral cortex became positive by P 14 (Figure 2.3). Astrocytes in the cerebellum, hindbrain and spinal cord showed a significantly lower intensity of staining than did astrocytes in forebrain (not shown). This pattern of developmental distribution of bFGF immunoreactivity is summarized in Table 2.1.

Table 2.1. Intensity of Immunohistochemical Staining for FGFs in Rat CNS at Different Ages

	E16	E18	P0	P7	P14	P21	P28	Adult
Location of aFGF-positive neurons								
Subcortical telencephalon								
caudate	-	-	-	-	++	++	++	+++
ventral pallidum	-	-	-	-	+	++	++	++
diagonal band of Broca	-	-	+/-	+	++	++	++	++
nucleus basalis	-	-	-	-	+	++	++	++
globus pallidus	-	-	-	-	+	+	++	++
Thalamus*	-	-	-	+	++	++	++	++
Hypothalamus *	-	-	-	+	++	++	++	++
Midbrain								
substantia nigra	-	-	-	-	+	+	++	++
motor nucleus III	-	-	+	++	+++	+++	+++	+++
sensory, mesencephalic V	-	+/-	++	+++	++++	++++	++++	++++
red nucleus	-	-	-	-	+	+	++	++
Brainstem								
all motor nuclei	-	-	+	++	+++	+++	+++	+++
sensory, mesencephalic V	-	+/-	++	+++	+++	++++	++++	++++
raphe nuclei	-	-	-	-	+	+	++	++
reticular nuclei	-	-	+	++	++	+++	+++	+++
deep cerebellar nuclei	-	-	-	-	+	++	++	++
Location of bFGF-positive cells								
hippocampal CA2 neurons	-	-	+	++	++	++	++	++
hippocampal astrocytes	-	-	-	+	++	++	+++	+++
neocortical astrocytes	-	-	-	-	+	+	++	+++
subcortical astrocytes	-	-	-	+	++	++	+++	+++

+, detectable staining; ++, moderate staining intensity; +++, strong staining intensity; ++++, most intense staining.

* aFGF immunoreactivity is present in some, but not all, neurons in these areas see (Stock *et al.*, 1992). All groups show similar developmental regulation of aFGF immunoreactivity.

Western blot analysis of aFGF and bFGF

The immunohistochemical analysis described above indicated that FGF levels were significantly different between forebrain, mid/hindbrain, cerebellum and spinal cord, and that FGFs became first detectable after birth. These tissues were dissected from animals ranging from P 0 through adult, and FGF expression in extracts prepared from these tissues was investigated using western blot techniques.

aFGF immunoreactive bands (about 18 and 16 kD, representing a full length and a proteolytic degradation product, respectively (Burgess and Maciag, 1989)) were more abundant in spinal cord and mid/hindbrain than in cerebellum and and forebrain. In all these areas, immunoreactivity was strongest in adult tissues, and not detected in P 0 tissues. Distinct aFGF immunoreactivity was detectable in spinal cord and hindbrain at P 14, and in cerebellum and cerebral cortex by P 21 (Figure 2.4). These observations agree with the immunohistochemical data presented above, although the onset of aFGF expression was detected at an earlier stage by immunohistochemistry than by western blot. It is thus likely that the immunohistochemical methods used here are more sensitive than the western blot method.

Three bFGF immunoreactive bands (approximately 22.5, 21.5 and 18 kD), originating from differential initiation of translation of bFGF mRNA (Florkiewicz and Sommer, 1989) were observed in forebrain, mid/hindbrain and spinal cord. No bFGF was detectable in cerebellum. In the three areas that contained bFGF immunoreactivity, the larger forms of bFGF appeared to predominate, occasionally making it difficult to detect the 18 kD form. The 22.5 and 21.5 kD are known to be localized to the nucleus of cells producing bFGF, while the 18 kD form appears to be cytoplasmic (Bugler *et al.*, 1991; Florkiewicz and Sommer, 1989; Renko *et al.*,

1990; Woodward *et al.*, 1992). Thus, the relatively higher abundance of the larger forms is in good agreement with the mainly nuclear distribution of bFGF immunoreactivity shown above. Levels of bFGF immunoreactive bands at all developmental time points analyzed were significantly higher in forebrain than in mid/hindbrain and spinal cord. Basic FGF immunoreactivity was first detected in forebrain at P 0, and in mid/hindbrain and spinal cord at P14, and showed a gradual increase to their final adult levels (Figure 2.4). Again, these observations agree with the immunohistochemical data presented above.

Determination of mitogenic activity

FGFs are potent mitogens for the mouse AKR-2B cell line, and a previously described mitogenesis assay (Eckenstein *et al.*, 1991) was used to quantify the levels of FGF-like biological activity in extracts of adult and developing nervous tissue. In this assay aFGF is active only when heparin is added, while bFGF is active both in the presence and absence of heparin, making it possible to use the heparin-dependence of mitogenic activity to estimate relative contributions of bFGF-like and aFGF-like activity to the overall mitogenic activity. Estimates of the levels of bFGF- and aFGF-like bioactivities were made using the same extracts that were analyzed for aFGF and bFGF immunoreactivity by western blots.

Data obtained generally correlated well between the two methods. For example, aFGF bioactivity was highest in adult spinal cord, intermediate in mid/hindbrain and low in cerebellum and forebrain, and activity was barely detectable in all tissues at P 0, a distribution of bioactivity that overall is very similar to the pattern of aFGF expression observed by western blot (Figure 2.4). However, no aFGF was detected by western blot in P 7 spinal cord, although levels of heparin-dependent mitogenic activity in this tissue were moderately high.

The levels of bFGF-like bioactivity also showed a good general correlation to data observed by western blot. For example, in adult tissues bFGF-bioactivity and immunoreactivity were highest in forebrain, intermediate in mid/hindbrain and lowest in cerebellum (Figure 2.4). Both methods also demonstrated a significant increase in bFGF during the developmental time span studied. However, moderate levels of heparin-independent activity were present at P 0 in all areas, sometimes in levels higher than those detected at P 7, but no bFGF immunoreactivity was observed in these samples using the western blot technique.

One unit of biological activity as defined here (see methods) corresponds roughly to 250 pg of either aFGF or bFGF. Thus, the highest levels of aFGF (about 150 units / mg protein in adult spinal cord), were estimated to be 37.5 ng / mg of protein, and the highest levels of bFGF (about 40 units / mg), were estimated to be 10 ng / mg of protein.

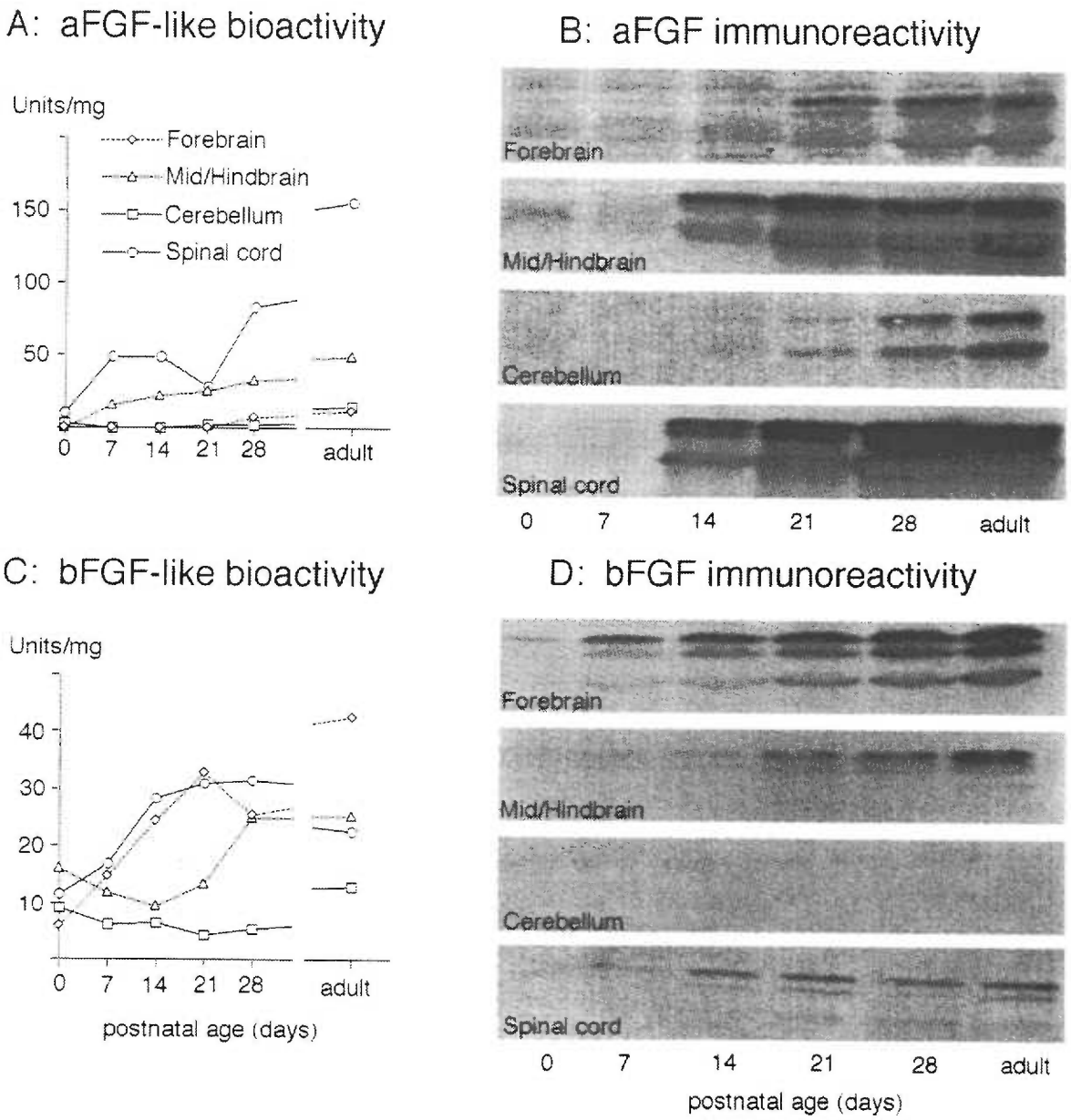


Figure 2.4: Determination of the levels of aFGF- and bFGF-like mitogenic bioactivity (A and C, respectively) and of the levels of aFGF and bFGF immunoreactivity (B and D, respectively) in four areas of the CNS between P0 and adulthood. The molecular weight of bands detected on Western blots probed with bFGF antibodies (22.5, 21.5 and 18 kD) or aFGF antibodies (18 and 16 kD) are known forms of these factors. Note that levels of bioactivity and immunoreactivity in general show good correlation, but that some early developmental time-points show detectable levels of bioactivity, while no immunoreactivity is observed. This suggests that mitogenic growth factors different from aFGF and bFGF may be present in significant levels at these earlier time points. Biological activity is expressed as ED50 units per mg of protein (see methods for definition of units). Each data point is the mean of triplicate determinations (associated standard errors were generally less than 15% of total).

Northern blot analysis of FGFR expression

It is of interest to correlate the timing of aFGF and bFGF expression with that of their receptors in CNS. Antibody probes useful for the specific immunohistochemical localization of individual members of the rat FGFR-family were not available to us, thus we chose to analyze the pattern of FGFR-1 and FGFR-2 mRNA expression by northern blot methods. The four known members of the FGFR family share substantial sequence homology, making it necessary to demonstrate that the probes used will selectively detect a given family member. FGFR-1 and FGFR-2 mRNA are known to be both expressed in brain, but to show a different pattern of expression across other tissues (Johnson and Williams, 1993; Partanen *et al.*, 1992). Figure 2.5 demonstrates that the FGFR-1 and FGFR-2 probes and methods used here result in the expected pattern of tissue specific expression of the two receptor mRNAs. Interestingly, both FGFR-1 and FGFR-2 mRNA were present in moderate to high levels in all areas of CNS at all time points studied, including at E 16 and E 18 (Figure 2.6). This suggests that these receptors are present significantly before their most abundant ligands (aFGF and bFGF) are detected in the same tissue.

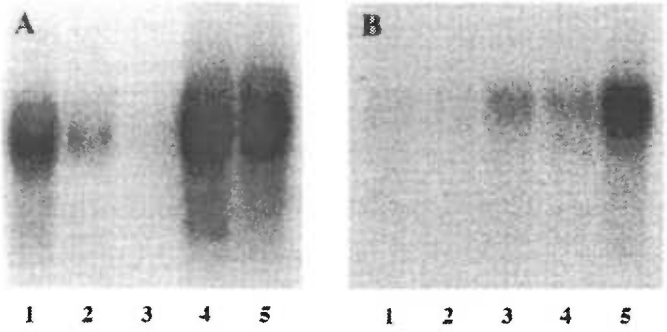


Figure 2.5: Tissue specific expression of FGFR-1 (A) and FGFR-2 (B) mRNA. Autoradiographic detection of FGFR mRNAs (at about 4.4 kB molecular weight) on Northern blots is shown. Lanes were loaded with equal amounts (15 μg) of total RNA isolated from adult rat heart (1), spleen (2), liver (3), kidney (4) and brain (5). Note that both FGFR-1 and FGFR-2 are expressed at high levels in brain, but that they show a differential pattern of expression in the other tissues.

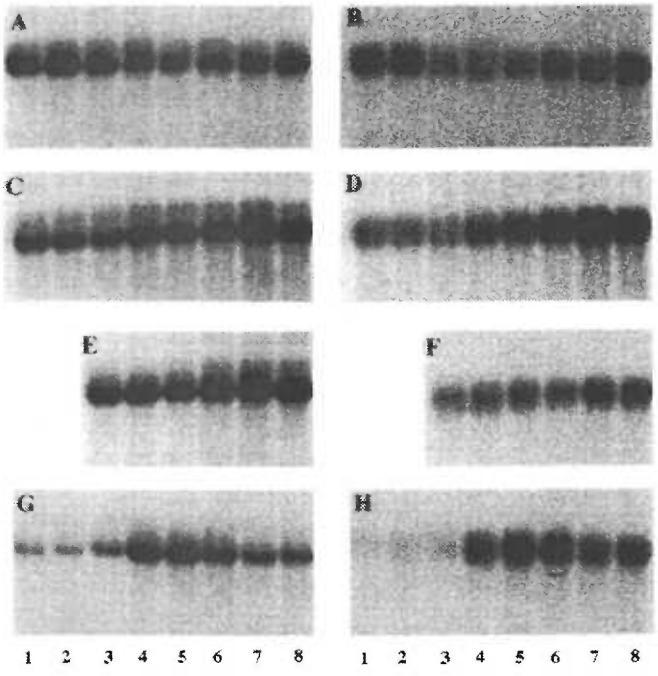


Figure 2.6: Developmental expression of FGFR-1 (A,C,E,G) and FGFR-2 (B,D,F,H) in four areas of the CNS by autoradiographic detection on Northern blots (A and B: Forebrain; C and D: Mid/Hindbrain; E and F: Cerebellum; G and H: Spinal cord). Lanes were loaded with equal amounts (15 μg) of total RNA isolated from E 16 (1), E 18 (2), P 0 (3), P 7 (4), P 14 (5), P 21 (6), P 28 (7) and adult (8) CNS areas. FGFR mRNA expression significantly precedes the expression of aFGF and bFGF in all areas. The only change in level observed is an increase in both FGFR-1 and FGFR-2 mRNA in spinal cord between E 16 and P 7.

Discussion

This study describes at a cellular level the time course of aFGF and bFGF expression in the developing rat CNS. This type of information is of importance for correlating FGF expression patterns with specific developmental events in which FGFs may play a physiological role. In fact, the widespread mitogenic and neurotrophic effects of FGFs *in-vitro* have been the basis for hypotheses that FGFs may regulate neurogenesis or gliogenesis, developmental programmed cell death, neuronal maintenance, regeneration and recovery from injury (see (Eckenstein, 1994) for review). Due to limits of sensitivity, the present study only analyzed tissues from animals of the age E16 and older. Within this time frame, the main observation was that both aFGF and bFGF are detectable in specific sets of neurons or astrocytes, at relatively late developmental (postnatal) times only. This suggests that the role of these factors in CNS likely is restricted to regulating maturation and maintenance, including injury responses. The data of the present study cannot rule out, however, that transient expression of bFGF may affect earlier events in the rat CNS development, as suggested by the detection in early rat CNS of bFGF mRNA using polymerase chain reaction methods (Nurcombe *et al.*, 1993).

Interestingly, FGFR mRNAs were found to be expressed in all areas of the developing CNS (see Figure 2.6 and (Heuer *et al.*, 1990; Peters *et al.*, 1993; Wanaka *et al.*, 1991)), significantly before aFGF and bFGF become detectable. This suggests the possibility that members of the FGF-family different from aFGF and bFGF could be expressed and act at times between E16 and P0, when the levels of FGFR message are high and the levels of aFGF and bFGF are not detectable.

There is currently no general agreement about the developmental patterns of expression of FGFs in CNS and the observations reported here are consistent with

only a subset of earlier work. For example, northern blot studies have revealed the apparent presence in embryonic rat CNS of high levels of several mRNAs of different sizes that hybridized with a bFGF probe (Ernfors *et al.*, 1990; Weise *et al.*, 1993). On the other hand, a more specific nuclease protection assay indicated that bFGF mRNA was quite low at prenatal time points and increased to adult levels over the first three weeks after birth (Riva and Mocchetti, 1991). A similar time course of postnatal increase of expression of aFGF and bFGF proteins has been reported in a study using western blot and mitogenic assay methods (Caday *et al.*, 1990). However, other studies, using immunohistochemical or hybridization techniques, have reported the presence of significant levels of bFGF immunoreactivity or in the embryonic rat CNS (Gonzalez *et al.*, 1990; Weise *et al.*, 1993). In addition, *in-situ* hybridization studies have indicated widespread (Wilcox and Unnerstall, 1991) or more defined (Elde *et al.*, 1991; Schnurch and Risau, 1991) expression of aFGF mRNA. Our own past studies (Eckenstein *et al.*, 1991; Stock *et al.*, 1992; Woodward *et al.*, 1992), using a combination of immunohistochemical, western blot and bioassay methods, agree with the set of studies indicating a defined and restricted cellular distribution of aFGF and bFGF.

These conflicting observations may be due, in part, to the fact that the FGF family consists of at least nine members which exhibit significant sequence homologies (around 50% amino acid sequence homology in core regions). Thus, if no specific precautions are taken, probes to one member of the FGF family may crossreact with several family members (see (Stock *et al.*, 1992), for review), leading to possible concommitant detection of several members of the family. The present study employs several well characterized antibodies to aFGF and bFGF, which show negligible cross reactivity between these two most homologous members of the FGF family (Eckenstein *et al.*, 1991; Stock *et al.*, 1992; Woodward *et al.*, 1992). Definite proof for the lack of crossreactivity among all family

members, however, is very difficult to obtain. We have therefore performed western blot and bioassay experiments to independently confirm the immunohistochemical observations. Overall, results obtained using the three different techniques showed good correlation. However, the bioassay also detected a transient increase in heparin dependent mitogenic activity in spinal cord between postnatal days 7 and 14, and moderate amounts of heparin independent activity in newborn tissues, both of which were not observed using the other two methods. This bioassay is not specific for FGFs however (see (Eckenstein *et al.*, 1991; Stock *et al.*, 1992; Woodward *et al.*, 1992) for detailed discussion) suggesting that mitogens different from aFGF or bFGF may be relatively more abundant in newborn CNS, and especially in early postnatal spinal cord.

The overall levels in the adult nervous system of bFGF (about 10 ng per mg protein in cerebral cortex) or aFGF (about about 37.5 ng per mg protein in spinal cord) are markedly higher than that of nerve growth factor (NGF, about 0.04 ng per mg of protein in cerebral cortex (Korsching *et al.*, 1985)), the best characterized neurotrophic factor thus far. This is all the more impressive as aFGF and bFGF, on a molar basis, appear to be about ten fold more potent than NGF. It is important in this regard to note that high resolution immunohistochemical localization of both aFGF and bFGF has found the factors to be localized in intracellular compartments (Elde *et al.*, 1991; Woodward *et al.*, 1992). This intracellular localization is consistent with the known lack in both aFGF and bFGF of a hydrophobic signal peptide, which is thought to be necessary for sorting of proteins into the secretory pathway (Walter and Lingappa, 1986). Together, these observations demonstrate that the bulk of aFGF and bFGF resides in intracellular compartments, but they cannot rule out that a minor proportion (below the detection limit of methods used) of the factors reaches the extracellular space. Our own preliminary observations, using cultured cerebral astrocytes, show that roughly 1% of the intracellular amount of bFGF is found in the medium conditioned by these cells. This low level may be

due to cell lysis as a similar percentage of the cytoplasmic marker enzyme lactate dehydrogenase is also present in the medium (A. Ho and F. Eckenstein, unpublished).

Together, the observations that aFGF and bFGF are detectable only relatively late in CNS development and that the bulk of the factors reside in intracellular compartments lead us to hypothesize that a major function of these factors is to initiate responses to cellular injury. This hypothesis may be best illustrated using primary motor neurons for an example, as these cells have been intensely studied in the past. This provides an excellent opportunity to correlate changes in the expression of aFGF with specific stages in motor neuron development. No aFGF is detectable in motor neurons during the period of cell death, which occurs mainly prior to E 17 (Harris and McCaig, 1984). In addition, the application *in-vivo* of exogenous bFGF during this period appears to fail to rescue the dying motor neurons (Oppenheim *et al.*, 1992). The period of programmed cell death is followed by a perinatal period when the developing motor neurons will die in response to a nerve crush. However, older, mature motor neurons will survive the nerve crush and will regenerate (LaVelle and LaVelle, 1958). The appearance of aFGF in the motor neurons appears to correlate with this transition from being highly vulnerable to injury to becoming able to survive the insult. In addition, FGFs are known to support the survival of motor neurons *in-vitro* (Hughes *et al.*, 1993; Sendtner *et al.*, 1991). Together, this suggests that aFGF in mature motor neurons may confer on these cells an increased resistance to injury and may initiate regeneration. In this model, the extraordinarily high levels of intracellular aFGF in mature motor neurons will become available in the extracellular space after an injury to the cells compromises their plasma membrane. This extracellular aFGF then is likely to stimulate both gliosis and neuronal survival in affected areas.

An analogous hypothetical model for the role of bFGF released from damaged

astrocytes can also be formulated. The only difference in function between the aFGF and bFGF in these models thus is that bFGF will specifically report damage to astrocytes, while aFGF will report damage to select groups of neurons. To us, the most attractive features of the injury model are that first, it is consistent with the high levels of aFGF and bFGF observed in mature brain, as the model predicts that in uninjured brain the factors are not available to the cell surface receptors; and second, that it is consistent with the varied neurotrophic and mitogenic activities of these FGFs, as the model predicts that the factors serve as initiators of an injury response by activating a variety of cell types. The model does not rule out, however, that a minor proportion (probably less than 1% of total cellular aFGF or bFGF) may become available in the extracellular space from non-injured cells, serving possible functions in the maturation and maintenance of the postnatal CNS.

Clearly, significant additional information is needed to test the model elaborated above. Most important will be to determine whether indeed high levels of aFGF and bFGF become available in the extracellular space only after plasma membrane compromise, or whether additional, more specific release mechanisms exist for these factors. It is also currently unclear whether the specific cellular expression patterns of FGFRs and heparan sulphate proteoglycans indicate that aFGF and bFGF may exhibit more of a cellular specificity of action than what is predicted by the injury hypothesis. This question may be addressed by correlating dose-response curves to either aFGF or bFGF with a detailed information on FGFR and HSPG expression in purified CNS cell types.

Chapter 3 : Motor Neuronal Survival Following Injury: Relationship to Fibroblast Growth Factor Levels

Given the knowledge concerning FGF developmental expression contributed in Chapter 2, the historical observation that neuronal survival following a crush injury increased proportionally with age became of great interest. Is it possible that this survival is somehow connected to FGF levels? In particular, is there an association between post injury survival and aFGF levels within motor neurons? In order to define better the potential association, the injury system required further examination.

The bulk of the investigations presented in this chapter has been recently accepted for publication in (Kuzis *et al.*, 1999).

"Time course and age dependence of motor neuron death following facial nerve crush injury: Role of fibroblast growth factor."

Abstract:

Peripheral nerve crush injury (PNCI) has been used for many years in adult animals to study central and peripheral changes related to regeneration across the injury site. While these adult animals experience full recovery with no neuronal cell loss following PNCI, it has been noted that the injury in perinatal animals is followed by retrograde neuronal cell death. The present study determines, in mice of different postnatal ages, the degree to which motor neurons are vulnerable to PNCI induced cell death, and examines the rate of neuronal loss. Animals of 4 days of age and younger were found to be significantly more vulnerable to motor neuron cell death following PNCI. There also was a proportional relationship between age at injury and final motor neuronal survival and an inverse relationship between age at injury and rate of neuronal cell death following injury. In addition a proportional relationship was observed between the expression level of acidic fibroblast growth factor within motor neurons and resistance to PNCI induced neuronal death. It was also found that PNCI in an environment which contained higher levels of FGFs (either in mice treated with aFGF or in transgenic mice that overexpress basic FGF) significantly decreases neuronal cell death following early postnatal injury.

Introduction

For many years peripheral nerve crush injury (PNCI) has been used in adult animals to study central and peripheral changes related to regeneration across the injury site. The complete survival and regeneration of motor neurons seen in injured adult animals is in stark contrast to the significant retrograde neuronal cell death seen in animals injured at less than one week of age (Bueker and Meyers, 1951; Gudden, 1870; La Velle and La Velle, 1959; Lowrie *et al.*, 1987; Romanes, 1946). These divergent results provide an opportunity to explore the factors which influence the survival observed in the adult animals. The present study provides a detailed description of the time course and rate of neuronal loss following PNCI in mice of different postnatal ages.

Age related survival appears correlated with the availability of neurotrophic factors. The complex cellular environment of the motor neuron offers many potential sources and molecules for neurotrophic support during the period when a crushed axon is attempting to regenerate. For motor neurons, such as those of the facial nucleus, important factors are derived from the Schwann cells at the injury site (ciliary neurotrophic factor, CNTF (Stockli *et al.*, 1991), neurotrophins (Heumann *et al.*, 1987a; Meyer *et al.*, 1992) and glial cell-line derived neurotrophic factor, GDNF (Henderson *et al.*, 1994; Naveilhan *et al.*, 1997)).

Motor neurons themselves contain extremely high levels of acidic FGF (Elde *et al.*, 1991; Stock *et al.*, 1992). Acidic FGF is a member of the fibroblast growth factor family which consists of over fifteen structurally related proteins (Eckenstein, 1994). FGFs are characterized by their selective binding to the sulfated glycosaminoglycan heparin and by their overlapping mitogenic or neurotrophic actions on a variety of mesodermal, ectodermal and neuroectodermal cell types (Burgess and Maciag, 1989; Eckenstein, 1994). The biological effects of the FGFs

are mediated by a family of at least four transmembrane receptors which contain intracellular tyrosine kinase domains. All four FGF-receptor genes are expressed in a cell type specific pattern in the CNS, which includes expression of FGFR-1 by motor neurons (Heuer *et al.*, 1990; Wanaka *et al.*, 1991). Acidic and basic FGF were share about 55% amino acid sequence homology, and both can act as mitogens for nonneuronal cells, including astrocytes (Pettmann *et al.*, 1985), Schwann cells (Davis and Stroobant, 1990), and oligodendrocytes (Eccleston and Silberberg, 1985), and as neurotrophic factors for a large variety of peripheral (Eckenstein *et al.*, 1990; Schubert *et al.*, 1987; Unsicker *et al.*, 1987) and central (Engele and Bohn, 1991; Morrison *et al.*, 1986; Walicke *et al.*, 1986) neuronal populations. Both aFGF and bFGF appear to be able to bind and stimulate the FGF receptor variants most common in CNS (see (Eckenstein, 1994; Jaye *et al.*, 1992; Johnson and Williams, 1993) for reviews). In contrast to several other members of the FGF family, both aFGF and bFGF lack an amino terminal hydrophobic signal sequence, which appears required for the sorting of proteins into the secretory pathway (Walter and Lingappa, 1986).

Acidic FGF is present in large quantities in the cytoplasmic compartment of select neuronal populations, including motor neurons, where it may represent nearly 0.1% of soluble protein (Elde *et al.*, 1991; Schnurch and Risau, 1991; Stock *et al.*, 1992). These extremely high tissue concentrations of aFGF and bFGF contrast with the high affinity of FGF receptors, which mediate the full range of FGF biological effects at FGF concentrations of a few ng/ml. We propose that the high intracellular stores of aFGF present in motor neurons are not normally available to the high affinity surface receptors, but will become available after damage to the motor neuron, possibly through a simple breach of the plasma membrane. In order to determine whether endogenous aFGF expression in motor neurons is correlated

with the degree of motor neuron vulnerability to injury, we determine here the age-dependence of the extent to which mice are vulnerable to PNCI - induced facial motor neuron loss, and examine the developmental expression of aFGF within the motor neurons of the mouse facial nucleus. The effects of treating PNCI lesioned mice with recombinant aFGF and of transgenic overexpression of bFGF are also reported.

Experimental Procedures

Facial Nerve Anatomy

The facial motor nucleus innervates the musculature of the face. The motor neuron cell bodies reside in the ventral lateral portion of the hindbrain just rostral of the pons. In an adult mouse the nucleus extends over a distance of approximately 800 μm and has well defined boundaries. The axons of these motor neurons leave the CNS at the caudal margin of the pons via cranial nerve VII. The facial nerve exits the cranial cavity via the Stylomastoid foramen. The main trunk courses under the external ear and out onto the face musculature until it furcates into its primary divisions as it passes under the external jugular vein (Figure 3.1A). The main trunk of the nerve is easily accessible by a simple surgical procedure (Figure 3.1B). An injury at this point damages approximately 90% of the motor neuronal fibers which originate within the borders of the facial motor nucleus. The remaining 10% whose cell bodies map to the ventral medial subnucleus within the facial nucleus, are accounted for by the retroauricular branch (Komiyama *et al.*, 1984) which leaves the main trunk at the Stylomastoid foramen prior to the site of injury (see Figure 3.1A).

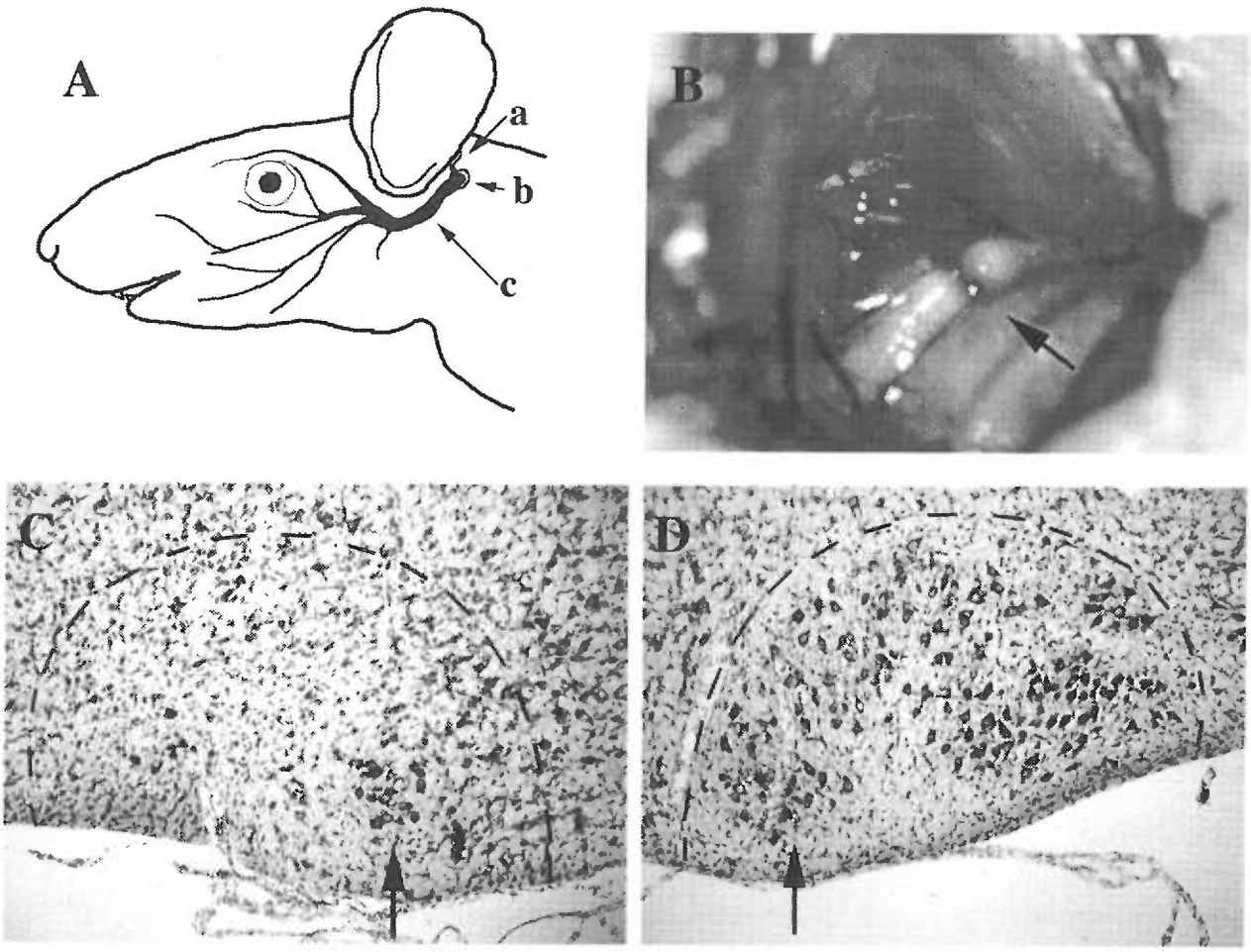


Figure 3.1 Panel A: Line drawing of the anatomical positioning of the mouse facial nerve. Note the path of the nerve trunk, the retroauricular branch and the site of lesion (a: Retroauricular branch of the facial nerve; b: Stylomastoid foramen; c: Site of facial nerve crush).

Panel B: Photograph of a Peripheral Nerve Crush Injury (PNCI) of the facial nerve in an 21 day old mouse. Note the gap between the nerve stumps, which is bridged by a barely visible thin epineural connection.

Panels C and D: Photomicrographs of the left and right facial nuclei, taken from the same coronal, toluidine blue stained section through the brainstem of an animal that suffered facial nerve crush at 2 days of age and was harvested 14 days after the operation. The broken line defines the approximate extent of the facial nucleus, and the arrows point to the the ventral medial facial subnucleus that supplies axons to the retroauricular branch of the facial nerve, which is not lesioned in this model. Note the significant decrease in the number of motor neurons in the facial nucleus ipsilateral to the lesion (Panel C), compared to the number of neurons present in the contralateral control nucleus (Panel D).

Facial Nerve Injury and Tissue Processing

BALB/c mice of selected ages (Days after birth; 0, 2, 4, 7, 14, 21, 28, or 45) were anesthetized and the facial nerve on the right side exposed through a 2-5 mm incision dorsal and caudal to the external ear. All operations were performed under a stereo dissecting microscope (Leitz 0.7-3x zoom). The nerve trunk was released from the surrounding connective tissue by gentle dissection and then injured. The nerve trunk was crushed distal to the auricular branch of the nerve (Figure 3.1) with a slight shearing motion, between the tips of jeweler's forceps (Dumont model PP/45 selected for their size and resistance to deformation) for a count of five seconds, resulting in an approximately 3 mm gap between the nerve stumps. The crush site distal to the auricular branch can readily be examined at all postnatal ages for clear separation of the nerve stumps and retention of the overlying epineurium. If the site was not visible to inspection, for example due to bleeding, or the stumps were freely separated with no epineural connection, the animal was excluded from the study. The skin incision was closed with cyanoacrylate adhesive. The animals were monitored in a temperature controlled environment until they regained consciousness, then returned to their mother. The surgical protocols used followed approved animal care guidelines.

After the selected survival interval (1, 2, 3, 5, 7, 14, or 30 days after injury) the animals were sacrificed by an overdose of anesthetic, followed by perfusion through the heart with 0.5-5 ml (depending on age) of Phosphate Buffered Saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.2) then by 5-20 ml (depending on age) of phosphate buffered formalin (5% formalin, 100 mM sodium phosphate, pH 7.2). The brain was rapidly dissected and post fixed in the same fixative at room temperature for 30 mins. The tissue was soaked in PBS overnight at 4˚C, then cryoprotected by sinking in 15%, then 30% sucrose in PBS at 4˚C.

Serial 25 µm cryostat sections were cut from the rostral pyramids to the inferior colliculus and every fourth section collected onto the same slide. This resulted in four sets of slides, each set containing sections representing the complete facial nucleus. Each slide set is separated from the adjacent sets by 25 µm and each section on a single slide is separated by 75µm from the adjacent sections on that slide. The slides were dried flat overnight at room temperature and then stored in desiccated slide boxes at -70°C until staining.

Cellular Identification and Counting

A single set of slides from each animal was stained by standard methods for Nissl substance with Toluidine Blue. The slides were examined to determine the location of the facial nucleus within the set. These sections were labeled, with the first and last section labeled being the section directly before or after the facial nucleus where no motor neurons were found. Motor neurons were identified as cellular profiles with a diameter larger than 10 µm (all other cells within the facial nucleus have smaller profiles). Motor neuron diameters are also smaller than the 75 µm separation between sections analyzed, thus double counts of the same neuron are avoided. The motor neuron profiles within the boundaries of the facial nucleus were counted at 160x using an etched grid reticle, (Grid squares are 60 µm on a side at 160x). As the average neuronal profile area was found to change by less than 5% following PNCI no correction for cell shrinkage was applied (Kuzis unpublished results). Total counts recorded for each side of the brain stem were adjusted to account for the neurons from the retroauricular branch of the facial nerve whose axons were not injured. Retroauricular neurons were taken as ten percent of the total count from the uninjured side. For each animal, the percent of injured motor neurons surviving the crush (survival percentage) was calculated by

dividing the adjusted motor neuronal profiles from the injured side by the adjusted motor neuronal profiles from the uninjured side. From these individual survival percentages, mean survivals for each data point (by age at injury and post injury interval) were calculated. Statistical significance was assessed using t-test and ANOVA as calculated using the "Statistica Mac" software tool. Significance was tested using a p value of 0.05 as the upper limit. All counts were performed blinded to the animals age at injury and survival time.

Acidic Fibroblast Growth Factor Immunohistochemistry

Tissue was collected from BALB/c mice raised in our breeding colony of the selected ages (Days after birth; 0, 3, 5, 7, 11, 14, 21 and 30) as described above. Coronal 50 µm thick frozen sections were cut from the rostral pyramids to the inferior colliculus and processed for immunohistochemical staining of aFGF.

For aFGF immunohistochemistry, selected sections were incubated for 30 min in 30.0% (v/v) ethanol and 5.0% (v/v) hydrogen peroxide in 65 mM Tris, pH 7.3, to inactivate endogenous peroxidases, followed by two five minute washes in Tris Buffered Saline (TBS: 100 mM Tris, 150 mM NaCl, pH 8.2). The sections were then incubated for 90 minutes in blocking solution (100 mM sodium phosphate, 10.0% (v/v) horse serum, 0.5% (v/v) Triton X-100, 0.2% (w/v) sodium azide), followed by overnight incubation (minimum of 12 hrs) in rabbit antiserum to aFGF or rabbit pre-immune serum (both diluted 1:1000 in blocking solution). Sections were washed three times for ten minutes each in TBS, incubated for one hour in goat anti-rabbit antiserum (Sternberger-Meyer) diluted 1:250 in blocking solution containing 5% (v/v) mouse serum), washed as described above, incubated for one hour in rabbit peroxidase-anti-peroxidase (Sternberger-Meyer) diluted 1:100 in blocking solution containing 5% (v/v) mouse serum and no sodium azide, washed and reacted with

0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% (v/v) hydrogen peroxide in PBS. The reaction was stopped by washing twice for five minutes each with PBS. The stained sections were mounted on gelatin coated glass slides and coverslipped according to standard procedures. The rabbit polyclonal anti-aFGF serum used here for immunohistochemistry has been characterized previously (Stock *et al.*, 1992). For anatomical identification of stained cells, the atlas by Sidman was used (Sidman *et al.*, 1971).

Acidic Fibroblast Growth Factor Treatments

Human recombinant acidic fibroblast growth factor was expressed and purified in our laboratory (Stock *et al.*, 1992). The biologically active purified factor was diluted to a concentration of 25 ng/µl. Treated animals were provided with a 2 mm cube of gel foam which was soaked in 5 µl of the aFGF solution. The soaked gel foam was placed upon the crush site immediately following verification of the lesion. This resulted in a maximal application of 125 ng per lesion site. Control animals received gelfoam soaked with either PBS alone or PBS containing 25 ng/µl of bovine serum albumin. The neuronal survival observed following these two control treatments were found to be equivalent with those observed in untreated animals.

Basic Fibroblast Growth Factor Transgenic Animals

Mice which carry the human bFGF gene under the control of the phosphoglycerokinase (pgk) promoter have been developed (Coffin *et al.*, 1995). These animals were created in the FVB/N strain, due to its suitability to transgenic manipulation (Taketo *et al.*, 1991).

The transgenic line used to generate the offspring for this study contains a

stably integrated construct which, driven by the pgk promoter, produces all four forms of human bFGF. Basic FGF is normally expressed in four molecular weight forms, arising from alternative initiation sites for translation (Florkiewicz and Sommer, 1989). Use of the pgk promoter, leads to constitutive expression in all cells throughout development. Western blot analysis of tissue extracts indicate that the central nervous system of the transgenic mice contains approximately 40 fold more bFGF immunoreactivity than their nontransgenic littermates. The normal appearance of the central nervous system in these bFGF transgenic animals, lends evidence to the hypothesis of limited bFGF release (Hicks and Eckenstein, 1994). Injuries were performed as described above in transgenic animals and their non-transgenic littermates. Transgene status was determined as described (Coffin *et al.*, 1995).

Figure 3.2

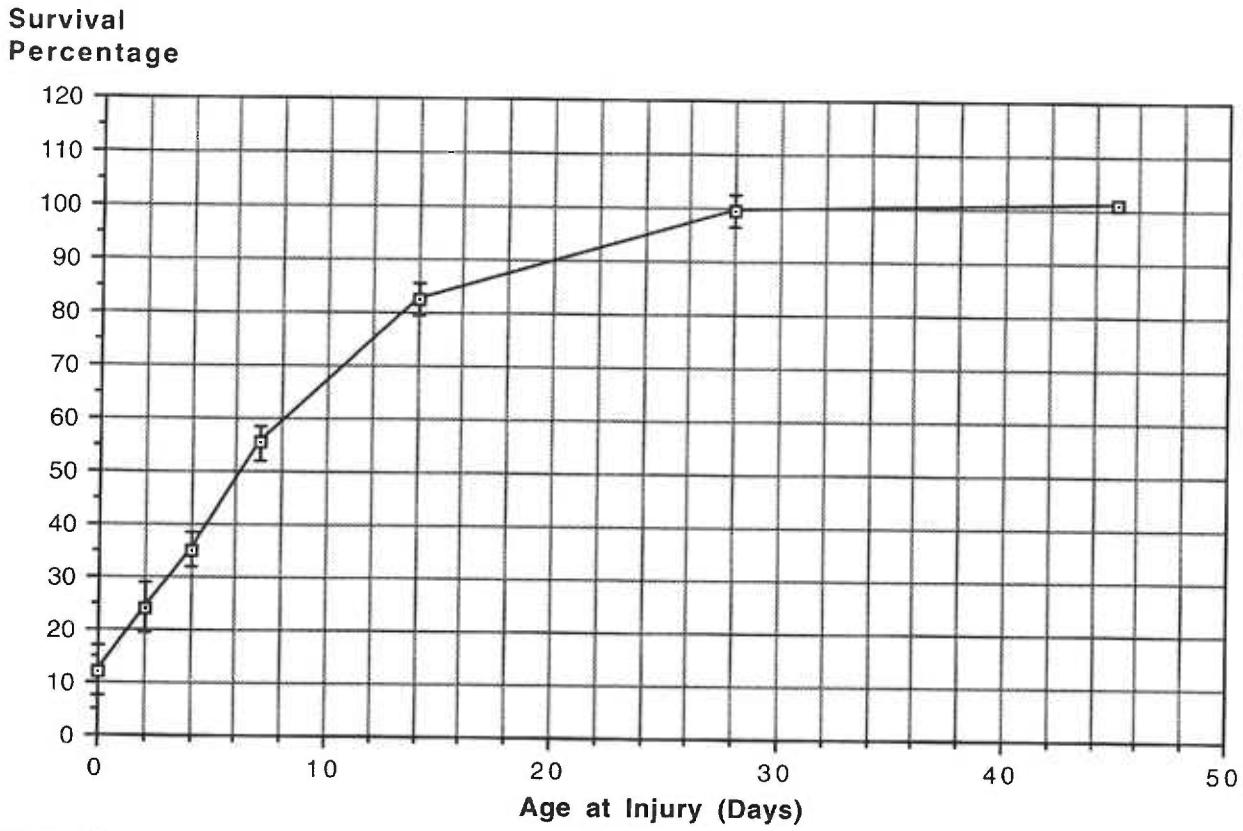


Figure 3.2
End point analysis of the extent of neuronal survival after PNCI performed at different postnatal ages. Neuronal profiles were counted after a 30 day survival interval. Survival percentages are calculated by dividing the number of motor neurons in the facial nucleus on the injured side by the number on the contralateral, non-injured side (see methods section for details). Age indicated are days after birth (day of birth = 0).

Table 3.1: Summary of the extent of facial motor neuron survival at different post-PNCI survival times in mice of different ages.

Age (days)	Postop (days)	n	Mean % Survival	Standard Error	Mean % Loss	% Maximal Loss
0	1	3	72.2	4.1	27.8	31.9
0	2	3	36.2	2.4	63.8	73.3
0	3	4	23.4	2.9	76.6	88.0
0	5	2	09.3	3.0	90.7	104.2
0	7	3	06.4	1.9	93.6	107.5
0	14	4	10.3	1.7	89.7	103.0
0	30	2	12.2	4.6	87.8	100.0
2	1	2	67.2	6.6	32.8	43.3
2	2	3	56.9	1.4	43.1	56.8
2	3	4	25.9	4.1	74.1	97.7
2	5	3	12.2	3.8	87.8	115.7
2	7	3	14.0	5.0	86.0	113.3
2	14	3	24.3	2.6	75.7	99.7
2	30	3	24.1	4.8	75.9	100.0
4	1	2	76.5	1.3	23.5	36.2
4	2	4	63.3	1.3	36.7	56.5
4	3	4	58.3	2.3	41.7	64.2
4	5	4	44.7	1.0	55.3	85.1
4	7	4	40.3	1.3	59.7	91.8
4	14	4	43.6	1.6	56.4	86.8
4	30	2	35.0	3.3	65.0	100.0
7	1	3	96.0	1.6	04.0	8.9
7	2	3	73.7	3.0	26.3	58.8
7	3	5	72.4	5.0	27.6	61.8
7	5	4	61.8	1.6	38.2	85.5
7	7	3	54.2	2.1	45.8	102.5
7	14	3	59.1	2.4	40.9	91.5
7	30	2	55.3	3.2	44.7	100.0
14	1	3	96.4	1.8	3.6	20.6
14	2	2	96.4	1.9	3.6	20.6
14	3	3	95.9	1.4	4.1	23.4
14	5	3	93.6	3.2	6.4	36.6
14	7	3	88.9	0.8	11.1	63.5
14	14	3	80.1	2.2	19.9	113.8
14	30	3	82.5	3.2	17.5	100.0

Table 3.1: The time course of the extent of motor neuron survival after PNCI in mice of different postnatal ages is shown. Mean % survival is calculated by dividing the number of identifiable facial motor neurons on the injured side by the number of neurons on the contralateral uninjured side, followed by multiplying with 100. Mean % loss is calculated by subtracting mean % survival from 100. Maximal loss is defined for each age group to equal the mean loss of motor neurons 30 days after injury. Percent maximal neuronal loss at post-PNCI survival times shorter than 30 days is defined as the extent of neuronal death observed at that time, divided by the the maximal death characteristic for the appropriate age group, and multiplied by 100. Note that the total extent of cell loss after 30 days of survival is about 5 times lower in 14 day old animals than in newborn, and that this reduction in vulnerability occurs in a graded fashion during this period of postnatal development.

Time Course of Motor Neuron Death at Different Ages.

Animals 0, 2, 4, 7 and 14 days of age were used and the extent of neuronal survival was evaluated at 1, 2, 3, 5, 7, 14, 30 days following PNCI. As expected from the end point analysis presented above, there were large differences in neuronal loss between different ages at injury. Consistent with the determination that ultimate motor neuronal survival is proportional to age at injury, it can be seen that younger animals have lost a significantly higher percentage of their facial motor neurons than older animals at every survival interval analyzed (Table 3.1). In addition, motor neuron loss appeared to proceed more quickly in younger animals than in older ones. For example, when the maximum of motor neurons loss is defined as the loss at 30 days after the injury for any given age group, animals injured on the day of birth will reach 85% of maximal loss within 3 days after the injury. In contrast, 14 day old animals reach only about 25% of maximal loss within the first three days, while the remaining 75% of motor neurons destined to die will more slowly disappear over the next 10 days (Table 3.2 & Figure 3.3).

Table 3.2: Percent Maximal Neuronal loss observed at 3 days post-PNCI, and Postoperative days required to reach 85% of Maximal Loss. Presented by age at facial nerve crush.

Postnatal Age at Injury (Days)	% Maximal Loss at 3 Days Postoperative	Days Required to Reach 85% of Maximal Loss
0	88.0	3
2	97.7	3
4	64.2	5
7	61.8	5
14	23.4	14

Table 3.2: Percent maximal facial motor neuron death in mice of different postnatal ages in the first three days versus the next 27 days post PNCI. Note that the vast majority of maximal cell death in 0 and 2 day old animals occurs within the first three days after PNCI, while most of cell death in 14 day old animals occurs between 4 and 30 days post-PNCI in 14 day old animals. Animals which were injured at a postnatal age of 4 and 7 days show significant cell death both before and after three days post-PNCI. Numbers given in Table 3.1 were used for to calculate the data shown.

Figure 3.3A

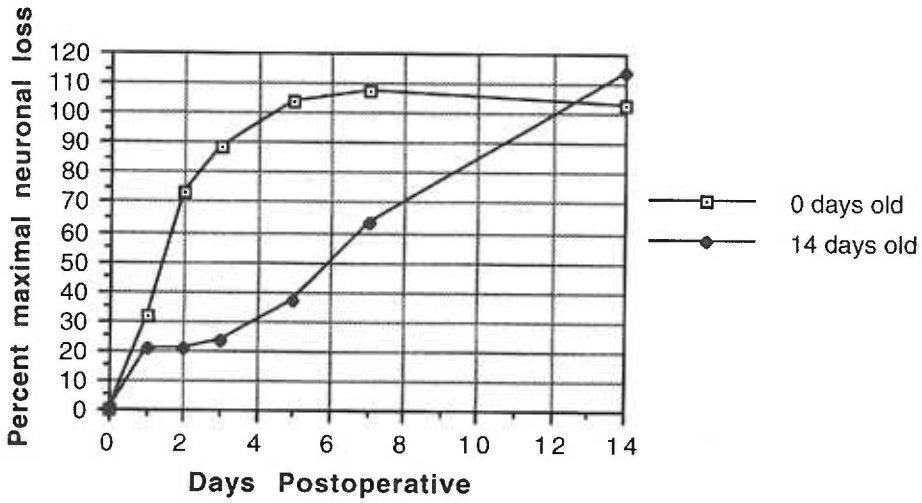


Figure 3.3B

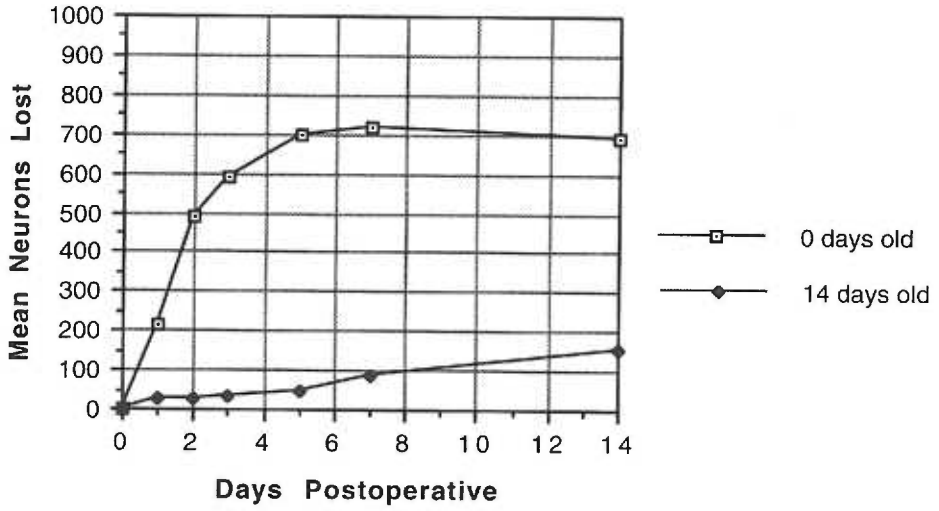


Figure 3.3
Analysis of the time course of motor neuron loss following PNCI in 0 and 14 day old mice.
Panel A: Percent of maximal neuronal loss (maximal neuronal loss= loss observed at 30 postoperative days). Note that motor neuron death in 14 day old animals appears to proceed more slowly than in newborns.
Panel B: Mean neuronal loss (mean uninjured neuronal count = 770 motorneurons per facial nucleus). Note that motor neuron death in 14 day old animals appears to proceed more slowly than in newborns.

Rate of Motor Neuron Death

Tables 3.1 and 3.2 generate the observation that both extent of MN loss and rate of disappearance are related to age at PNCI. In order to better define the relationship between motor neuronal death following PNCI and age at injury linear regression analysis was performed. From the data for animals injured at ages 0,4, 7 and 14 days, the point of neuronal loss 50 % (NL50) was determined. This is defined as the postoperative data point which demonstrated greater than or equal to 50% of the maximal neuronal loss seen for that particular age at injury. Each regression analysis considered all the data points from postoperative day 0 through the first postoperative data point greater than NL50 for that age. The regression analysis was used to generated a best fit linear equation where the slope of this equation represents the initial rate of neuronal loss. This data is presented in Table 3.3. When plotted, as in Figure 3.4, these slopes can be fit with a straight line indicating that the initial rate of cell loss seen after PNCI is inversely proportional to the age at PNCI.

Table 3.3

Age	NL50	Limit	n	Slope	SEM of Slope	R^2
0	2	3	13	27.15	0.99	0.98
4	2	3	13	15.56	0.82	0.968
7	2	3	14	9.73	1.03	0.87
14	7	14	20	1.45	0.97	0.92

Table 3.3
Results from regression analysis of neuronal survival following PNCI at various ages. Standard regression analysis was performed for each age at injury. Data points from post operative day 0 through the first sample after NL50 were included in the analysis.
Age = age at PNCI in days. NL50 = first postoperative day at which neuronal loss was greater than or equal to 50% of total loss for that age at injury. Limit = Next postoperative day sampled past NL50. SEM = Standard error of measurement.

Figure 3.4

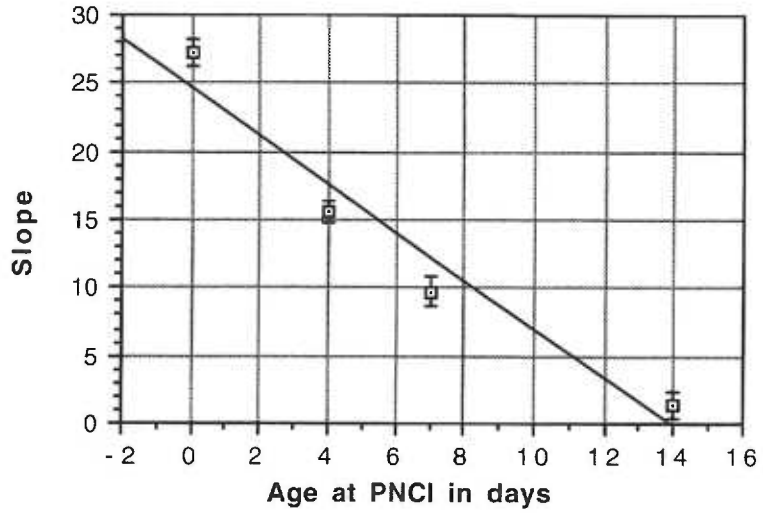


Figure 3.4
Plot of the results of regression analysis of PNCI neuronal survival as presented in table 3.3. Slope represents initial rate of neuronal loss.
Note the linear equation fitted to the slopes. $y=24.58-1.77x$; $R^2=0.945$

Correlation of Motor Neuron Survival with aFGF Expression.

In previous work we had determined the cellular localization and developmental time course of FGF expression in rats (Kuzis *et al.*, 1995). In order to confirm the temporal expression pattern in mice we performed immunohistochemical staining of brain stem sections containing the motor nucleus of cranial nerve VII from mice of different ages (days after birth; 0, 3, 5, 7, 11, 14, 21, 30). Most facial motor neurons begin to show very faint aFGF immunoreactivity on the day of birth. The staining at three days of age is of weak intensity, and increases only slightly until seven days of age. Beginning at day eleven the immunoreactivity gradually increases to reach adult levels by 21 days after birth (Figure 3.5). This gradual increase in aFGF immunoreactivity appears to proceed simultaneously in all facial motor neurons. These observations demonstrate that aFGF is present at high concentrations within motor neurons only at developmental ages when they are no longer highly vulnerable to death by PNCI.

Acidic FGF Treatment

The ability of exogenously supplied biologically active recombinant aFGF to increase the neuronal survival after PNCI was tested in animals of four days and seven days of age. At four days of age, (when endogenous aFGF levels are low), the observed motor neuron survival after PNCI is of sufficient extent to detect either a positive or negative influence of the treatments. When injured at this age the normal neuronal survival percentage seen after a post injury interval of seven days is 40.3% (Table 3.1). We found that in treated animals the motor neuron survival percentage rose to 56.5% which represents an increase of 16.2% as compared to the untreated animals. To see if the effects were altered with age we also treated animals injured at seven days of age, an age outside of the most vulnerable period for neuronal cell death stimulated by PNCI, when endogenous aFGF levels are moderate. In these animals we observed that neuronal survival in the treated animals rose to 67.7% as opposed to the untreated survival of 52.3%. This increase in motor neuron survival of 9.4% while smaller than the increase seen in the four day old animals was still statistically significant (p 0.05, Table 3.4).

PNCI in transgenic animals expressing a Basic Fibroblast Growth Factor Transgene.

FVB/N transgenic mice which overexpress bFGF were used to test the ability of endogenous, misexpressed FGF to reduce PNCI induced neuronal loss.

PNCIs were performed in four day old pups from transgenic mothers bred with nontransgenic FVB/N males, and it was observed that the transgenic offspring showed a small (9%), but statistically significant increase in motor neuron survival survival when compared to their non-transgenic littermates (Table 3.4). In addition, transgenic animals injured at seven days of age had survivals 7.8% higher than

their nontransgenic litter mates. These results indicate that endogenous FGF neurotrophic activity can support to some extent the survival of injured motor neurons. It is possible that the relatively small increase in survival observed is due to the fact that bFGF may not be able to fully replace aFGF *in-vivo*, or that the global overexpression of the bFGF transgene by the pgk-promoter is not entirely appropriate for obtaining maximum responses. Interestingly the nontransgenic FVB/N animals showed somewhat lower overall neuronal survival than the Balb/c mice used in the development of the PNCI model. A likely explanation for this observation is that individual strains of mice may vary in slightly in their reactions to PNCI.

Table 3.4
The effect of aFGF application and transgenic expression of bFGF on facial motor neuron survival after PNCI.

Age (days)	Calculated Condition	n	Mean %Survival	Difference in %Survival	P-Value
4	Untreated	5	40.45	16.03	0.0002
4	Treated	2	56.48		
7	Untreated	5	52.30	9.42	0.015
7	Treated	2	61.72		
4	Nontransgenic	9	30.77		
4	Transgenic	7	39.82	9.05	0.0038
7	Nontransgenic	2	43.82		
7	Transgenic	6	51.64	7.82	0.023

Table 3.4
The effect of exogenous application of aFGF and of transgenic over-expression of bFGF on the extent of facial motor neuron death induced by PNCI. Note that application of exogenous (25 ng) FGF-1 to the lesion site and global over-expression of bFGF lead to a similar extent of moderate increase in facial motor neuron survival after PNCI in 4 day old animals, while a smaller effect is observed in 7 day old animals.

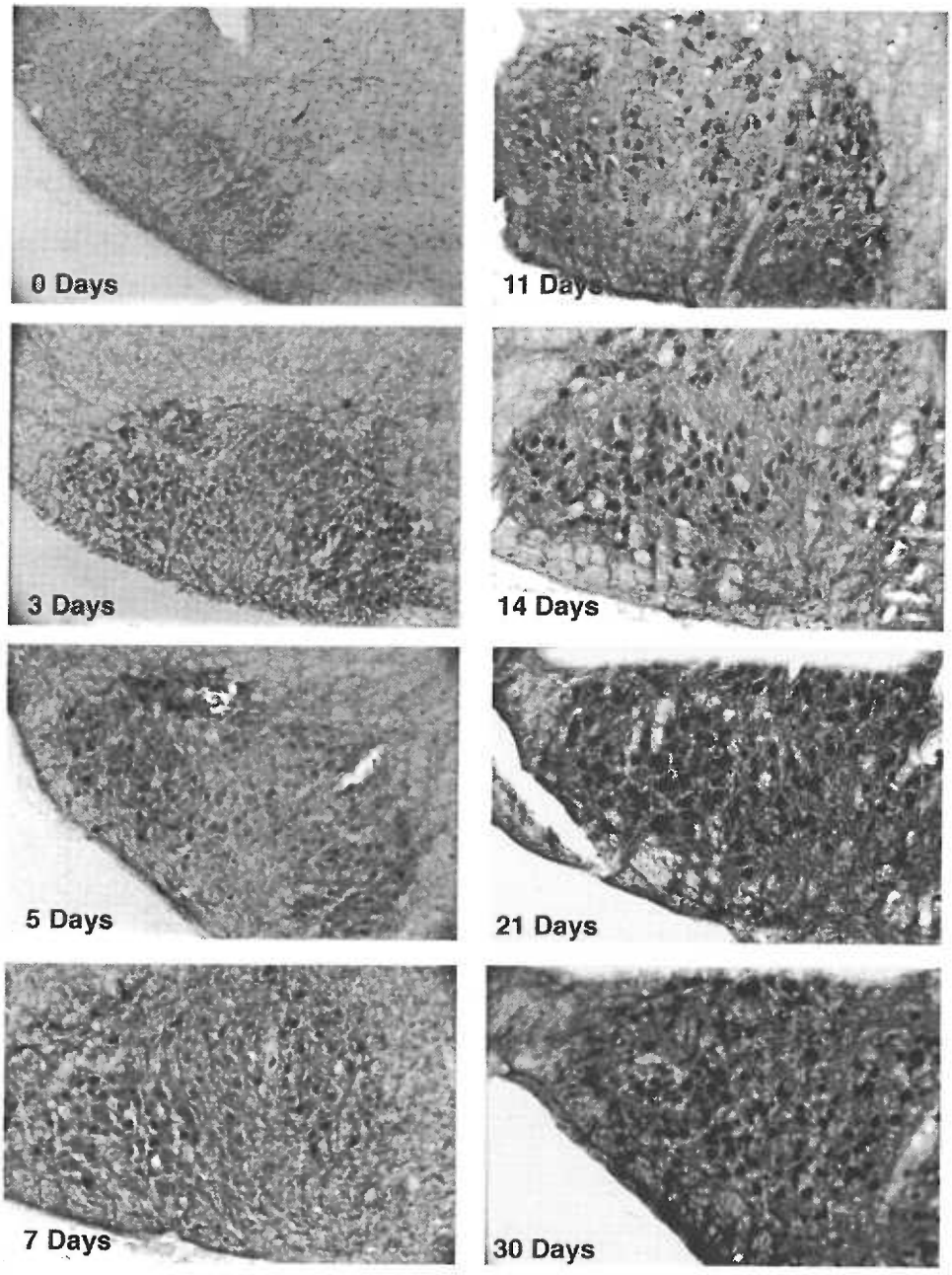


Figure 3.5
Timecourse of the intensity of aFGF immunoreactivity in facial motor neurons in animals of different postnatal ages. Section through the facial nucleus of 0, 3, 5, 7, 11, 14, 21, 30 day old mice were stained for aFGF. Note the very faint immunoreactivity visible on the day of birth. The staining intensity of motor neurons shows a gradual increase to adult levels by day 21. Bar = 20μm.

Discussion

The decision to investigate the developmental dynamics of motor neuron cell death following axotomy in a peripheral nerve crush injury (PNCI) model, instead of the somewhat more commonly used transection model, is based on the following two observations: First, while adult motor neurons survive an initial transection, a second slower phase of degeneration leads to the elimination of a significant number of neurons later on (Kou *et al.*, 1995). Thus, it is possible that in younger animals this second phase of cell death may overlap with earlier phases of injury induced cell death and confound results. This potential problem is of less concern in the PNCI model, where adult neurons (which successfully regenerate) demonstrate minimal cell death. Second, partial axonal regeneration can occur in some transection models. This additionally would confound efforts to quantify neuronal survival after the injury.

The present study demonstrates that the total extent of motor neuron survival following PNCI is related to the postnatal age at which injury occurs, shown in Figure 3.2. There appears to be a linear relationship for animals injured from 0 to 14 days of age followed by a trend which appears exponential at ages greater than 14 days. These observations are in good agreement with previous studies of the extent of cell death following PNCI in adult and newborn animals (La Velle and La Velle, 1959; Li *et al.*, 1994; Lieberman, 1971; Lowrie *et al.*, 1987; Snider *et al.*, 1992; Torvik and Skjorten, 1971). Also demonstrated in this investigation is the novel determination that the initial rates of neuronal cell death following PNCI are inversely related to age at injury, as in Table 3.3 and Figure 3.4.

The present study also demonstrates that the gradual developmental increase of expression of the motor neuron trophic factor aFGF within motor neurons parallels the development of resilience to PNCI induced motor neuron cell death. Similarly, it confirms that application of exogenous aFGF after PNCI leads to moderate increase in motor neuron survival (Cuevas *et al.*, 1995), and that global overexpression of a bFGF transgene has a like effect. The relatively modest extent of this effect may be due to the fact that adult peripheral nerves (where PNCI does not induce motor neuron death) normally contain exceedingly high levels of aFGF, which may only partially be mimicked by the application of limited amounts of exogenous FGFs, or by transgenic expression of FGFs. Alternately the hypothesis of aFGF action following PNCI may be incorrect. Although, in that case, it would be difficult to account for even the modest increase in neuronal survival seen in the supplementation experiments. Together, these data suggest that aFGF, derived from injured or dying motor neurons, promotes the survival of motor neurons affected by the injury. It is important to integrate this conclusion into the current understanding of how other neurotrophic factors regulate motor neuron survival.

Historically, it has been thought that developing motor neurons, like most other neurons, depend among other influences on the supply of trophic factors from their target (Oppenheim, 1996). These target derived factors likely include members of the family of neurotrophins (Koliatsos *et al.*, 1993), GDNF (Henderson *et al.*, 1994), cardiotrophin-1 (Pennica *et al.*, 1996) and FGF-5 (Hughes *et al.*, 1993). It is also known that cells present in the adult peripheral nerve begin to express neurotrophic factors after injury. These induced factors include members of the family of neurotrophins (Heumann *et al.*, 1987a; Heumann *et al.*, 1987b; Meyer *et al.*, 1992) and GDNF (Naveilhan *et al.*, 1997). More recently, it has become clear that additional trophic factors are constantly present in the mature motor

neuron (aFGF, (Elde *et al.*, 1991; Stock *et al.*, 1992)) or in neighboring Schwann cells (CNTF, (Stockli *et al.*, 1991)). The levels of aFGF and CNTF present in mature peripheral nerves are significantly more abundant (from 100 to 10,000 times higher in concentration) than those of neurotrophins present in the target tissues or inducible by injury even though all these factors are fully active at concentrations of a few ng/ml. In addition CNTF and aFGF lack hydrophobic signal peptides and are largely present in intracellular, non-secretory, compartments.

How might the role of aFGF relate to the role played by neurotrophins and other factors in promoting the successful survival and regeneration of motor neurons? The long term survival of both adult and developing motor neurons is ultimately dependent on access to target derived secreted factors. In adult animals, trophic factors induced in injured nerve likely act to rescue motor neurons from death after nerve crush. However, significant time is required for the *de novo* expression of trophic factors and to allow for their secretion and action. We propose that aFGF is released in sufficient quantities immediately after injury from lesioned axons to provide a rapid neurotrophic and mitogenic signal in the vicinity of the injury. In this model, the aFGF signal is necessarily transient, as the source of the factor disappears, after the axon has either sealed or degenerated. If this view is correct, the main physiological role of aFGF in the peripheral nerve is to promote neuronal survival for a short period after injury, and to initiate Schwann cell responses to the injury, until other, induced factors can perform these functions. A similar role may be proposed for CNTF released from injured Schwann cells (Sendtner *et al.*, 1997).

The data presented here support this model. The developmental time course of the ability of motor neurons to survive PNCI parallels the raise in levels of aFGF, and also of CNTF in Schwann cells (Stockli *et al.*, 1991). Thus, the action of these pre-existing factors, which are stored in high levels in non-secretory compartments

may be crucial in providing a window of time for the induction of secreted trophic factors. Additional support comes from observations that both STAT3 and ERK phosphorylation (which mediate CNTF and FGF action) are observed in nerves within 30 min after the injury (Sheu *et al.*, 1997). It is also known that aFGF and CNTF, besides exhibiting neurotrophic activity, can act on a large number of different non-neuronal cell types, and the action of aFGF or CNTF may include providing an early stimulus for the induction of other neurotrophic factors by Schwann cells. It is of interest in this regard that cells which are probable targets for injury - released aFGF in the nerve, such as non-myelinating Schwann cells, continue their proliferation and migration into the second month after birth (Jessen and Mirsky, 1991).

Obviously, many aspects of this model of trophic factor action after peripheral nerve injury remain to be tested. The PNCI model provides a stable, well characterized tool for the examination of the regulation of neuronal survival and neuronal death following injury.

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Chapter 4: Ciliary Neurotrophic Factor's Influences

The main thrust of my research has centered on the role aFGF may play in the response to motor neuronal injury. In Chapter 1 I discuss the complex environment of the motorneuron and mention several trophic factors which have the potential to be available to injured motor neurons. One source of these factors is the mature Schwann cell. The best characterized factor derived from Schwann cells is Ciliary Neurotrophic Factor (CNTF). This chapter provides additional background on CNTF as published in the recent invited review (Kuzis and Eckenstein, 1996). The review is presented here in its final form.

"Ciliary neurotrophic factor as a motor neuron trophic factor"

Introduction

The survival of developing parasympathetic ciliary neurons depends on a target derived neurotrophic activity. This activity has been characterized and appears to share most biological and molecular characteristics with ciliary neurotrophic factor (CNTF), which was initially isolated and sequenced from sciatic nerve. Ciliary neurons provide cholinergic innervation to the musculature of the iris, which immediately suggested that CNTF may serve as a motor neuron trophic factor (see (Richardson, 1994) for review).

A multitude of initial studies provided convincing data that application of CNTF indeed promoted motor neuron survival both *in-vitro* and *in-vivo.* In addition, abundant CNTF expression was found in sciatic nerve, indicating that endogenous CNTF may serve as a Schwann cell - derived motor neuron trophic factor. These observations caused great excitement as they provided a a rational for the experimental treatment with CNTF of degenerative motor neuron diseases, such as amyotrophic lateral sclerosis (ALS). Disappointingly, first clinical trials showed that treatment with CNTF alone is not highly efficient in arresting the progress of ALS, and that injections of high doses of CNTF cause deleterious effects (Barinaga, 1994). This review article surveys the current literature in order to re-evaluate the proposed motor neuron trophic role of CNTF.

Definitions

A significant number of molecules have been shown to affect the survival, growth and differentiation of neurons, and varying terms are used in the literature to classify such molecules. We propose (and use in the remainder of this review) the following terminology, which is mainly based on spatial and temporal distribution:

Neurotrophic Activity: The promotion of neuronal growth or survival, *in-vivo* or *in-vitro,* by defined molecules, extracts or conditioned media. Molecules/extracts that are essential for the survival or growth of all cell types are excluded from this definition.

Neurotrophic Factor: A polypeptide that exhibits neurotrophic activity at low concentrations (less than 10 ng/ml) and acts through a specific high affinity transmembrane receptor.

Target Derived Neurotrophic Factor: A specific neurotrophic factor released in limiting amounts by cells innervated by neurons sensitive to this factor. A characteristic of Target Derived Neurotrophic Factors is that the initial action of the factor occurs in the vicinity of neuronal terminals, thus retrograde axonal transport of the factor signal is required for the promotion of cell body survival. Nerve Growth Factor (NGF), produced by the targets of sympathetic and sensory neurons, represents an excellent example of a Target Derived Neurotrophic Factor (Thoenen and Barde, 1980).

Local Neurotrophic Factor: A specific Neurotrophic Factor produced in the vicinity of neuronal cell bodies sensitive to this factor. Retrograde axonal transport is not

required for the action of LNtFs. Basic fibroblast growth factor, produced by astrocytes in the CNS, represents a good candidate for a Local Neurotrophic Factor (Eckenstein, 1994).

Pathway Neurotrophic Factor: A specific Neurotrophic Factor produced in the vicinity of axons sensitive to this factor. Retrograde axonal transport is required for the action of Pathway Neurotrophic Factors. Neurotrophic factors induced in Schwann cells after peripheral nerve injury (such as NGF or Brain Derived Neurotrophic Factor (BDNF)) represent good examples of Pathway Neurotrophic Factors (Heumann *et al.*, 1987a; Meyer *et al.*, 1992).

Target Derived, Local and Pathway Differentiation Factors: Polypeptides that regulate the expression of specific sets of neuronal genes without directly promoting survival. Differentiation Factors act at low concentrations (less than 10 ng/ml) and through specific high affinity transmembrane receptors. A good candidate for a Target Derived Differentiation Factor is activin, which regulates somatostatin expression in the ciliary ganglion (Coulombe *et al.*, 1993).

Additional classification of neurotrophic factor function is provided by temporal parameters. For example, whether they limit the extent of programmed developmental neuronal cell death (Developmental Neurotrophic Factor), are required for the normal function of adult neurons (Maintenance Neurotrophic Factor), or become available after injury (Repair Neurotrophic Factor).

Overall, we feel that the value of the above classification is to allow a precise grouping of the different functions of neurotrophic factors. However, all such classifications must allow for the fact that a specific neurotrophic factor can play

multiple biological roles. A good example is NGF, which may function both as a Developmental Target Derived Neurotrophic Factor and as a Repair Pathway Neurotrophic Factor.

Motor neuron development and environment

The development and cellular environment of rodent motor neurons (MN) have been studied intensely in the past. A brief summary of salient observations is given below, as background for considering the possible role of CNTF in regulating motor neuron function.

The final mitosis of MN precursors occurs between embryonic days 10 and 11, (E10-E11; (Phelps *et al.*, 1990a; Phelps *et al.*, 1990b). They then soon begin to extend their axonal process towards their targets. Prior to arrival at their target, the MNs begin to express detectable levels of the transmitter synthesizing enzyme choline acetyltransferase (Chen and Chiu, 1992; Phelps *et al.*, 1991). The MN axons arrive at the target muscle between E13 and E17 during which time they begin to form polysynaptic connections with the target (Dennis *et al.*, 1981). Interestingly, this time of synapse formation overlaps with the process of programmed developmental cell death which eliminates more that 50% of the starting MNs (Harris and McCaig, 1984; Lance-Jones, 1982). During this time MNs express high levels of the low affinity NGF receptor (NGFR, (Raivich *et al.*, 1985). This period of cell death is followed by the normal elimination of polysynaptic connections, which extends into the first postnatal weeks. (Lance-Jones, 1982). Maturation of MNs during the first two postnatal weeks is characterized by a cessation of the expression of the low affinity NGFR (Eckenstein, 1988) and a steady increase to adult levels in neurotransmitters and in acidic FGF (Kuzis *et al.*, 1995). Interestingly, during these two weeks the response of developing MNs to

axotomy also undergoes a marked change in that MNs in newborn rodents will die within a few days after transection of their axons, while MNs in older animals will survive for significantly longer periods (Kou *et al.*, 1995).

Many of the developmental changes listed above are the results of specific interactions of the MN with other cell types. Of particular interest for this review are astrocytes, Schwann cells and myotubes, which are known to produce neurotrophic activities that promote the survival of MNs *in-vitro* (Dohrmann *et al.*, 1986; Yin *et al.*, 1994). Additional cellular interactions may occur between MNs and activated microglia or infiltrating leukocytes after injury (Heumann *et al.*, 1987b).

Molecular properties of CNTF

Mammalian CNTF protein about 200 amino acids length, with a molecular weight of about 23,000 dalton, iso-electric point slightly above pH 6 and for soluble molecules, relatively hydrophobic. Amino acid sequence homology among mammalian CNTFs is roughly 85% (Richardson, 1994). A similar protein isolated from chicken also supports ciliary neuron survival, shows about 50 % sequence homology with mammalian CNTF (Leung *et al.*, 1992) and is slightly more acidic (pI around 5) and even more hydrophobic. It is currently unclear whether this molecule represents chick CNTF (as referred to from now on) or a close member in a CNTF family. Mammalian and chick CNTF lack traditional hydrophobic amino terminal signal sequences thought to be essential for the sorting of proteins to the secretory pathway. Consistent with this are reports showing that mammalian CNTF, expressed by standard transfection methods, accumulates in the cytoplasm of producing cells, while no CNTF bioactivity is found in the medium conditioned by these cells (Lin *et al.*, 1990; Stockli *et al.*, 1989). However, similar experiments

with chick CNTF found substantial amounts of CNTF bioactivity in the medium (Leung *et al.*, 1992). Interestingly, chick CNTF contains an internal string of hydrophobic amino acids, while the homologous sequence in mammalian CNTFs is not as hydrophobic. Thus, this string is a good candidate for allowing chick CNTF to be released.

No other molecules with close sequence homologies to CNTF are currently known, but computer assisted sequence analysis has shown that CNTF shares structural homology with an expanding number of cytokines, including interleukin-6 (IL-6), IL-11, Leukemia Inhibitory Factor (LIF), Oncostatin M and Cardiotrophin-1 (Bazan, 1991; Bruce *et al.*, 1992; Pennica *et al.*, 1995). Validation for the view that this structural similarity was of functional importance came from studies showing that these cytokines all signal through receptor complexes that contain gp 130 as the common transmembrane transducer (Hirano *et al.*, 1994; Stahl and Yancopoulos, 1994). A specific CNTF-binding component (termed CNTFRa) of the receptor has been identified, and the presence of CNTFRa in the complex is necessary for CNTF action (Davis *et al.*, 1991). Interestingly, the binding component for LIF, termed LIFRb likely also has to be present in the complex to allow CNTF signalling (Davis *et al.*, 1993b). On the other hand, LIF, Oncostatin M and Cardiotrophin-1 appear to be able to signal through a LIFRb-gp130 complex in the absence of CNTFRa (Baumann *et al.*, 1993). To us, the most straightforward interpretation of these observations suggests that cells which respond to CNTF will show an identical response to LIF, but that cells which respond to LIF will respond to CNTF only if they express CNTFRa. In addition, IL-6, IL-11, Oncostatin M, and Cardiotrophin-1, by signalling through gp 130 may have effects similar to CNTF on cells that express the appropriate complement of receptors. It is of interest to note that CNTFRa can exist in both membrane bound and soluble forms, and that the addition of the soluble form of CNTFRa *in-vitro* can confer CNTF responsiveness

to cells that express only LIFRb and gp130 (Davis *et al.*, 1993a). Thus, it is possible that cells which do not express CNTFRa *in-vivo* may still be able to respond to CNTF, if their environment contains sufficient amounts of soluble CNTFRa.

Temporal and spatial distribution of CNTF and its receptor

Adult sciatic nerve is an exceedingly rich source of CNTF, where large stores of the protein are found within the cytoplasm of Schwann cells (Dobrea *et al.*, 1992). Levels of CNTF in newborn sciatic nerve are very low, and the time course of induction to adult levels parallels that of the appearance of myelin associated proteins (Stockli *et al.*, 1991). Transection of peripheral nerves leads to a marked reduction in the level of CNTF, which slowly recovers as regenerating axons are re-myelinated (Friedman *et al.*, 1992; Sendtner *et al.*, 1992b; Seniuk *et al.*, 1992; Smith *et al.*, 1993), suggesting that CNTF expression in Schwann cells requires contact with axons. Interestingly, both LIF and IL-6 mRNAs are strongly induced in transected nerves (Bolin *et al.*, 1995; Curtis *et al.*, 1993), suggesting that regenerating axons may be exposed primarily to LIF and IL-6, and and less to CNTF.

CNTF immunoreactivity has also been demonstrated in the olfactory bulb and in astrocytes of the optic nerve (Dobrea *et al.*, 1992; Stockli *et al.*, 1991). CNTF levels in astrocytes in other areas of the CNS normally appear to be below the level required for detection, but increase significantly in astrocytes that respond to injury (Ip *et al.*, 1993b). CNTF mRNA, as detected by northern blot, is expressed throughout the adult central nervous system. This may reflect the low level synthesis of CNTF in non-injured astrocytes. A significantly weaker signal for CNTF mRNA can be detected in the head as early as at embryonic day 11 (E11, (Ip *et al.*,

1993b)), however, no CNTF immunoreactivity or bioactivity can be detected in the embryonic rodent nervous system (Dobrea *et al.*, 1992). On the other hand, the developing chicken eye is a rich source for chicken CNTF, similar in abundance to adult sciatic nerve (Barbin *et al.*, 1984; Eckenstein *et al.*, 1990), suggesting a role as target derived neurotrophic factor for ciliary neurons.

Distribution of CNTFRa mRNA has been most extensively studied by *in-situ* hybridization and northern blot techniques. These studies demonstrated that, in rat, close to all adult sympathetic, parasympathetic and dorsal root ganglion neurons express CNTFRa message. Similarly, in the adult central nervous system, all motor neurons, many hippocampal neurons, neurons in layer V of cerebral cortex, in the substantia nigra, and in several additional areas express significant levels of CNTFRa mRNA. Developing motor neurons also express the receptor, and northern blots show that CNTFRa mRNA is expressed in the developing CNS early on (E11) at an adult level (Ip *et al.*, 1993a). Similarly, LIFRb and gp130 mRNAs are also widely in the CNS expressed from E11 through adulthood (Ip *et al.*, 1993a). Interestingly, axotomy increases the expression of CNTFRa mRNA in motor neurons and leads to an increased retrograde transport of LIF and CNTF (Curtis *et al.*, 1993; Curtis *et al.*, 1994; Mata *et al.*, 1993).

Biological effects of CNTF and LIF

Actions *in-vitro*: CNTF and LIF, as suggested by the properties of their receptors (see summary above), are expected to have overlapping biological activities. Yet, the two factors were initially discovered and isolated based on two distinct and separate *in-vitro* activities on neurons: CNTF being a survival promoting factor for ciliary neurons (Adler *et al.*, 1979; Nishi and Berg, 1981), and LIF being a factor promoting cholinergic differentiation in sympathetic neurons. It is clear by now,

however, that CNTF can exhibit, in cultures of sympathetic neurons, differentiation promoting activities highly similar to those of LIF (Fukada, 1985; Yamamori *et al.*, 1989). Mammalian LIF, on the other hand, seems unable to promote the the survival of chick ciliary neurons (Richardson, 1994), but this lack of effect may simply be due to a general inability of mammalian LIF to activate the chick LIF receptor. LIF and CNTF can act as survival promoting factors for postnatal, but not embryonic, rat sympathetic neurons *in-vitro* (Kotzbauer *et al.*, 1994). The developmental acquisition of this responsiveness is qualitatively identical for the two factors, which strongly supports the notion that LIF is able to reproduce most, if not all CNTF effects. It is possible, however, that more complex and subtle differences exist between CNTF and LIF responses. For example, it has been reported that depolarization of sympathetic neurons blocks the induction of neuropeptides by LIF, but not that by CNTF (Rao *et al.*, 1992). It is unclear what causes this differential effect of depolarization, but it could be speculated that stoichiometric differences in the number of functional CNTFRa and LIFRb expression might make the signal transduction of the respective factors differentially susceptible to depolarization.

CNTF has also been shown to promote the *in-vitro* survival of motor neurons purified from embryonic day six (E6) chicken spinal cord (Arakawa *et al.*, 1990), while no survival promoting activity is seen in cultures prepared from E 4.5 spinal cord (Bloch-Gallego *et al.*, 1991). It is possible that this difference is due to a developmental change in the responsiveness to CNTF (similar to that reported for sympathetic neurons, see above). Alternatively, this difference may be due the specific culture conditions employed by the two groups. Support for this latter possibility comes from observations that CNTF alone does promote significant, but sub-optimal motor neuron survival, and that other growth factors, such as bFGF or

(Clatterbuck *et al.*, 1993), septal (Hagg *et al.*, 1992), retinal ganglion (Mey *et al.*, 1993) and nigral neurons (Hagg *et al.*, 1992) from axotomy induced degeneration and cell death. The expression of CNTFRa by many of these populations suggests that the survival promoting effect of CNTF injections into the CNS may be due to a direct action of CNTF on the neuronal population under study. Alternatively, as such injections have been shown to activate astrocytes (Kahn *et al.*, 1995), it is possible that CNTF may promote neuronal survival also indirectly, by stimulating astrocytes to produce additional neurotrophic factors. Of central interest to this review are experiments performed in newborn rats, where about 80% of facial motor neurons die within seven days after transection of the facial nerve. These experiments demonstrated that application onto the transected nerve of a small pellet soaked in 5 µg of CNTF reduced motor neuron cell death by about 60% (Sendtner *et al.*, 1990). Similar, more recent experiments demonstrated a similar motor neuron survival promoting effect of LIF (Cheema *et al.*, 1994). Final incentive for the conclusion that CNTF may have therapeutic potential came from observations in mutant mouse strains showing signs of motor neuron disease and progressive loss of motor neurons. In the pmn/pmn mouse, intraperitoneal implantation of a cell line engineered to secrete CNTF (Sendtner *et al.*, 1992a) was reported to greatly reduce motor weakness, loss of motor axons, loss of facial motor neurons and to prolong survival of the mutant animals. Similarly, injection of CNTF into the wobbler mouse was shown to reduce motor weakness. However, this effect was not due to enhanced motor neuron survival, as the CNTF injections did not lead to an increase in the number of motor neurons (Ikeda *et al.*, 1995; Mitsumoto *et al.*, 1994a), while co-treatment with CNTF and BDNF was able to promote motor neuron survival (Mitsumoto *et al.*, 1994b). The effect of CNTF on motor strength in this model is possibly in part due to muscle hypertrophy, which appears to be caused by a direct effect of CNTF on the muscle (Forger *et al.*, 1993;

Helgren *et al.*, 1994), which in turn may cause motor neuron axon sprouting (Gurney *et al.*, 1992). Thus, application of endogenous CNTF appears to have the potential to increase motor strength via trophic actions on both muscle and motor neurons.

These data, taken together with the recognized high level expression of CNTF in adult sciatic nerve, form a solid basis for the hypothesis that one of the physiological roles of CNTF is to serve as a pathway derived maintenance factor for motor neurons. It came therefore somewhat as a surprise when different CNTF-knockout mice were found to show no motor neuron losses at birth and only very mild losses and small signs of motor weakness at an advanced adult age (DeChiara *et al.*, 1995; Masu *et al.*, 1993), and that humans homozygous for an apparent CNTF null-mutation showed no detectable neurological symptoms (Takahashi *et al.*, 1994). A possible explanation for the weak or non-detectable effects of the CNTF-knockout or the null mutation might be that LIF (or possibly another cytokine that is able to activate gp130) may provide sufficient trophic support to overcome the lack of CNTF. It is interesting in this regard that LIF-knockout mice do not show any overt motor deficits (Escary *et al.*, 1993; Stewart *et al.*, 1992). The LIF knockout animals, however, show striking changes after axotomy in the regulation of neuropeptide expression in peripheral ganglia (Rao *et al.*, 1993). This observation is in good agreement with findings that LIF and IL-6 levels are not detectable in normal peripheral nerve and are highly induced in injured nerves (Bolin *et al.*, 1995; Curtis *et al.*, 1993; Curtis *et al.*, 1994). The absence of the two cytokines from normal nerves makes it unlikely that they replace CNTF function in individuals that lack CNTF. More recently, mice that lack the CNTFRa-gene have been generated. At birth these animals show significant neuronal deficits, including loss of motor neurons (DeChiara *et al.*, 1995). Mice

lacking the LIFRb gene show a host of developmental abnormalities, including changes in the spinal cord (Ware *et al.*, 1995), but more work is needed to define whether these changes include the loss of motor neurons.

In the hands of other investigators, CNTF injections and implantation of CNTF-secreting cell lines into rodents had additional and troubling effects, such as causing the induction of acute phase proteins by the liver, rapid weight loss, including loss of skeletal muscle, and death (Dittrich *et al.*, 1994; Henderson *et al.*, 1994; Nesbitt *et al.*, 1993; Schooltink *et al.*, 1992; Ulenkate *et al.*, 1995). Some weight reductions had also been noted in one studies showing trophic effects of CNTF (Mitsumoto *et al.*, 1994a), but clearly in other studies (see above) the beneficial effects of CNTF application were more pronounced than the deleterious side effects. It is likely that the precise method, route, timing and dosage of CNTF application greatly influences whether beneficial or deleterious effects predominate.

Conclusions

A commonly held view is that CNTF serves the role of a repair pathway derived neurotrophic factor, which becomes available to motor neurons from damaged Schwann cells after peripheral nerve injury. As described above, this interpretation is mainly based on observations of CNTF's trophic effect on motor neurons *in-vitro* and *in-vivo*, on the high intracellular levels of CNTF found in mature Schwann cells, and on findings that motor neurons express CNTFRa and retrogradely transport CNTF. While this view represents a reasonable conclusion, aspects of this interpretation are based on studies that leave open two main questions of potential functional importance, as discussed below.

First, the interpretation that CNTF is not released from healthy cells is mainly

based on the absence of a traditional signal peptide in CNTF, and on findings that CNTF biological activity and immunoreactivity accumulates in intracellular stores after transfection of mammalian cell lines with CNTF expression vectors. This is strong evidence that in these cell lines CNTF is not efficiently released when overproduced to very high levels. Minor reservations remain as to whether pronounced instability of CNTF in the medium conditioned (CM) by the transfected cells may interfere with the detection of CNTF in CM. In addition, the transfection studies do not address whether specific cell types that produce CNTF *in-vivo* are able to release some of the factor. Interestingly, it has been shown for bFGF, which also lacks a signal peptide and appears to mainly reside in intracellular stores (Eckenstein, 1994), that transformation of fibroblasts is associated with the appearance of an efficient release mechanism for bFGF (Kandel *et al.*, 1991). It can thus not be ruled out that cell type specific or regulated mechanisms for the release of CNTF exist. If such mechanisms do exist, the low levels of CNTF mRNA found in the developing CNS and in muscle might be of biological relevance. It is also unclear how efficient CNTF release would have to be in order to cause the appearance of biologically significant levels of CNTF in the extracellular space. In this regard, it can be argued that release over 24 hours of 0.1% of the high levels of CNTF present in sciatic nerve should be sufficient to promote motor neuron survival. Current technology is unlikely to be able to differentiate release efficiencies that low from release due to cell lysis.

Second, it is obvious that extensive crosstalk of cytokines can occur through the CNTRa-LIFb -gp130 or LIFb-gp130 receptor complexes, with CNTF, LIF, cardiotrophin and oncostatin M being known active ligands. To further complicate matters, additional active ligands may also exist (see the effects of the CNTFRa knockout mouse, above). The *in-vivo* availability of some of these cytokines to

gp130 receptor complexes expressed on motor neuron cell bodies and axons is not well known. In addition, it is unclear whether the stoichiometry of receptor subunit expression on the protein level may influence the precise biological response of a cells to these ligands. Solving these questions would greatly help to provide a rational explanation for the moderate phenotype of the CNTF-knockout mouse, which may simply be due to the fact that low levels of other cytokines acting through LIFb receptors on motor neurons may be sufficient to substitute for the majority of CNTF action.

These two sets of questions by themselves do not appear significant enough to weaken the rationale that CNTF may have clinical potential for the treatment of ALS, especially when considering the inevitable progress and death of this devastating disease. However, the complexity of the gp130 containing receptor systems, and the wide array of biological effects of their ligands, indicates that unexpected side effects may occur when injecting high doses of gp130 ligands. In addition, the complexity of this cytokine system suggests that the precise effects and side effects of a given dose of cytokine may vary among species, or possibly even individuals. Thus, in hindsight it is clear that more effort should have been made to characterize side effects and efficiency of CNTF injection in several species, including primates, prior to conducting clinical studies. One additional reservation concerns the clinical use of a likely Repair Neurotrophic Factor, such as CNTF. By our definition, repair factors become available in a transient burst to injured neurons. Yet a continued application of CNTF appears necessary for the treatment of an ongoing neurodegenerative disease such as ALS. Thus, the clinical use of a repair factor in such a scenario far extends the physiological time limit during which the factor is normally acting. Thus, prolonged exposure to CNTF could result in a down-regulation of CNTF sensitivity and the appearance of

additional confounding side effects. On the other hand, one has to consider that the treatment of human disease using recombinant cytokines and growth factors is a novel endeavor, and optimal treatment protocols and regulatory mechanisms still are being developed. It is likely the clinical trials of CNTF will contribute to the establishment of such protocols and mechanisms.

Chapter 5 - Additional Experiments & Observations

Introduction

The focus of this chapter is to document the additional experimental avenues which I explored in the process of testing my hypothesis of aFGF's role following injury. The first segment describes my approach to examining the contribution of Schwann cells (and factors derived from them) to the neuronal survival following PNCI.

The second segment of this chapter records the behavioral observations made in adult mice who underwent PNCI.

The Contribution of Schwann Cells to the post PNCI neuronal survival.

Experiment Introduction

As pointed out in the body of the review presented in Chapter 4, there is considerable evidence that mature Schwann cells are the source of neurotrophic factors. With this in mind it became of interest to test the response to PNCI in an environment devoid of mature Schwann cells. The tool selected for this is the dystrophic mouse model.

Mutant Mice Background

The dystrophia muscularis mutant is a strain retained from a spontaneously occurring mutation at Jackson Labs. The homozygotes suffer from progressive weakness and paralysis which begins at about one month of age. The heterozygous animals are unaffected. The condition progresses in a caudal to rostral fashion ending in death of the homozygotes at about six months of age. This model has been well characterized and the functional deficits are due to Schwann cell abnormalities. These include a high number of undifferentiated Schwann cell precursors, (Perkins *et al.*, 1981), a lower total number of mature cells yielding naked axons, (Okada *et al.*, 1976), and segments of polyaxonal myelination, (Brown and Radich, 1979). The molecular deficit for the phenotype appears to be due to abnormal splicing and expression of mRNA from the laminin alpha 2 chain gene resulting in reduced protein expression (Xu *et al.*, 1994a; Xu *et al.*, 1994b). The requirement of this basement membrane signal in controlling Schwann cell development is the key to the phenotype seen.

Experimental Approach

The fact that these animals have a lower number of mature Schwann cells allows an evaluation of the motor neuron reaction to injury with one component of the complex environment essentially removed. By performing lesions in animals which are old enough to have a full complement of other factors in place (such as aFGF within the MNs) we can get an initial view of the relative importance of Schwann cells and the factors derived from them.

For this set of experiments I performed the standard facial nerve crush injury in a series of 30 day old dy/dy homozygotes and a series of wt/? littermates. These animals were allowed to survive for thirty days, and then their motor neuron survival were evaluated.

Results

The control animals were seen to have 95 ± 5% neuronal survival as expected from my prior work on adult PNCI. The homozygous mice had a mean neuronal survival of 55 ± 2%. This difference in survival was found to be significantly lower as evaluated by the t-test. ($p=0.003$) These results are presented in Figure 5.1.

Discussion

While using naturally occurring mutant animals has the same risks to interpretation that all *in-vivo* experiments have, it was felt that these animals provided a useful tool to reinforce the importance of mature Schwann cells in the post injury survival of motor neurons. These experiments demonstrated that animals deficient in mature Schwann cells suffer significantly higher levels of retrograde neuronal cell death following PNCI. There are several interpretations which could be assigned to these results.

First of these is the thought that perhaps the lack of mature Schwann cells in this mutant turns the crush injury into more of a transection model. In other words because no Schwann cell / degenerating axon tube exists the death is due to disorganized regrowth much like in the transection injury model. If we examine the recently published report examining 30 day survival following transection injury (Kou *et al.*, 1995) it is clear that even the neurons which will never regenerate functionally survive past 30 days.

Additional options include the secondary lack of neurotrophic factors, such as CNTF, which are normally derived from mature Schwann cells. Based upon the well described reaction of young animals supplemented with CNTF and other Schwann cell derived factors (Arakawa *et al.*, 1990; Reynolds and Woolf, 1993; Sendtner *et al.*, 1991) the lack of these factors no doubt contributes to the poor outcome following injury in these animals. In fact, the neuronal survival seen in the dy/dy mice following PNCI is quite similar to those seen after injury in the CNTF knockout animals (Sendtner *et al.*, 1997).

An alternative explanation for the results is that the defect in the alpha-laminin 2 gene yields an as yet uncharacterized defect in motor neurons which causes them to be more sensitive to PNCI. While always possible, this explanation is viewed to be less likely than the explanations which account for the loss of the well known Schwann cell / MN interactions both in development and after injury.

The fact that there is a moderate level of survival in these animals, as opposed to complete MN death following PNCI, can be explained by two trains of thought. First it is possible that the remaining mature Schwann cells provide sufficient neurotrophic support. While no hard data exists on the levels of Schwann cell derived neurotrophic factors in the dy mouse, the histologic evidence suggests that there are only one sixth of the normal number of mature Schwann cells exist in the

affected animals (Brown and Radich, 1979). These observations combined with the survival data make this a less likely explanation. The second train of thought is that factors from sources other than mature Schwann cells play a role in post injury survival. This thought fits well with the previously presented observation that neurotrophic factors exist in many locations within the MN's environment.

While the complete characterization of the reaction of dy mice to injury is beyond the scope of my research it appears that Schwann cells play a crucial role in post injury events. All these observations suggest that following injuries such as the peripheral nerve crush model there is an overlapping response which makes use of neurotrophic factors and cells surrounding the injury site. These factors and cells act in a concerted manner to provide the full motor neuron survival seen in adult animals following PNCI.

Figure 5.1

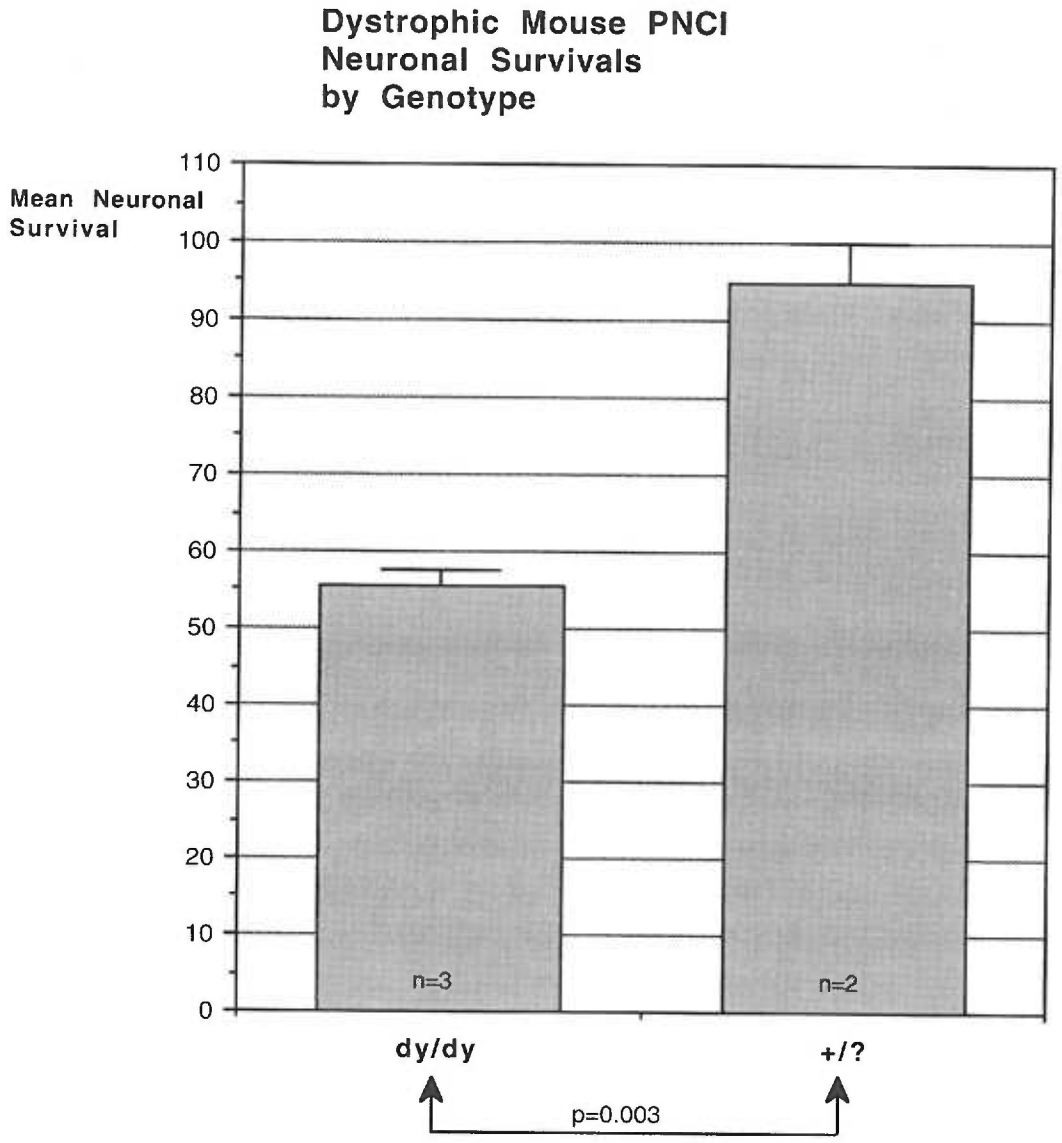

Figure 5.1 - Motor neuronal survival following adult PNCI in dy/dy and wt/? genotype mice. Neuronal profiles were counted after a 30 day survival interval. Survival percentages are calculated by dividing the number of motor neurons in the facial nucleus on the injured side by the number on the contralateral, non-injured side (see methods section for details). p value as determined by T-test.

Observations on Post PNCI Functional Recovery

Functional Recovery Background

The motor branches of the facial nerve provides innervation to the facial musculature. In the mouse these include not only the muscles of facial expression but also the orbicularis oculi responsible for closing the eyelids, and the musculature which control movement and angle of the whiskers. It is these latter two functions which have proved useful in following post PNCI recovery.

In an uninjured adult mouse the blink reflex is highly responsive. A simple puff of air is stimulus enough to force the lids tightly closed for an instant. Following facial nerve injury this reflex is interrupted. The blink reflex immediately after injury is completely absent. The reflex returns by passing through four distinct stages of recovery. First there is absolute absence of reflex, next there is a hint of movement but no reliably reproducible lid movement, followed by partial closure where the lid clearly moves yet it is not able to meet the lower lid and finally there is full closure.

In the uninjured adult animal, the whiskers are in almost constant motion. They project rostrally from the face and move in coordinated small groups which are independent from other groups on either the ipsilateral or contralateral side. Immediately following injury whisker motion is completely absent. The control and movement of the whiskers recovers in a predictable fashion. After injury the whiskers assume a distinctive caudal angle with a complete absence of movement other than that seen when the facial skin is manipulated. The first stage of recovery is realignment of a rostral whisker angle without any consistent movement of the whiskers, followed by recovery of random movement of whisker fibers independent of any grouping. The third stage is recovery of large group coordination where the whiskers move together in large group movements Finally there is full recovery of independent small group motion as is seen in the uninjured animal.

Observational Approach

In order to get an appreciation of the regeneration and reinnervation which takes place normally following a PNCI in adult animals, a group of animals was carefully observed at 24 hr intervals following PNCI. The elements recorded were spontaneous whisker movement, coordination and response to a puff of air directed at the animals cornea. These animals all tested ++ for both blink reflex and whisker movement prior to PNCI. The observations recorded were the best responses, in 3 observation periods each day. These observation periods were typically separated by less than five minutes.

Recovery of Function

The earliest signs of functional recovery occur at about the same time for whisker function and blink reflex, (6.7 vs 7.3 days). (Table 5.1 and Fig. 5.2). Furthermore both functions recover to normal by 12 days following injury.

Table 5.1 - Blink and Whisker movement recovery in adult mice following PNCI.

	Days to Blink Recovery			Days to Whisker Recovery		
Animal	+/-	+	++	+/-	+	++
1	8	9	13	8	10	13
2	7	8	12	6	8	12
3	7	8	11	6	7	8
Mean	**7.3**	**8.3**	**12**	**6.6**	**8.3**	**11**
Mean Err	**0.4**	**0.4**	**0.7**	**0.9**	**1.1**	**2.0**

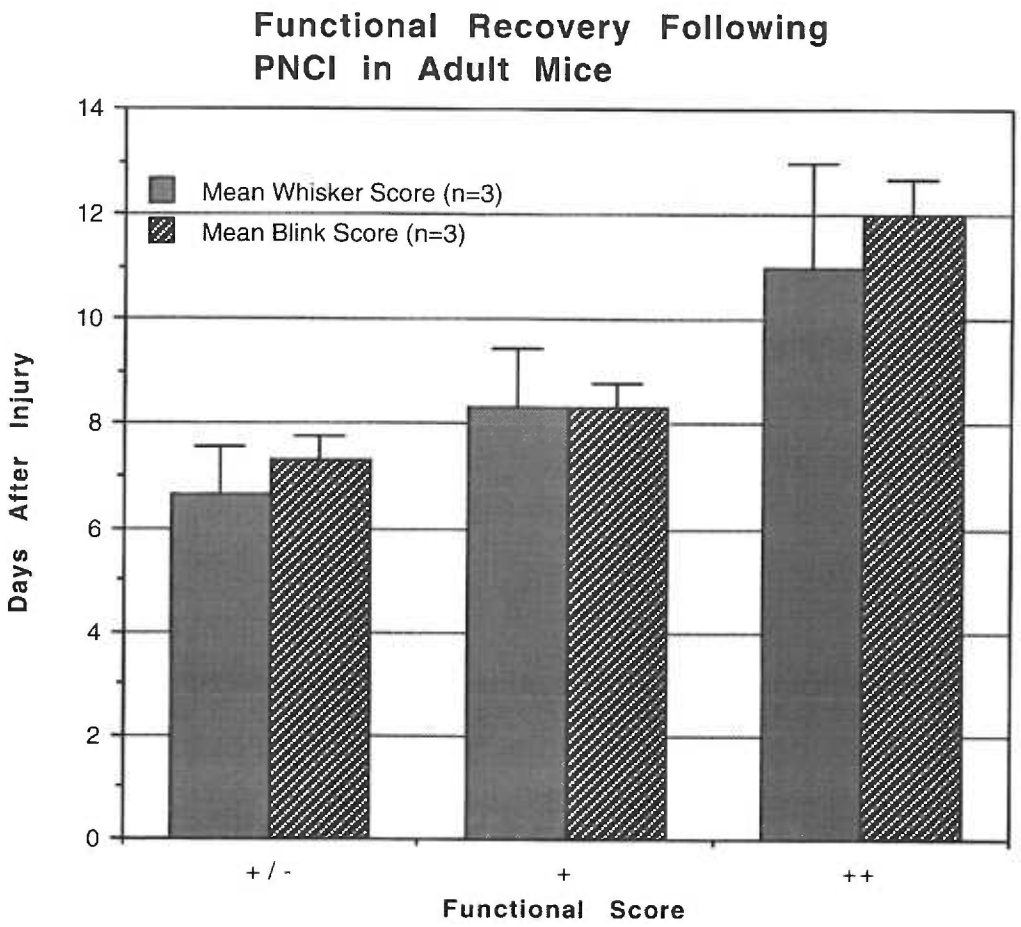

Figure 5.2 - Observations of whisker function and blink reflex at various times following PNCI in adult mice. Blink Scoring: +/- = Trace movement but not consistently reproducible; += Partial closure ; ++= Full closure.
Whisker Scoring: +/- = Forward angle without consistent movement ; + = Random independent movement of whisker fibers ; ++ = Full recovery.

Discussion

The delay in recovery of even trace function of either blink or whisker movement can be attributed to the time required for reorganization at the lesion site. The formation and extension of growth cones occurs rapidly with extension into the degenerating Schwann cell tube beginning shortly after injury. The time to initial recovery (7 days) corresponds to the time required for growth cone extension. The distance from crush site to target muscle was 12-18 mm, demonstrating a growth rate of 1.7-2.5 mm/day for the leading growth cones. The observed differences in recovery seen between animals is most likely accounted for by slightly different positioning of the PNCI. These observations were made in the course of experiments describing neuronal survival following PNCI which were presented in Chapter 3.

Chapter 6: Discussion

Introduction

In the process of these studies, the PNCI system was described in detail for the first time. The unique feature of the PNCI is that the peripheral nerve axons are crushed creating an axonal gap, yet this gap is bridged by the epineurial covering of the nerve. Briefly stated, here are the major events which occur during a PNCI.

Having been isolated from the surrounding fascial attachments, the intact peripheral nerve is crushed with a slight shearing motion between the nibs of a fine forceps (Fig. 6.1). As the nerve is crushed and structures are disrupted, there is a small amount of cytoplasm drainage within the zone of injury (primarily from damaged axons and Schwann cells). This drainage is presumably accompanied by minor bleeding within the fascial compartment containing the vascular bundle. Depending on the structural integrity of the perineurium this bleeding may or may not be isolated from the perineurial encased axonal segments. Once the axons are disrupted there is an influx of Ca^{2+} and synaptic transmission fails. As the axonal membrane seals, the severed stumps swell and retract from the lesion site (Jessell, 1991; Kiernan, 1979). Within the distal nerve stump the process of Wallerian degeneration begins. Axonal neurofilament begins to form whorls and clumps, as the intimate Schwann cell sheaths begin to dissipate and form a loose Schwann cell tube capable of directing the regrowing axons towards the target. (Jessell, 1991). Within a short time the proximal stump begins the formation of growth cones which will be delimited in their extension by the intact perineurial / epineurial membranes. Thus the projecting growth cones are presented with a restricted, directed area within which they will be "channeled" towards the distal Schwann cell tube which leads to the target muscle (Fig. 6.2).

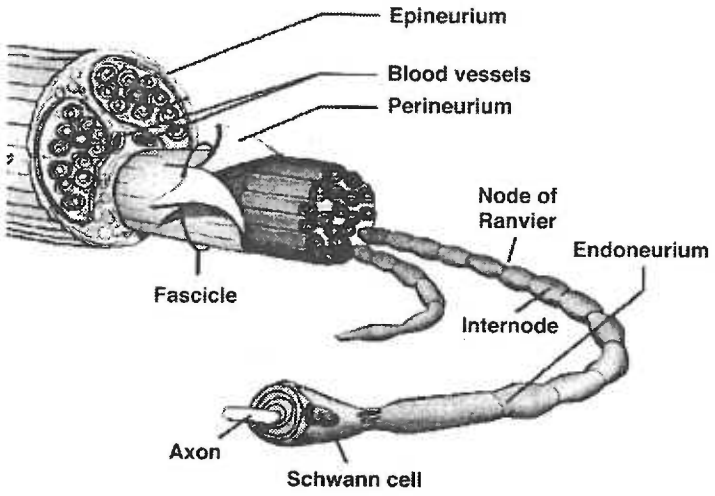

Figure 6.1
Diagram of the peripheral nerve anatomy. Note the relationship between individual axons, their associated Schwann cell covers, the encompassing perineurium, intrinsic vascular bundles and protective epineurium. Figure adapted from (Gartner and Hiatt, 1990).

It has long been observed that in adult animals there is little, if any, neuronal cell death induced by peripheral injury (Torvik and Skjorten, 1971a; Torvik and Skjorten, 1971b; Torvik and Soreide, 1975). This resistance to injury induced death is confirmed by the studies described in Chapter 3. Chapter 5 extends these observations by demonstrating that functional recovery is complete in adult animals within 14 days. Acknowledging the dependence of motor neurons on target derived trophic factors for long term survival, it stands to reason that during the interval of time when the neurons are separated from their targets they must receive trophic support from other sources.

Cellular sources for these unknown trophic factors may be located at the site of lesion, perhaps derived from axons, Schwann cells, endoneurial fibroblasts, blood borne cells such as lymphocytes or they may arise from CNS located sources such as astrocytes, oligodendrocytes or microglia.

The well described localization of the neurotrophic factors aFGF and CNTF within cellular populations at the site of PNCI makes these factors good candidates to exert their neurotrophic activity at the time of injury. Interestingly, aFGF and CNTF are both potent neurotrophic factors, capable of providing neurotrophic support at very low (pg/ml-ng/ml range) concentrations. They are both sequestered in the cytoplasmic compartment of their respective cells yet require access to extracellular receptors to initiate their neurotrophic support (aFGF (Elde *et al.*, 1991; Schnurch and Risau, 1991; Stock *et al.*, 1992); CNTF (Dobrea *et al.*, 1992; Friedman *et al.*, 1992; Gupta *et al.*, 1992; Rende *et al.*, 1992; Stockli *et al.*, 1989)). It is feasible that these two factors are made available to exert their action by the same mechanistic process.

Upon breach of the plasma membrane, axonal and Schwann cell cytoplasm is released in limited quantities within the injury zone. The released cytoplasm contains concentrated aFGF and CNTF. As the released cytoplasm is limited in it's ability to diffuse by the intact epineurial and perineurial membranes, the relative concentration may remain at levels sufficient to activate pre-existing receptors located on either axonal segments or on Schwann cell membranes (Fig. 6.2).

Figure 6.2A

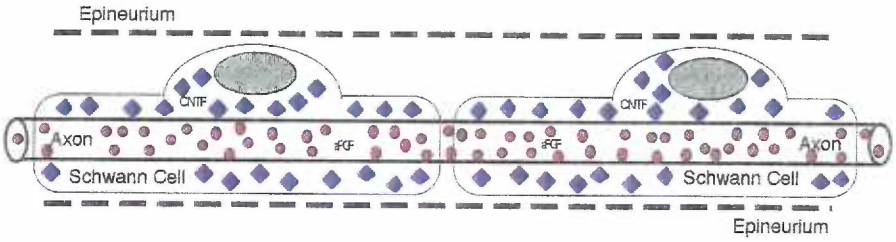

Figure 6.2B

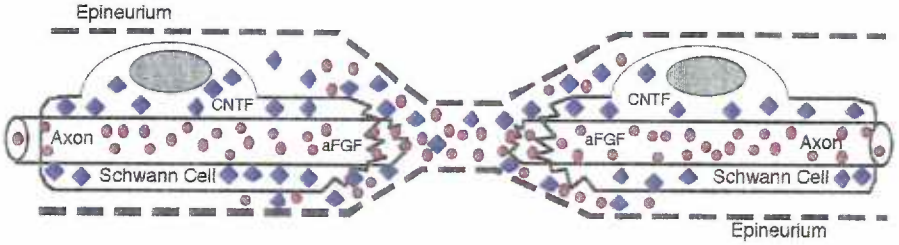

Figure 6.2
Molecular model of neurotrophic factor action at the site of PNCI.
Panel A: Uninjured system with aFGF (red balls) sequestered in the axoplasm, and CNTF (blue diamonds) sequestered in Schwann cells.
Panel B: Cytoplasm leakage from damaged axons and Schwann cells provides free aFGF and CNTF within the injury zone. Neurotrophic support (both direct and Schwann cell mediated) is provided by activation of pre-existing FGFRs and gp130/CNTFa complexes located on axonal and Schwann cell membranes.

Under this model of neurotrophic action following injury, it is necessary that aFGF be present within motor neurons in order to be released with the cytoplasmic leakage.The investigations in Chapter 2 use immunohistochemistry, western blots and a mitogenic cell bioassay to establish the developmental time course of aFGF expression within the rat CNS. These observations are supported by the immunohistochemical data presented in Chapter 3 which confirm the developmental time course of aFGF expression within mouse motor neurons. Similar observations have been made about the developmental expression of CNTF within Schwann cells (Stockli *et al.*, 1991).

As Chapters 2 and 3 demonstrate a dramatic rise in aFGF expression within motor neurons during the postnatal period and the model postulates a neurotrophic role for aFGF, the prediction can be made that neuronal survival following PNCI will increase with age as does aFGF expression. This prediction makes the assumption that while the volume of cytoplasm which escapes through cellular membranes disrupted during PNCI is not dependent on age at injury, the concentration of neurotrophic factor within the cytoplasm released is. Chapter 3 presents PNCI data showing that neuronal survival following injury does indeed increase with postnatal age as predicted. Moreover, these data are in good agreement with previously published work on PNCI (Bueker and Meyers, 1951; Romanes, 1946; Schmalbruch, 1984; Soreide, 1981).

These observations on developmental expression of neurotrophic factors and PNCI induced neuronal cell death have led to the development of the hypothesis that *Motor neuron derived acidic fibroblast growth factor acts to support motor neuronal survival following peripheral nerve crush injury.* While this hypothesis is limited in its scope to the role of aFGF, from the prior discussion as well as evidence previously published it is unlikely that aFGF is the single factor responsible for neuronal survival following PNCI (Gurney *et al.*, 1992; Henderson *et al.*, 1994a; Henderson *et al.*, 1994b; Mitsumoto *et al.*, 1994; Nishi, 1994; Sendtner *et al.*, 1992; Sendtner *et al.*, 1990). Undoubtably aFGF is but one of the important factors in the concerted response to neuronal injury which invokes neurotrophic assistance from nearly every cell which is in contact with the damaged neuron. Yet scientificly it is prudent to attempt to understand the contribution of the parts to the whole, so I set out to test aFGF's ability to act following PNCI.

Under this model, released aFGF may act in either an autocrine manner by binding to HSPGs and FGFRs on the membrane of damaged axons or in a paracrine manner by binding to HSPGs and FGFRs on Schwann cell membranes. (While it's possible that aFGF's paracrine role might be through the stimulation of FGFR on other cell types, such as endoneurial fibroblasts or blood borne lymphocytes, because their density is relatively low compared to Schwann cells and neuronal elements they are presumed to play only a minor role in post injury neurotrophic support). The autocrine role of this finite supply of aFGF would be to stimulate neurotrophic support for a limited time (presumably the half-life of free aFGF is short, on the order of minutes or seconds, therefore the effects of the single point activation of neuronal FGFRs will also be temporally limited). On the other hand, with its known ability to stimulate Schwann cell protein synthesis and mitogenesis (Davis and Stroobant, 1990) the paracrine effects of released aFGF on Schwann cells may last longer. It is unclear which manner of action, autocrine or paracrine, is predominant and this question is beyond the scope of this thesis, although a strategem to examine this question will be presented later in this chapter.

Discussion

In evaluation of a specific hypothesis such as: *Motor neuron derived acidic fibroblast growth factor acts to support motor neuronal survival following peripheral nerve crush injury,* it is useful to make and test predictions of the hypothesis, as well as to attempt to disprove directly the hypothesis.

The most basic of predictions are related to availability of aFGF. Recognizing the likely role of aFGF as part of a larger concerted neurotrophic injury response, it is difficult to make numerical predictions about the percentage of post PNCI neuronal survival which is attributable to aFGF as there is likely some ability to compensate between the roles of multiple factors.

A first prediction is that absence or sub-maximal levels of aFGF at the site of injury would lead to decreased neuronal survival. Presented in Chapter 3 and discussed above, we see that PNCI in animals less than 14 days old (where endogenous expression of aFGF is less than maximal) result in substantial neuronal loss. While these observations support the hypothesis, we know that other factors such as CNTF are also at low levels during those developmental ages so the percentage of the neuronal loss which is attributable to the lack of aFGF can not be estimated. The direct test of this prediction would be experimental blockade of aFGF to prevent activation of FGFRs. This specific prevention of intracellular aFGF stimulated signalling, if done in conjunction with PNCI in an adult animal where aFGF expression is maximal, would allow quantification of aFGF specific neurotrophic support. Unfortunately, repeated attempts to produce well characterized blocking tools, (high affinity blocking antibodies as well as trials with chemical blocking agents), were not successful in producing a reliable blocking strategy, this aim will have to be left for future study.

A second prediction of the hypothesis is that when FGF availability within the injury zone is increased following PNCI (and presumably then FGFR activation will be increased) neuronal survival should rise. Chapter 3 presents evidence that direct supplementation of aFGF, at ages where immunocytochemistry indicated that endogenous aFGF levels were low, does increase neuronal survival. It is unclear whether the submaximal survival increase is due to technical factors such as: limited diffusion across epineurium; application of too little factor; species difference between receptor systems and the human recombinant factor applied; or if the increased survivals were a true reflection of percentage attributable to aFGF's post injury role. It is clear that the increases in neuronal survival were in excess of the sample error attributable to the assay and were statistically significant as compared to untreated controls.

While these treatment experiments were limited in scope, (only one concentration of aFGF examined), the evidence is consistent with our hypothesis and agrees with similar investigations using the transection injury model (Cuevas *et al.*, 1995). This data also indicates that the FGFR system is able to respond to aFGF before aFGF expression reaches maximum *in-vivo* levels, which is consistent with previous reports (Jaye *et al.*, 1992; Kuzis *et al.*, 1995).

Additional support for the prediction is provided by the data from PNCIs in bFGF overexpressing transgenic animals. The use of transgenic animals which over-express a neurotrophic factor represents a novel approach to the study of PNCI. The genetic supplementation of aFGF resulted in increases in neuronal survival which were again submaximal yet in excess of the sample error attributable to the assay and were statistically significant from the nontransgenic controls. It is unclear if the submaximal survival increase is due to technical factors such as species difference between receptor systems and the human recombinant factor expressed

or if the increased survivals were a true reflection of percentage attributable to aFGF's post injury role.

Although this prediction testing has yielded data which is consistent with aFGF serving a neurotrophic function following PNCI, the inability to quantify the true contribution of aFGF to post injury survival limits the strength of the hypothesis. In the model presented, where aFGF is part of a larger concerted response, the developmental expression of CNTF suggests that a similar role exists for CNTF. The molecular model of PNCI includes neurotrophic effect of CNTF released by cytoplasmic leakage from Schwann cells (Fig. 6.2). Similar to the relationship between aFGF expression and neuronal survival following PNCI, the model predicts that neuronal survival will be reduced following PNCI in a system with reduced CNTF levels.

Chapter 5 provides just such evidence. Using the dystrophia muscularis mutant strain, which has a reduced number of Schwann cells (Brown and Radich, 1979) and presumably reduced endogenous level of CNTF, (while maintaining aFGF expression in motor neurons), represents a novel approach to testing CNTF action following PNCI. These experiments demonstrated a statistically significant 40% reduction in neuronal survival in adult animals following PNCI. This suggests that there is a post PNCI role for Schwann cell derived factors such as CNTF. As expected, this data is in good agreement with lesions studies which make use of the CNTF knockout mutant (Sendtner *et al.*, 1997).

Future Investigations

The proposed model for the action of aFGF following PNCI has been tested in several but not all aspects. One important experiment which remains is to block the action of aFGF following PNCI. As repeated attempts to produce well characterized blocking tools, (high affinity polyclonal and monoclonal blocking antibodies as well as trials with chemical blocking agents), were not successful in producing a reliable blocking strategy, this aim has been left for future study. Considering the difficulties experienced during the attempts to create agents capable of preventing the aFGF released during PNCI from binding to its high affinity tyrosine kinase receptor, I propose two different approaches: aFGF knockout mice and inducible, cell-specific dominant negative FGFR mice.

aFGF Knockout Mice

According to the presented model of neurotrophic action following injury, it is necessary that aFGF be present within motor neurons in order to be released with the cytoplasmic leakage. The establishment of a genetic mutant which is devoid of endogenous aFGF would provide the opportunity to test the post injury neuronal survival attributable to the action of aFGF. Although there are precedents for this type of genetic manipulation it is not without risk.

The primary risk to many, if not all, genetic knockout experiments is that with our incomplete knowledge of the developmental role of the factor in question the knockout may be an embryonic lethal mutation, such as the FGFR-2 knockout (Arman *et al.*, 1998). Additionally, the risk of non-lethal developmental abnormalities within the system under study also exists. This risk can be partially overcome by careful descriptive investigation of the target system before undertaking the definitive experiment.

With respect to the question of aFGF's role in neuronal survival following PNCI, a knockout animal would have to: survive to adulthood; demonstrate little if any aFGF expression (as evaluated by western blot and immunocytochemistry); have no gross abnormalities of the CNS; have normal facial motor neurons (in both number and in cell distribution); have normal Schwann cell number, distribution, and CNTF expression; have normal motor end plate architecture and function. A deficiency in any of these areas would suggest additional roles for aFGF beyond post injury neurotrophic support. It is possible that a knockout animal with a deficiency may still be useful for PNCI investigations although it would require that the deficiency be well understood to limit any confounding effects on PNCI results.

Specifically, the PNCI experiment to be done would consist of performing PNCI on adult knockout animals. The use of an adult animal, the age where wild type animals demonstrate full neuronal survival following PNCI, would allow any reduction in survival to be detected. The use of adult animals also increases the likelihood that other neurotrophic factors which have a role after PNCI will be at a mature level of expression.

The prediction for this experiment is that neuronal survival would be reduced following PNCI in these animals. The reduction would represent the minimum percentage of post injury survival attributable to aFGF as compensation by other neurotrophic factors is likely to occur. Western blot analysis of extracts from areas which would contain compensatory factors can be used to detect and quantify the level of compensation which occurs. Comparison of extracts from wild type and knockout animals should examine the following areas: peripheral nerve extracts probed for CNTF and LIF; hindbrain extracts probed for bFGF and FGF-9; and muscle extracts probed for BDNF.

The model predicts that axoplasmal leakage following PNCI provides free aFGF. The action of this free aFGF is mediated by activation of extracellular FGFR on either the axonal membrane or Schwann cell membranes. In wild type animals this activation can be witnessed by examining the peripheral nerve segments for phosphorylated tyrosine kinases as well as phosphorylation of down-stream signal transduction elements like STAT3 and ERK (Sheu *et al.*, 1997). The same type of analysis would be of use to determine the level of FGFR activation following PNCI in knockout mice. As FGFRs are presumably not the only tyrosine kinase receptors in this area, it would be expected that these measures of TK activity would be greatly reduced but not completely absent.

Interestingly, these experiments are underway in the laboratory of Dr. Felix Eckenstein. A colony of aFGF knockout mice has been established (a gift from Dr. C. Basilico at NYU). The animals have no phenotypic abnormalities, they survive to adulthood, they have no gross CNS abnormalities and they appear to have normal facial motor neurons as seen in Table 6.1. The analysis of aFGF expression, as well as characterization of peripheral nerve and motor end plate architecture remain to be accomplished prior to PNCI experiments.

Table 6.1

	aFGF Knockout (mm ±SEM)	Wild Type (mm ±SEM)	p Value
Brain Length	9.3 ± 0.1	9.2 ± 0.04	0.14
Brain Width	10.8 ± 0.1	11.0 ± 0.1	0.13
Facial MNs	2975 ± 55	3601 ± 346	0.15

Table 6.1
Comparison of aFGF knock out mice and wild type controls. Cortical brain width and length (in mm) determined by dissection analysis. Facial motor neuron counts determined by computer assisted stereological cell estimation methods (West, 1999). p Values as calculated from 2 tailed T tests. Data provided by Dr. Eckenstein.

While the analysis of these knockout animals will advance our knowledge of aFGF and its role following PNCI, these experiments will not provide a complete answer. The model of injury provides the possibility of both autocrine and paracrine actions of the free aFGF. The knockout experiments will provide information on the percentage of post PNCI neuronal survival which may be attributed to aFGF, yet these experiments will not address the mechanism of action. An experimental design which would address both these questions is to construct inducible, cell-specific, dominant negative FGFR animals.

Inducible, Cell-specific, Dominant Negative FGF Receptor Mice

The challenge in blocking aFGF action following PNCI is to make use of a system with specific characteristics: It must not interfere with normal development, (as an aFGF knockout might); It must not disrupt the normal anatomy of the PNCI system, (as an intra-nerve injection of blocking antibodies might); and It must be specific to the primary cell types at the lesion site, (unlike chemical blocking agents or a generalized aFGF knockout). I suggest creation of a transgenic animal which carries inducible, cell-specific, dominant negative FGF tyrosine kinase receptors.

A dominant negative (DN) tyrosine kinase (TK) receptor system takes advantage of the requirement for dimerization and cross phosphorylation of ligand bound TK receptors. The controlled over-expression of a "negative" receptor, a receptor form capable of binding ligand and dimerization but incapable of cross phosphorylation, renders the endogenous wild type receptors inactive. This action in effect blocks ligand specific TKs from being activated. This technique has been used successfully in the study of insulin like growth factor (Dunn *et al.*, 1998), transforming growth factor beta (Letterio and Bottinger, 1998), as well as fibroblast growth factors (Celli *et al.*, 1998; Zhang *et al.*, 1998). One refinement I propose is

the inclusion within the receptor construct of an inherently fluorescent peptide sequence (such as green fluorescent protein (Chalfie *et al.*, 1994)) which would allow for easy detection of the sites of dominant negative receptor expression.

As opposed to the generalized expression of DN FGFRs, I propose the creation of animals in which expression of DN FGFRs is limited to specific cell lines and is under the control of an inducible promoter. This would allow targeted interference of FGFR activity, in both cell-specific and time-specific manners. The specific experiment I propose would require creation of three transgenic animals. The first carrying a construct for DN FGFR controlled by a neuron specific promoter upstream of an inducible promoter; the second carrying a similar construct but with a Schwann cell-specific promoter; the third carrying both of these constructs. The definitive experiment would be PNCI in adult animals where transgene expression has been induced prior to the injury.

The power of this strategy is that be examining the neuronal survival following PNCI in adult dual transgenic animals, a survival percentage could be ascribed to the complete activity of FGFR activation (presumably attributable to aFGF released during PNCI). The use of the Schwann cell-specific animal will allow quantification of the post PNCI neuronal survival which occurs through paracrine action of FGFR stimulation by aFGF, while the neuron specific animals will identify the percentage of post PNCI neuronal survival which occurs through autocrine manner.

While this represents a novel approach to examining post PNCI action of FGFR activation (presumably mediated by injury released aFGF) there is considerable evidence to suggest that the methodology is feasible. The first aspect of the constructs is the ability to achieve cell-specific expression within neurons. There are published reports of generalized neuronal expression under the control of various neuron specific promoters such as: neuron specific enolase (Chen *et al.*,

1998; Farlie *et al.*, 1995; Twyman and Jones, 1997), microtubule-associated protein 1B (Liu and Fischer, 1996), presynaptic t-SNARE SNAP-25 (Ryabinin *et al.*, 1995). The best construct would be one which was more selective in expression. Use of the promoter region from the choline acetyltransferase gene would limit expression to cholinergic cells including motor neurons. While an example of this has yet to be seen in publication, the region has been extensively studied in drosophila melanogaster (Kitamoto *et al.*, 1995), in rats (Hahn *et al.*, 1992) in mice (Lonnerberg *et al.*, 1995; Lonnerberg *et al.*, 1996; Naciff *et al.*, 1999) and in humans (Bausero *et al.*, 1993). Similarly, obtaining Schwann cell-specific expression could be achieved by use of well described promoters such as: peripheral myelin protein-22 (Suter *et al.*, 1994), proteolipid protein (Lemke, 1993), protein zero (Lemke, 1993) or the Schwann cell-specific inducer region of the myelin basic protein gene (Gow *et al.*, 1992).

The second aspect of the construct is the ability to induce expression of the cell-specific DN FGFRs. Induction of gene expression is well described. Established induction systems previously used *in-vitro* and *in-vivo* include: heavy metal responsive systems (Peden *et al.*, 1989), dexamethasone-inducible promoters (Moss *et al.*, 1990), glucocorticoid-responsive elements (Lacal *et al.*, 1990), as well as a tetracycline-regulated expression system driving the neuron-specific enolase promoter (Chen *et al.*, 1998).

Once the constructs and transgenic animals are created, the level of DN FGFR expression within peripheral nerves would need to be determined in both non-induced and induced transgenic animals. This would be accomplished by fluorescent microscopy detection of the GFP segment. Additionally the time course of transgene expression once induction has begun will need to be determined so that PNCIs could be performed after expression levels have reached maximum.

The final descriptive step would be to demonstrate DN FGFR action in blocking FGFR activation. This can be accomplished following PNCI by examining the peripheral nerve segments for evidence of phosphorylated FGFRs as well as phosphorylation of down stream signal transduction elements like STAT3 and ERK (Sheu *et al.*, 1997).

This novel approach would require significant effort to achieve. The prediction is that neuronal survival would be reduced following PNCI in all three transgenic animals as the neurotrophic action of aFGF would be blocked at the receptor level. The use of cell-specific transgenics would not only address the question of what percentage of post PNCI neuronal survival was attributable to aFGF, but would also answer the route (autocrine or paracrine) of this action without interfering with normal development or PN anatomy.

Additional Investigations

During the course of investigating the model of aFGF action following PNCI, the mouse facial nerve crush system was described in detail. These observations revealed that cell death occurs rapidly and predictably in young animals following PNCI, thereby establishing the PNCI system as a new biological assay with which to study cell death.

In order to exploit this new system, the first objective is to define if the cell death is apoptotic or necrotic in nature. The standard evaluation of DNA fragmentation, to detect the characteristic DNA laddering seen with apoptotic cell death, should be done in both injured and uninjured facial nucleus tissue samples. This assay should confirm and extend our understanding of injury induced cell death.

Conclusion

This thesis set out to describe peripheral nerve crush injury in an attempt to understand the physical and biochemical interactions surrounding an injury event. The development of a hypothesis of the action of aFGF following injury and an associated cellular / molecular model has allowed further understanding of neuronal response to injury. While the efforts presented here are but a drop in the ocean of knowledge waiting to be understood, they have provided new approaches and new tools that were previously unavailable.

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Solutions **Page**

Animal Perfusion (Rat)

Anesthetic:

This can be obtained pre-made at Animal Care. It is light sensitive, so the tube must be wrapped in foil and kept in the dark.

0.5cc Rat Anesthesia Cocktail:
500 mg ketamine
50 mg xylazine
10 mg Acepromazine
1 ml/kg IM or IP

Fixative:

Made in phosphate buffer.
10% formalin = 4% formaldehyde

Phosphate Buffer:

12.68 g $NaH_2Po_4H_20$ (monobasic)
44.04 g Na_2HPo_4 (dibasic)
4 L dH_2O

1. Anesthetize animal with .5cc of the rat anesthesia cocktail.
   The rat can be held on the floor by grasping body with two fingers on either side of head and injecting intramuscularly in the hip/hind area. Before injecting, draw back on the syringe slightly to make sure a blood vessel was not hit. If uncomfortable with this procedure, you can first put the rat under with ether in a dessicator. After the rat's whiskers have stopped twitching, remove the rat and inject as above.

2. Wait until the rat is completely under before beginning; 10 to 20 minutes. Pinch the tail and feet to make sure there is no reaction.

3. Pin limbs onto styrofoam board with needles or pins.

4. Make a shallow cut under skin near abdomen. Cut up towards head to get the skin out of the way.

5. Cut below sternum through the muscles into the abdominal cavity. The dark red liver can now be seen. Cut to where the thin diaphragm can be seen, with the heart above it.

6. Cut through the diaphragm and up along sides of ribs to expose heart. Now you must work quickly because the animal can not breathe. Pull ribcage over head and clamp out of the way with hemostat forceps.

7. Holding the heart firmly with forceps, inject .3cc heparin + .3cc sodium nitrite into the left ventricle. Heparin is an anticoagulant and sodium nitrite is a vasodilator.

8. Snip left ventricle with small scissors and insert the blunt needle with the perfusion tubing connected into the heart. The needle should go far into the heart and into the aorta. Hold the needle firmly in place with forceps.

9. Begin 0.9% Saline flowing. Nick right atrium to allow blood to exit the circulatory system. Continue saline wash for 30 seconds.

10. Shut off saline flow and immediately begin fixative flowing. There should be gradual twitching in the neck, tail, legs, etc. After a few minutes, tilt rat on board into bin so blood and fix can drain. Continue fix flowing until the full 300 ml. has gone through the rat.

11. Decapitate rat and remove the brain and other desired organs. Post-fix for 90 minutes in scintillation vials on shaker.

12. Remove fix and wash in 1XPBS for two hours and then overnight.

13. Remove 1XPBS and add 15% sucrose for about 2-4 hours and then 30% sucrose + 0.1% sodium azide until ready to section.

Avidin- Biotin Complex Staining Protocol.

Time Needed

60 Min. - **BLOCKING**
Block Tissues in blocking solution for 60 Min.

12 Hours. - **PRIMARY**
Incubate Tissues in primary Antibody overnight.
(Minimum of 12 Hours, SAVE primary after incubation, record # of times stock used.) (bFGF @ 1:2000)

30 Min. - **WASH**
Wash Tissues in 1xPBS.
Repeat three times at 10 Min each wash.

60 Min. - **SECONDARY**
Incubate Tissues in biotinylated secondary Antibody for 60 Min.
(Typical dilution 1:250)
SAVE secondary after incubation, record # of times stock used.

30 Min. - **WASH**
Wash Tissues in 1xPBS.
Repeat three times at 10 Min each wash.

60 Min. - **ABC Complex (Vectastain Kit)**
Incubate Tissues in ABC solution, (1dp A 1dpB in 10 ml PBS).
Always make new ABC.

30 Min. - **WASH**
Wash Tissues in 1xPBS.
Repeat three times at 10 Min each wash.

15 Min. - **Color Reaction**
React Tissues in substrate solution for 15 Min.
(DAB 1ml/9mlPBS+100µl3%H2O2, All items that contact DAB MUST be soaked in bleach until the brown percipate dissipates.)

10 Min. - **WASH**
Wash Tissues in 1xPBS.
Repeat twice at 5 Min each wash.

ABC Staining Planning Sheet

Date:

Tissue to be stained:

Vial / Slide #	Tissue	Primary / Dilution	Secondary / Dilution
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			

Check List: -----

____ 60 Min. - **BLOCKING With Azide.**

1.5 Hr ____ 12 Hours. - **PRIMARY**

----- ____ 30 Min. - **WASH 3 @ 10 Min. 1x PBS.**

____ 60 Min. - **SECONDARY**

____ 30 Min. - **WASH 3 @ 10 Min. 1x PBS.**

4 Hr ____ 60 Min. - **ABC solution**

____ 30 Min. - **WASH 3 @ 10 Min. 1x PBS.**

____ 15 Min. - **Substrate**

----- ____ 10 Min. - **WASH 2 @ 5 Min. 1x PBS.**

Analysis of Staining:

Date:

Blocking Solution With Azide. (Omit Azide for NO AZIDE BS).

50.0 % 200 mM Phosphate Buffer =
10.0 % Horse Serum =
0.5 % Triton X 100, (10 % Stock) =
0.2 % Na Azide , (10 % Stock) =
Up to volume with dd H2O =

Primary Antibodies. **Subject.** **Control.**

___ % Antibody =
___ % Blocking Solution =
With AZIDE.

Secondary Antibodies.

___ % Antibody =
5.0 % Serum from same
Species as Tissue. =
___ % Blocking Solution
With AZIDE. =

ABC Complex

1 Drop "A",
1 Drop "B",
in 10ml 1xPBS

Diaminobenzene Solution. ***CAUTION CARCINOGEN.***

Makes 10 ml.
9.0 ml 1xPBS.
1.0 ml DAB - allioquots in freezer, 5.0 mg /ml.
100 ul H2O2 (3% Stock).

Coverslipping Protocol

1. Allow tissue to dry onto slides, (± 2Hr.)

2. Dehydrate Slides before coverslipping:
   In glass holders put slides into the following for 10 min each.

a. 50% Ethanol (EtOH)
b. 70% Ethanol
c. 95% Ethanol
d. 100 % Ethanol
e. 100% Ethanol
f. 100% Xylene
g. 100% Xylene
h. 100% Xylene

3. Using Pro-Texx mounting solution and a glass pipette, coverslip the slides.

4. Allow to air dry, carefully clean off excess Pro-Texx with xylene and a cotton swab.

Cresyl Violet Counter Stain

(Nissel Substance Stain, Stains RNA, & rER)

Procedure

30 min -Defat Tissues in 50/50 mix Chloroform / 100 % EtOH.
5 min - 95 % EtOH.
5 min - 70 % EtOH.
5 min - 50 % EtOH.
5 min - dd H2O.
30 min - Staining Solution (Filter Before Use)
5 min - dd H2O.

15-25 min - Destain in 70 % EtOH, Monitor Progress.

Quick rinse in 95% EtOH (< 10 sec)
Quick rinse in 95% EtOH (< 10 sec)
10 min - 100% Xylene
10 min - 100% Xylene
Coverslip

0.1% Cresyl Violet Solution

1.0 g Cresyl Violet Acetate
1000 ml dd H2O
Dissolve Completely, Stir Well.
Solution should be bright Blue NOT pink.
Keeps for 10 Weeks or so.
Filter before using.

Cryostat Procedure

1. Flip both switches on and set temp. at -18.
   The far left switch should always be left on - it controls the chamber temp.
   Everything should soon be at the same temp.

2. Insert anti-roll plate; screw in place.

3. Set blade angle at 3 degrees.
   Loosen the left knob and move blade holder to the third lowest mark on the right side. Do before blade in place.

4. Insert blade - pick up with chem wipes.
   Tighten knobs to hold blade in place.
   Wipe off oil - ALWAYS WIPE UP!

5. Clean mounting block.
   Scrape off frost with razor blade

6. Put platform on block and apply tissue tek to form a raised surface.
   Continue adding tissue tek after the previous layer begins to freeze to build up a surface.

7. Place tissue on tek and cover with one more drop to make sure entire tissue will be frozen.

8. Mount specimen.
   Lock down by turning black knob.

9. Pull blade all the way back.
   Turn front lever to right, and use black knob on right outside to move the blade back towards yourself.

10. Move specimen down to middle of blade - watch the shadow.
    Move blade forward to match shadow so it will be at a point ready to slice the tissue.
    Move front lever back to left to lock blade in place.

11. Crank wheel to cut specimen.
    Begin at 25 microns until you are close to the tissue, then cut 10 micron sections. (Set wheel on front of machine)

12. Adjust the anti-roll plate to get a good section without rolling.
    If the plate is too close, the tissue won't be cut by the blade; if it's too far, the tissue rolls up when cut.

13. When ready to make slide, briefly touch the slide to the blade - the section should quickly stick to the warmer slide.

CLEANING UP

1. Remove blade with chem wipes.
   Rinse under hot water.
   Dry with chem wipes.
   Drip oil onto blade and wipe all over - wipe UP!

2. Remove anti-roll plate.

3. Remove frozen samples with razor and rinse platforms under hot water.
   Leave setting on paper towel in cryostat room.

4. Take out tubs and dump.

5. Brush off any pieces inside machine.

6. Turn off 2 right switches.
   Set temps. at 15-17 degrees.

Facial Nuc Injuries; Tissue Handling Protocol

Setioning

25µm cryostat sections onto sets of four slides. So that this creates four sets of tissue separated by , at most, 75µm. The slides should be labeled with the animal # and a sequential slide number. NOT with set numbers or other designations.

Drying down

Slides should be dried flat, with a dust cover for 24 hrs. Three sets are then placed in storage boxes with drierite, and stored in either the -80 if the box is full, or at 4˚ until the next batch is ready to be added. The remaining set gets fixed and stained.

Fixation

The slides are then racked, and placed in in a staining boat containing paper towels soaked in 40% formlin for1 hour. They are then rinsed with slowly flowing ddH2O for10 min.

Toludine Blue Staining

The slides to be stained are rinsed in ddH2O for 5 min, then placed into the staining solution overnight. The next day, one at a time, they are swished through 70% EtOH (1 swish 1 sec), then swished through 95% EtOH, The slide edge is rapidly dabed on paper towels to remove excess EtOH, then diped three times in100% Isopropal Alcohol, the slide edge is rapidly dabed on paper towels to remove excess, then placed into 100% Xylene for at least 5 mins, moved to fresh100% Xylene for an aditional 5 mins then coversliped. The freshly coversliped slides are left to dry in the hood for 24 hrs. Then they are cleaned with a Xylene soaked kimwipe, air dried, then counted.

Counting

Toludine blue slides are examined to determine the location of the facial nucleus within the set. The sections containing the nuc are labeled with letters A-?. With the first and last section labeled being the section directly before or after the nucleus where no motor neurons are found. The sections are counted with the 16x objective, using 10x eyepeices with an etched grid reticule. Counts are recorded for both sides of the brain stem, as well as animal #, slide #, and section letter.

Neurons are counted based on size and morphology. A cell which is counted must be > 15µm across, and have motor neuronal morphology. While this yields "double counts" where a neuron is counted and would also be counted in the next serial section, this can be corrected for. Given an observed maximal cell diameter of 20-25µm, it is estimated that the portion of a cell which is 15µm across would represent a third of a cell. If so, there are five possible states for a counted cell in a section: One third in from top or bottom, two thirds in from top or bottom, completely in. Using the cells centered nucleous location as the determinant of which section the cell "belongs" to, three out of the five counted cells "belong" to the section in question. So using a 3/5 correction factor, multiplied by the number of slide sets collected, 4, we get a final correction factor of 2.4. This factor when applied to the counted cell number in a single set will yield the number of countable cells in the complete nucleus.

Holmes' Silver Technique for Neurons

(From "Handbook of Histopathological and Histochemical Techniques", 3ed, Culling CFA, 1974, Butterworths pub. pp440-442)

Results: Neurones Red-Black; Myelin(if Luxol blue used) Blue.

Method

1) Bring formalin fixed paraffin sections to water.
2) Place in 20% aqueous silver nitrate, in the dark, at room temp for 1 Hr.
3) Wash in ddH2O (3x@10min)
4) Place in impregnating solution, cover container and leave overnight at 37°C. There sould be no less than 20ml of solution for each slide.
5) Remove slides from impregnating solution, shake off excess fluid and place in reducing solution for 2-3 minutes.
6) Wash in running water for 3 minutes.
7) Rinse in ddH2O.
8) Tone in 0.2% gold chloride for 3 minutes.
9) Rinse in ddH2O.
10) Place in 2%oxalic acid for 3-10 minutes. The impregnation of the neurones is controled at this stage, they become progressively pale red, deep red, then black. If Luxol fast blue is being used to counterstain mylein the impregnation should be stopped while the axons are reddish-black.
11) Rinse in ddH2O.
12) Place in 5% sodium thiosulphate for 5 minutes.
13) wash in Tap-water.
14) Dehydrate, Clear, & Mount.

OR

14) Rinse in 95% alcohol.
15) Stain in Luxol fast blue overnight at 37°C.
16) Wash in 95% alcohol, then in ddH2O.
17) Commence differentiation by immersing sections in lithium carbonate solurtion for not more than 20 seconds.
18) Differentiate in 70% alcohol until grey and white matter are clearly distinguishable, 30 seconds-1 minute.
19) Rinse in ddH2O and examine under microscope. If differentiation is not complete repeat steps 17-19 with reduced times (lithium carbonate 2-3 seconds).
20) Wash well in ddH2O.
21) Dehydrate, Clear, & Mount.

Solutions

Impregnating Solution - Dilute 100 ml of Holmes' boric acid-borax buffer pH 8.4 to 494ml with ddH2O. Add 1ml of 1% aqueous silver nitrate, the 5 ml of 1 % aqueous solution of pure pyridine. Mix well. This solution should be freshly prepared.

Reducing Solution -

Hydroquinone	1g
Sodium sulphite (crystals)	10 g
ddH2O	up to 100 ml

Luxol Fast Blue Solution

Luxol fast blue	1g
95% Alcohol	1000 ml
10% acetic acid	5ml

Alternative Luxol Fast Blue Solution

Isopropanol	100 ml
Luxol fast blue	0.1 g

Lithium Carbonate Solution
0.005% aqueous solution lithium carbonate

Holmes' Boric Acid-Borax Budder (pH Range 7.4-9.1)
Stock Solutions:
M/5 boric acid
Prepared by dissolving 12.368 g of boric acid up to1L of ddH2O.
M/20 borax (sodium tetraborate)
Prepared by dissolving 19.071 g of borax up to1L of ddH2O.

pH	M/5 boric acid (ml)	M/20 borax (ml)
7.4	**90**	**10**
7.6	85	15
7.8	**80**	**20**
8.0	70	30
8.2	**65**	**35**
8.4	55	45
8.7	**40**	**60**
9.0	20	80

Microglial Lectin Staining Protocol

Time Needed

10 Min. - **WASH**
Wash tissues twice in 1xPBS, for 5 Min each time.

15 Min. - **CATION Infusion**
Incubate in cation solution for 15 Min.

2 Hours - O/N. - **Lectin**
Incubate in biotinylated Lectin solution,
(Minimum of 2 Hours, can go overnight, usu. 20µg/ml in cation solution).

30 Min. - **WASH**
Wash Tissues in 1xPBS.
Repeat three times at 10 Min each wash.

60 Min. - **ABC Complex**
Incubate Tissues in ABC solution.

30 Min. - **WASH**
Wash Tissues in 1xPBS.
Repeat three times at 10 Min each wash.

15 Min. - **DAB**
React Tissues in Diaminobenzene solution for 15 Min.
All items that contact DAB MUST be soaked in bleach until the brown percipate dissapates.

10 Min. - **WASH**
Wash Tissues in 1xPBS.
Repeat twice at 5 Min each wash.

Lectin Staining Planning Sheet.

Date:

Samples to be stained:

Sample#	Sample Name	Lectin / Dilution
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		

Check List:

---	____	10 Min.	- WASH 2 @ 5 Min. 1x PBS.
2.5Hr - O/N	____	15 Min.	- Cation Infusion 15 Min. Cation Solution.
—	____	2 Hours - O/N	- Lectin
---	____	30 Min.	- WASH 3 @ 10 Min. 1x PBS.
	____	60 Min.	- ABC Complex.
2.5 Hr	____	30 Min.	- WASH 3 @ 10 Min. 1x PBS.
	____	15 Min.	- DAB
—	____	10 Min.	- WASH 2 @ 5 Min. 1x PBS.

Analysis of Staining:

Cation Solution g/500ml

PBS +

0.1mM CaCl2	0.0074
0.1mM MgCl2	0.010
0.1mM MnCl2	0.010
0.1% Triton x-100	0.5ml

Lectins

___ ml of _____mg/ml biot. Lectin = ___µg/ml

___ ml of _____mg/ml biot. Lectin = ___µg/ml

___ ml of _____mg/ml biot. Lectin = ___µg/ml

ABC Complex

1 Drop "A",
1 Drop "B",
in 10ml 1xPBS

Diaminobenzene Solution. ***CAUTION CARCINOGEN.***

Makes 10 ml.
9.0 ml 1xPBS.
1.0 ml DAB - allioquots in freezer, 5.0 mg /ml.
100 ul H2O2 (3% Stock).

Microglia Nonspecific Esterase Staining Protocol

- Fix samples in cold 10% formaldehyde in 0.1M Phosphate buffer to pH7.0, for 24 Hrs.

- Rinse twice for 5 min in ddH2O

- Place samples in staining solution for 1-5 Hrs.,

- Wash gently in tap water for 30 sec,

- Counterstain if desired,

- Mount and examine.

Staining Solution(s):

Grey-Black Product
- 100mg of a-naphthyl acetate (Sigma N-8505)
- 45 mg Fast Blue BB salt (Sigma F-0250)
- into 14ml ethylene glycol monomethyl ether in a 125 ml flask,
- Add 50 ml 0.1M Tris-maleate buffer pH7.8,
- Stopper and shake vigorously,
- Filter and use immediately.

Blue Product
- Substitute 5mg naphthol AS-E acetate (Sigma N-7139) for the a-naphthyl acetate.

Red Product
- Substitute 5mg naphthol AS-E acetate (Sigma N-7139) for the a-naphthyl acetate,
- and 35mg Fast Garnet GBC salt (Sigma F-0875) for the Fast Blue BB salt.

From: "Cytochemical Identification of Mononuclear Macrophages"; Leonard S. Kaplow; pg 199-207, in *Manual of Macrophage Methodology*, Ed. H.B. Herscowitz, HT Holden, JA Bellanti, and A Ghaffar; 1981, Marcel Dekker, Inc. NY, NY.

Osmium Amplification

CAUTION: Osmium Tetraoxide is a very strong reducing agent.
ALL activities take place well into the hood, use gloves.

OsO_4 is used as 0.4% , mixed from 4% stock in 1xPBS. The 4% stock is kept in a sealed vial within a sealed jar in the refrigerator.

Mix a maximum of 5ml as it can be used on a number of samples without a loss of amplification.

Setup:

- 1 small (±150 ml) Beaker 1/4 full of corn oil. This will be the OsO_4 waste collection.
- 1 Beaker of 1xPBS.
- Scintillation vial with mixed OsO_4.

Protocol:

- Place 0.4% OsO_4 solution on the samples for 1-5 mins. (Based on amount of amplification desired, which varies with sample).
- Remove OsO_4 and return to vial.
- For already mounted slides, rinse excess off of samples with PBS directly into waste beaker, then soak samples in PBS for five miniutes.
- For floating sections 2 five miniute rinses with PBS.

Cleanup:

- When complete, OsO_4 0.4% mix is disposed of into the corn oil containing waste jar, which is kept in the hood. The vial is rinsed with corn oil then water into the waste container after which the vial can be trashed.
- The beaker of corn oil is used to rinse any pippettes/tips used before their disposal. When this is complete, consolidate the waste into the jar in the hood. (Including the first rinse water from the waste beaker.)

Penfield's Modification of Hortega's Method for Oligodendroglia

(From "Handbook of Histopathological and Histochemical Techniques", 3ed, Culling CFA, 1974, Butterworths pub. pp 456-457)

Results: Oligodendroglia (& microglia) Black.

Method

1) Remove traces of formalin by washing sections overnight in 1% ammonia in ddH2O.
2) Transfer directly to 5% hydrobromic acid in ddH2O for 1 hour at 37°C..
3) Wash in ddH2O (3x@10min)
4) Transfer to 5% sodium carbonate solution for 1 hour (up to 6 hours OK).
5) Transfer to silver carbonate solution for 3-5 minutes, until sections turn light brown in colour.
6) Transfer to 1% formalin and agitate sections by blowing on the surface of the fluid for 2-3 minutes.
7) Wash in ddH2O.
8) Tone in 0.2% gold chloride for 10-15 minutes.
9) Rinse in ddH2O.
10) Fix in 5% sodium thiosulphate.
11) wash in Tap-water.
12) Dehydrate, Clear, & Mount.

Solutions

Silver Carbonate solution

20% Silver nitrate	10 ml
5 % sodium carbonate	20 ml

Mix and add strong ammonia (.880) drop by drop until the preciptate, which is first formed, is dissolved. Make up to total volume of 75 ml with ddH2O.
For microglia as well try 5 ml of 10% silver nitrate solution in the mix.

Peroxidase-Anti-Peroxidase Staining Protocol

Time Needed

30 Min. - **H2O2 Kill**
Soak Tissues in 5% Peroxide solution for 30 Min.

10 Min. - **WASH**
Wash tissues twice in 1xTBS, for 5 Min each time.

90 Min. - **BLOCKING**
Block Tissues in blocking solution for 90 Min.

12 Hours. - **PRIMARY**
Incubate Tissues in primary Antibody overnight.
(Minimum of 12 Hours, SAVE primary after incubation, record # of times stock used.)

30 Min. - **WASH**
Wash Tissues in 1xTBS.
Repeat three times at 10 Min each wash.

60 Min. - **SECONDARY**
Incubate Tissues in secondary Antibody for 60 Min.
SAVE secondary after incubation, record # of times stock used.

30 Min. - **WASH**
Wash Tissues in 1xTBS.
Repeat three times at 10 Min each wash.

60 Min. - **Peroxidase-Anti-Peroxidase**
Incubate Tissues in Peroxidase-Anti-Peroxidase solution.
Use PAP from the same species as the primary.
Always make new PAP.

30 Min. - **WASH**
Wash Tissues in 1xTBS.
Repeat three times at 10 Min each wash.

15 Min. - **DAB**
React Tissues in Diaminobenzene solution for 15 Min.
All items that contact DAB MUST be soaked in bleach until the brown percipate dissapates.

10 Min. - **WASH**
Wash Tissues in 1xPBS.
Repeat twice at 5 Min each wash.

PAP Staining Planning Sheet

Date:

Tissue to be stained:

Vial / Slide #	Tissue	Primary / Dilution	Secondary / Dilution
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			

Check List: -----

		____	30 Min.	- H2O2 Kill
	2.25 Hr,	____	10 Min.	- WASH 2 @ 5 Min. 1x TBS.
		____	90 Min.	- BLOCKING With Azide.
	-----	____	12 Hours.	- PRIMARY
	-----	____	30 Min.	- WASH 3 @ 10 Min. 1x TBS.
		____	60 Min.	- SECONDARY
		____	30 Min.	- WASH 3 @ 10 Min. 1x TBS.
	4 Hr	____	60 Min.	- Peroxidase-Anti-Peroxidase
		____	30 Min.	- WASH 3 @ 10 Min. 1x TBS.
		____	15 Min.	- DAB
	-----	____	10 Min.	- WASH 2 @ 5 Min. 1x PBS.

Analysis of Staining:

Date:

Peroxidase Kill Solution

30.0 % Ethanol =
5.0 % Peroxide (30 % Stock) =
65.0 % 0.1 M Tris Buffer pH 7.3 =

Blocking Solution With Azide. (Omit Azide for NOAZIDE BS).

50.0 % 200 mM Phosphate Buffer =
10.0 % Horse Serum =
0.5 % Triton X 100, (10 % Stock) =
0.2 % Na Azide , (10 % Stock) =
Up to volume with dd H2O =

Primary Antibodies. **Subject.** **Control.**

___ % Antibody =
___ % Blocking Solution With AZIDE. =

Secondary Antibodies.

___ % Antibody =
5.0 % Serum from same Species as Tissue. =
___ % Blocking Solution With AZIDE. =

Peroxidase Anti Peroxidase Solution.

1.0 % PAP from same species as primary. =
5.0 % Serum from same Species as Tissue. =
___ % Blocking Solution **NO AZIDE.** =
(Filter Before Use)

Diaminobenzene Solution. ***CAUTION CARCINOGEN.***

Makes 10 ml.
9.0 ml 1xPBS.
1.0 ml DAB - allioquots in freezer, 5.0 mg /ml.
100 ul H2O2 (3% Stock).

Slide Subbing Protocol

1. Soak slides in Nitric Acid solution for 20 min using metal or glass slide boats. (Solution 3:1, Acid : Water, Water first then add acid)

2. Soak in soap/hot tap water for 10-15 min.

3. Rinse : Hot tap water
   Twice in distilled H20
   Twice in house deionized H2O

4. Dip slides in boats into subbing solution, careful to avoid bubbles.

5. Drain on paper towels and allow to dry. Keep loosely covered. Subbing solution should not be kept for more than 48 hours. Filter before using if not used fresh.

6. If desired, slides can be dipped again after drying for 45 min.
(2 dip recipe)

Subbing Solution:
Dissolve 5.00 g Gelatin in 500 ml heated dd H2O
When cooled add 0.5g Chromium potassium Sulfate (Chrom Alum)

Slide Subbing Protocol (Rapid Method)

1. Soak slides in 95 % Ethanol for 10 min.
2. Allow to air dry.
3. Dip slides for 10 sec into subbing solution, careful to avoid bubbles.
4. Drain on paper towels and allow to dry at least 45 min.
5. Dip slides again for 10 sec into subbing solution, avoiding bubbles.
4. Drain on paper towels and allow to dry overnight.

Subbing Solution:
Dissolve 10.00 g Gelatin in 1000 ml heated dd H2O
When cooled add 1.0g Chromium potassium Sulfate (Chrom Alum)

Subbing solution should not be kept for more than 48 hours.
Filter before using if not freshly mixed.

Toluidine Blue Counter Stain

(Nissel Substance Stain, Stains RNA, & rER)

Procedure

- Bring Slides to ddH2O
5 min - dd H2O.
Overnight - Staining Solution
(48 Hr works well, haven't tried 24, 2Hr is too short)

1 Swish - 70% EtOH (1 swish 1 sec)
1 Swish - 95% EtOH (1 swish 1 sec)
- Dab slide edge on paper towels to remove excess EtOH
3 Dips - 100% Isopropal Alcohol
- Dab slide edge on paper towels to remove excess
5 min - 100% Xylene
5 min - 100% Xylene
Coverslip

Solutions:

1% Toluidine Blue Stock Solution

1.0 g Toluidine Blue-O
(C.I. 52040, Basic Blue 17)
100 ml dd H2O
Dissolve Completely, Stir Well.

Acetate Buffer

0.1M pH 3.5
0.303g Na Acetate Anhydrous
2.67 ml Glacial Acetic Acid
Mix to 500 ml with dd H2O

Toulidine Blue Working Solution

2.6 ml 1% Toulidine Blue
100 Ml Acetate Buffer

Thionin Counterstain Protocol

Function:

This counterstaining will bind to nucleic acids. Primarilly DNA some RNA binding seen.

Stain Solution:

500ml:

0.5 g Thionin
500 ml dd H_2O
10 drops glacial acetic acid

Procedure:

1. Stock Stain Solution:	25-30 sec for 50um sections.
2. 50% Ethanol:	3 Minutes
3. 70% Ethanol:	3 Minutes
4. 100% Ethanol:	3 Minutes
5. 100% Ethanol:	3 Minutes
6. 100% Xylene:	3 Minutes
7. 100% Xylene:	3 Minutes

Note: Steps 2 & 3 are destaining; time can be increased as needed. If necessary, slides may be left in the last xylene bath up to 30 min.

Weil-Davenport's Method for Microglia

(From "Handbook of Histopathological and Histochemical Techniques", 3ed, Culling CFA, 1974, Butterworths pub. pp 457-458)

Results: Microglia & Oligodendrocytes --- Black.

Method

1) Bring formalin fixed sections to water.
2) Transfer to silver solution for 15-20 seconds.
3) Transfer to 15% formalin; gently agitate section until it is coffee-brown in colour.
4) Rinse in ddH2O (3x @ 10 min).
5) Tone in 0.2% gold chloride for 10-15 minutes.
6) Rinse in ddH2O.
7) Fix in 5% sodium thiosulphate for 5 minutes.
8) wash in Tap-water.
9) Float onto clean slides, Dehydrate, Clear, & Mount.

Solutions

Silver Solution

To 2-3 ml of strong ammonia (.880) in a flask, add 10% silver nitrate, drop by drop, until about 18 ml have been added and the solution is still slightly opalescent. This solution gives the best results if it is prepared in a silvered flask (with a deposit of silver on the inside) which should be kept for the purpose.

NOTE:

Toning (steps 6&7) is optional and sections may be mounted untoned.

AKR2B Mitogenic Assay - 24 Well Version

24-well Plate Setup:

Take a flask of cells, aspirate off medium, rinse with solution A or EBSS, and add 1.5 ml - 2 ml of 0.05% trypsin in Solution A. Allow to sit until attachments are weakened (flask becomes opaque < 2min.), remove trypsin, tighten lid and mechanically dislodge cells (ie. whack flask). Add 10 mls of fresh McCoy's 5A + 5%FBS and put cell suspension on ice. Pippette up and down in a 10 ml pippette in order to break up clumps. Count cells with Hemacytometer. The number of cells counted within one 16 sq. field on the Hemacytometer is that number of cells X 10 4 per ml of your cell suspension. For accuracy count at least 4 fields of 16 squares for a total of more than 50 cells. Cells should be added to a volume of McCoy's 5A + FBS and brought up to the final volume to be plated (ie. 10 ml of 15 X 10 4 added to 140 mls medium to get total of 150 ml of 1 X 104 per ml of cell suspension to go into 6 , 24 well plates.) . Cells should be well stirred between each plating.

Cells should reach confluency in 5 days. DO NOT REFEED/CHANGE THE MEDIUM DURING THIS TIME.

- After 5 days, the cells should be switched to a serum free 402 plus pen/strep.

Sample Addition:

-2 days later, Cells should be re-fed with fresh 402 + pen/strep & Insulin must be added to all wells to get proper stimulation. The plates should be allowed to equilibrate pH in the incubator, and then sample added.

^{3}H Pulse:(1Hr)

After 22 hours of stimulation, add 1 uCi /well of 3H-thymidine. This is prepared by diluting high specific activity 3H-thymidine (NET-027Z, usually 1mCi/ml) 1:10 in Solution A = 100 uCi /ml (an addition of 10 ul/well).

Fixation:

At 23 hrs, the hot medium is aspirated, replaced with 1 - 1.5 mls of COLD 10% TCA and allowed to stand 10 min. Aspirate and wash two more times with 10% TCA and then aspirate dry. At this point , the assay can be interrupted if needed.

Solubization, Collection & Counting:

Add 400 ul of a 0.2 M NaOH containing 50 ug/ml Herring sperm DNA. Allow to stand 30 min. (with occasional, gentle rocking to dissolve DNA). Take 100 ul aliquot of sample and add to 20 ml scintillation vial. Add 8 mls of Ecoscint A (or H - just be consistent. H is more efficient than A) and 50 ul 0.4 M HCl and count on a 3H channel (1 - 3 min., 3 reps).

Stocks & Supplies:

EGF : Frozen stock : 20 ug/ml in Solution A (50 ul aliquots)
Dilute 1 : 20 in sterile Sol'n A +1 mg/ml BSA = 1 ug/ml (50µl:.95ml)
- Add 10 ul/well (final conc. of 10 ng/ml)

HEPARIN: Frozen stock: 1 mg/ml in Solution A (400 ul aliquots)
Dilute 1 : 5 in sterile Sol'n A = 200 ug/ml (400µl:1.6ml).
- Add 10 ul/well (final conc. of 2 ug/ml).

INSULIN: Frozen stock : 5 mg/ml in 10mM HCL (100 ul aliquots)
Dilute 1 : 100 in sterile Sol'n A = 50 ug/ml (20µl:1.98ml)
Add 10 ul/well (final conc. of 2 ug/ ml)

Needed for 6 Plates
- 402 + Insulin = 150ml + 1.5ml
- 402/I + Heparin = 75ml + 750µl

AKR2B 48 Well Assay

48-well Plate Setup:

- Aspirate off medium,
- Rinse with solution A or EBSS,
- Add 1.5 ml - 2 ml of 0.05% trypsin in Solution A.
- Allow to sit until attachments are weakened (flask becomes opaque < 2min.),
- Remove trypsin,
- Tighten lid and mechanically dislodge cells (ie. whack flask).
- Add 10 mls of fresh McCoy's 5A + 5%FBS and put cell suspension on ice.
- Pippette up and down in a 10 ml pippette in order to break up clumps.
- Count cells with Hemacytometer.

(The number of cells counted within one 16 sq. field on the Hemacytometer is that number of cells X 10 4 per ml of your cell suspension. For accuracy count at least 4 fields of 16 squares for a total of more than 50 cells).

- Cells should be added to a volume of McCoy's 5A + FBS and brought up to the final volume to be plated (ie. 10 ml of 15 X 10 4 added to 140 mls medium to get total of 150 ml of 1 X 104 per ml of cell suspension to go into 6 , 48 well plates.). Pippette 0.5ml/well, cells should be well stirred between each pippette during plating.
- Cells should reach confluency in 5 days. DO NOT REFEED/CHANGE THE MEDIUM DURING THIS TIME.
- After 5 days, the cells should be switched to a serum free 202 plus pen/strep.

Sample Addition:

24 Hours later, Cells should be re-fed with fresh 202 + pen/strep & Insulin must be added to all wells to get proper stimulation. The plates should be allowed to equilibrate pH in the incubator, and then sample added.

^{3}H Pulse:(1Hr)

After 22 hours of stimulation, add 0.5 µCi/well of 3H-thymidine. This is prepared by diluting high specific activity 3H-thymidine (NET-027Z, usually 1mCi/ml) 1:20 in Solution A = 50 µCi /ml (an addition of 10 µl/well).

Fixation:

- After 1 hour of 3H, the hot medium is aspirated, replaced with 0.5 - 0.75 mls of COLD 10% TCA and allowed to stand 10 min.
- Aspirate and wash two more times with 10% TCA and then aspirate dry.
- At this point , the assay can be interrupted if needed.

Solubization, Collection & Counting:

- Add 200 ul of a 0.2 M NaOH containing 50 ug/ml Herring sperm DNA. Allow to stand 30 min. (with occasional, gentle rocking to dissolve DNA).
- Take 100 ul aliquot of sample and add to 7 ml scintillation vial.
- Add 4 mls of Ecoscint H Acidified solution (300µl Glacial Acetic Acid / 1L of Ecoscint H).
- After all samples sealed and racked, invert racks 5 times in order to mix solution.
- Count on a 3H channel (1 - 3 min., 3 reps).

Stocks, Supplies, & Notes:

EGF : Frozen stock : 20 ug/ml in Solution A (50 ul aliquots)
Dilute 1 : 20 in sterile Sol'n A +1 mg/ml BSA = 1 ug/ml (50µl:.95ml)
- Add 10 ul/well (final conc. of 10 ng/ml)

HEPARIN: Frozen stock: 1 mg/ml in Solution A (400 ul aliquots)
Dilute 1 : 5 in sterile Sol'n A = 200 ug/ml (400µl:1.6ml).
- Add 10 ul/well (final conc. of 2 ug/ml).

INSULIN: Frozen stock : 5 mg/ml in 10mM HCL (100 ul aliquots)
Dilute 1 : 100 in sterile Sol'n A = 50 ug/ml (20µl:1.98ml)
Add 10 ul/well (final conc. of 2 ug/ ml)

aFGF: Frozen Stock : 20ng/ml (100µl aliquots)
Use 1ng/well, 50µl, to provide standardized maximum in (+) Heparin conditions.

bFGF: Frozen Stock : 20ng/ml (100µl aliquots)
Use 1ng/well, 50µl, to provide standardized maximum in (-) Heparin conditions.

202 Mixture from Incomplete Stock

974ml 202 incomplete
10ml Stock J
5ml Stock K1
1ml Stock K2
10ml Stock L

PH to 7.7 with 4.0M NaOH

# Plates	Wells	Minnium Cells	Round Volume	202		I		202/I	Hep
1	48	24 E4	25	25	0.25	13	0.13		
2	96	48 E4	50	50	0.5	26	0.26		
3	144	72 E4	75	75	0.75	39	0.39		
4	192	96 E4	100	100	1.0	52	0.52		
5	240	120 E4	125	125	1.25	65	0.65		
6	288	144 E4	150	150	1.5	78	0.78		
7	336	168 E4	175	175	1.75	91	0.91		
8	384	192 E4	200	200	2.0	104	1.04		
9	432	216 E4	225	225	2.25	117	1.17		
10	480	240 E4	250	250	2.50	130	1.30		

Planning Charts

Time Line Chart

(202 Time Min. is 24Hrs Can go 48Hrs Without Harm)

```
   M    T    W    Th   F    S    S    M    T    W    Th   F    S
X  P=================> 202    St   Pulse
-       P=================> 202    St    Pulse
+            P=================> 202     St  Pulse
+                 P=================> 202   St   Pulse
+                      P=================> 202    St   Pulse
```

P = Plate Cells, 202 = Serum Free, St = Stimulation, Pulse = ^{3}H Pulsing/Fixation/Collection.

Well Planning Chart

(3x Blanks & 3x +Control on each Plate).

Plates	Open Wells	3xSamples	2xSamples
1	42	14	21
2	84	28	42
3	126	42	63
4	168	56	84
5	210	70	105
6	252	84	147
8	336	112	168
9	378	126	189
10	420	140	210

Original Refs.

Shipley GD (1986); A serum-free [3H] thymidine incorporation assay for the detection of transforming growth factors; J Tiss Cult Methods 10:117-123.

Care & Maintenance of The AKR2B Cell

Startup of AKR2B Cells :

Frozen vials of AKR2B cells are stored in the liquid Nitrogen container. Cells should be thawed by air in the hood, resuspended in 25 ml McCoy's 5A+ 5% FBS and placed in a 150 cm flask.Medium should be changed within 48 Hrs.

Feeding:

Cells should be given new medium every 2-3 days. AKR2B cells are maintained in Gibco McCoy's 5A* + 5% FBS (*430-1500EB).

Doubling time is every 18-24 hrs. Dividing cells that are used as source material for setting up plates for assay should never be grown to confluency. This encourages transformants which are no longer contact inhibited. If a plate grows to confluency it can still be used to set up plates for immediate assay but should never be used to carry on the line for further assays.

Spliting:

Take a flask of cells, aspirate off medium, rinse with solution A or EBSS, and add 1.5ml - 2 ml of 0.05% trypsin in Solution A. Allow to sit until attachments are weakened (flask becomes opaque < 2min.), remove trypsin, tighten lid and mechanically dislodge cells (ie. whack flask). Add 10 mls of fresh McCoy's 5A + 5%FBS .

Plate cells (Fri: 1:6, 1:10; Mon. 1:3, 1:6) in 150 cm2 flasks in 25 - 30 mls McCoy's 5A + 5% FBS .

Earle's Balanced Salt Solution

Date:

A. Put 10 liters dd H_2O into Nalgene Carboy(1)

(Rinse Carboy and Grad. cylinder several times in dd H_2O)

add entire contents of EBSS powder (from GIBCO #450-1100EG w/o bicarbonate: makes 10 liters)

Add 35.75 g HEPES (i.e. 15mM) **actual wt.:**_____

Add 6.43 g NaCl (i.e. 11mM)(2) **actual wt.:**_____

Stir with large stir bar until mix is dissolved.

Rinse weighing containers into solution.

Measure pH. Optimal pH = 7.3

If not 7.3, then adjust with con. HCl, or NaOH. **actual pH:** _____

amount HCl/NaOH added: _____

B. Filter the solution through Millipore Millipak 20 to sterilize

Filter solution into 500 ml bottles; discard the first 500 ml bottle.

1) Run ddH_20 for a few minutes while measuring the resistivity. The optimal purity is with a resistivity of 20 megaohms.

2) The NaCl is added to adjust the molarity to .026, which is the concentration of bicarbonate added in the original GIBCO protocol.

Microglia Isolation - Shaking Method

Starting with confluent mixed glial cultures from P0 rat cortex.
Shake for 1.5 hr at 260rpm in a temp controled incubator.

Pool the shook off cells and media,

Spin down cells 1500 rpm for 10 min,

Resuspend in half the starting volume of McCoy's 5A/112 (5A),

Plate at 10 ml / T-150 flask,

Let set in incubator for 1-3 hrs,

While observing the cells, shake/whack the flask gently to remove loosely adhearing cells,

Rinse several times with the existing media,

Draw off and discard the media,

Rinse once with EBSS,

Add 2ml of 0.2% trypsin in solution A,

Allow to weaken adhesion for 1-5 mins,

Whack flask to break loose microglia,

Collect cells with 8mls of 5A + 10 % FCS,

Count cells and go on to experimental plating.

(Typical medium density for MG cells is $20x10^4$ cells per ml.)

Monoclonal Growth Protocol

Standard Medium:
L-15CO2 +10 % FCS + 1:1:2 (1ml/25ml L-15)

Starting up Hybridomas
From original fusion
From frozen stock

Prepare 10mls of cold medium in 15ml c-fuge tube for each line to be thawed.

Partially thaw tube in bath or hand, complete thawing with 1 ml of media.
Quickly transfer to lc-fuge tube, spin 4min @1000rpm.
Resuspend in 1ml media.
Distribute to 3 wells of 24 well plate (70%, 20%, 10%).
Bring wells up to 0.5 ml volume.

Cloning by Limiting Dilution

Set up a 96 well round bottom plate for each line, using L-15CO2 +15 % FCS + 1:1:2 (1ml/25ml L-15) + mø conditioned medium (1:20).
The plate will be arranged so that the first column will contain 25 cells/well (guesstimate or count with hemocytometer) the columns to the right will be 1:1 dilutions of the previous adjacent column.
Using a multipipetter place 100µl in all columns except the first,
Prepare a cell stock that will give you 25cells/200µl (=125c/ml)
Place 200µl of cell stock in the first column,
Using a prewetted100µl multipipetter gently pipet up/down three times in the first column then move 100µl to the next column, repeat this across the plate moving from the newly seeded column to the adjacent empty column.
Feed these plates every three days by carefully removing half of the medium being careful not to suck up any cells.
The column furthest right on the plate which grows up can then be assured of being clonal.
Test the sups for activity and expand selected wells, freezing down as soon as possible.

Expansion

When cells in 24 well plate begin to turn medium orange/yellow, double medium in well to 1.0 ml. When they begin to acidify 1.0 ml transfer cells into $25cm^2$ flask in a total volume of 5 mls (lay flask down). When they begin to acidify this medium double volume and stand up. Keep doubling as they grow and acidify. Once a day agitate medium by pipetting up/down with a 2ml pipet so that medium gets well mixed and oxygenated.

Preparation of Complete 402 from Frozen Incomplete Medium

1. Thaw stock 402 medium - (fridge overnight or warm in waterbath)

2. Measure volume in graduated cylinder. Pour into a clean beaker + stir bar.
   VOLUME = _____ml

3. Check pH and adjust to 7.4 if neccessary, using no more concentrated than 1M NaOH

4. Divide this volume by 49 = _____ml
   This is the volume of STOCK 7 to be added.
   402 stock 7 = CaCl2 (8 X 10 -2M)

5. Add stock 7 while stirring

6. Add volume of 402 + stock 7 = _____ml

7. Divide this number by 1000 = volume in liters = _____liter

8. Multiply the number (in 7) by 1.176 = _____.
   This is the amount of NaHCO3 in grams that must be added to the 402 as a buffer. (ex: one liter of complete requires 1.176g).

9. Add the bicarbonate (8) while stirring. After it is dissolved, sterile filter with a .22 micron and store at 4 degrees C.

Dot Blot Protocol

Objective:

Test of newly made antibody probes. Achived by spotting down antigen & negative control material on nitrocelloulose, then staining with the new antibody's.

Protocol

- On nitrocelloulose strips in staining lanes, spot down 2-4 ul of positive antigen(s) and negative control (BSA 1mg/ml). One lane per antibody to be tested.

- Air dry for 30 min.

- Block overnight with blot block @ room temp.

- Primary antibody, incubate in 1:1000 AB:Blotblock for 2 hr @ RT.

- Wash 3X 5 min in TBS/Tween

- Secondary antibody, incubate in Biotinylated anti producer 1:250 in blotblock

- Wash 3X 5 min in TBS/Tween

- Thirtiary antibody, Streptadvidin alkaline phosphatase for 1 hr @ RT
  Mixed 1:5000 in blotblock (2ul:10 ml)

- Wash 3X 5 min in TBS/Tween

- Wash 1X5 min in AP buffer

-React, 5 min or till well developed
  33ul NBT
  16.5ul BCIP in 5ml AP Buffer Mix within 30 min of use.

- Quench with dd H2O

Dot Blot Planning Sheet

Date:

Project :

Spot # 1 2 3 4 5

Antigen:

Plate # ___ Results Plate # ___
Results

Lane	Antibody	1	2	3	4	5
A						
B						
C						
D						
E						
F						
G						
H						

Results: +++ Strong Staining,
++ Moderate Staining,
+ Faint Staining,
0 No Staining

Check List

1 Hr. ±	___	40 Min	- Spotting
===	___	Overnight	- Blocking in Blotblock
===	___	2 Hrs	- Primary
|	___	15 Min	- Wash 3 @ 5 min TBS/Tween
|	___	1 Hr	- Secondary
|	___	15 Min	- Wash 3 @ 5 min TBS/Tween
5 Hr.	___	1 Hr	- 3°AB : Streptavidin
|	___	15 Min	- Wash 3 @ 5 min TBS/Tween
|	___	5 Min	- Wash 1 @ 5 min AP Buffer
|	___	5 Min	- Reaction; NBT & BCIP in AP Buffer
===	___	1 Min	- Quench in dd H2O

Speedvac Protocol

- Check collection chamber, (Clean if needed)
- Start cooling coils
- Seal main chamber and start pump
- Place samples in c-fuge and spin
(W/WO heating)
- Create vacume in C-fuge chamber

Shut down:

- Vent C-fuge
- Vent main chamber
- Stop pump
- Stop C-fuge
- Stop cooling

Tissue Extraction Protocol; Balanced for HBGF Westerns

- Weigh each sample.
- Homogenize in ice cold 20mM Tris, 600mM NaCl pH 8.0 (10 ml/g Tissue).
- Spin for 15 min @ 20,000 x G.
- Collect supernatant, Discard pellet.
- Dilute supernatants 1:1 in 20mM Tris pH 8.0
- Gravity filter through Whatman #1 filter paper.
- Protein Analysis to allow balancing of column loading.
  (Keep sample sups on ice in cold room)
- Apply filtrate onto heparin-affigel column *
  - Run through twice
  - Wash column with 2 volume 20mM Tris, pH 8.0
  - Wash column with 1/2 volume 0.5x Treatment Buffer, blot away flow thru.
  - Cap column, 60°C water bath for at least 5 min.
  - Elute column with 2 volume HOT 0.5x Treatment buffer
- Concentrate by Speedvac

* **Column prep:**

Load volume, allow gravity to pack column
Strip with 2 volume 20mM Tris, 2M NaCl pH 8.0
Wash with 3 volume 20mM Tris, pH 8.0

FGF/Western Tissue Extraction Work Sheet **Date:**

Extraction Description:

Elute Sample Volume Description	Mass (mg)	C-Fuge #(s)	Diluted Vol Sup (ml)	Protein Conc./Total (mg/ml) / (mg)	Bal. Load (mg)	Column Load (ml)	(ml)

Tissue Extraction Protocol; General for HBGFs

- Homogenize in ice cold 20mM Tris, 600mM NaCl pH 8.0 (10 ml/g Tissue).
- Spin for 15 min @ 20,000 x G.
- Collect supernatant, Discard pellet.
- Dilute supernatants 1:1 in 20mM Tris pH 8.0
- Gravity filter through Whatman #1 filter paper.
- Save aliquot of filtrate for AKR2B / Protein assays
- Apply filtrate onto 1ml heparin-affigel column *
    - Save aliquot of flow thru for AKR2B / Protein assays
    - Wash column with 5ml 20mM Tris, 300mM NaCl pH 8.0
    - Wash column with 2ml 20mM Tris, 600mM NaCl pH 8.0
    - Elute column with 3ml 20mM Tris, 2M NaCl pH 8.0
    - Collect elute, discard washes
    - Save aliquot of elute for AKR2B / Protein assays
- Dilute eluate 1:4 in 20mM Tris pH 8.0
- Apply diluted eluate onto 0.3ml heparin-affigel column **
    - Run through twice
    - Wash column with 1ml 20mM Tris, pH 8.0
    - Wash column with 150µl 0.5x Treatment Buffer (1/2 column volume), blot away flow thru.
    - Cap column, 60°C water bath for at least 5 min.
    - Elute column with 600 µl 0.5x Treatment buffer
- Concentrate by Speedvac

* **1.0ml Column prep:**

Load 1.5ml allow gravity to pack column
Wash with 2ml 20mM Tris, 2M NaCl pH 8.0
Wash with 3ml 20mM Tris, pH 8.0

** **0.3ml Column prep:**

Load 0.45ml allow gravity to pack column
Wash with 0.6ml 20mM Tris, 2M NaCl pH 8.0
Wash with 0.9ml 20mM Tris, pH 8.0

Tissue Extraction Protocol; Rocker Method With Salt Elution Step

- Homogenize in ice cold
  20mM Tris,600mM NaCl pH 8.0 (10 ml/g Tissue).
- In approiate sized capable column, (10 or 20 ml) Prepare Bed material.(500µl Hep-Affigel to 1.0g Starting tissue)
  Wash bed for 10 min with 2ml 20mM Tris, 2M NaCl pH 8.0
  Wash bed for 10 min with 3ml 20mM Tris, pH 8.0
- Spin homogenant for 15 min @ 20,000 x G.
- Collect supernatant, Discard pellet.
- Dilute supernatants 1:1 in 20mM Tris pH 8.0
- Save aliquot for AKR2B / Protein assays
- Incubate with Hep-Affigel for 90 min.
- Collect Flow Through
- Save aliquot of Flow Through for AKR2B / Protein assays

Salt Elution Step

- Wash for 10 min with 2ml 20mM Tris, 300mM NaCl, pH 8.0
- Drain off Supernatants
- Wash for 10 min with 2ml 20mM Tris, 600mM NaCl, pH 8.0
- Drain off Supernatants
- Elute for 20 min with 3ml 20mM Tris, 2M NaCl, pH 8.0
- Collect Elute
- Dilute eluate 1:4 in 20mM Tris pH 8.0
  (For several samples dilute to starting volume, MUST be at least 1:4).
- Save aliquot of diluted Eluate for AKR2B / Protein assays
- Reincubate with Hep-Affigel for 90 min.
- Drain off Supernatants

- Wash for 10 min with 2ml 20mM Tris, pH 8.0
- Drain off wash
- Incubate to elute with 1.5ml of 1x 1% SDS Sample buffer
  60˚C water bath for at least 5 min, RT for 5 min with shaking.
- Concentrate by Speedvac
  (Concentrate a sample of 1x 1% SDS Sample Buffer for gel running)
- Resuspend all samples to 10% speedvac starting volume.

Total Protein Analysis Protocol

- In a 96 well plate arrange:
  - Zeros
  - Standards, BSA 0.1mg/ml from 1mg/ml stock
    (0, 5, 7.5, 10, 15, 20, 30, 40 µl)
  - Sample range (diluted out to match standard range)
    i.e. 5, 10, 20 µl of 1:20 for assumed 0.5mg/ml
  - Equlibrate volumes in all wells

- Add 200µl dye solution (4ml dye : 16ml H_2O)
- Read on microplate reader @ 570nm
- Dilution factor for microplate reader:
  Dilution of stock solution BEFORE Added to wells.
  i.e. 1:10 = 10

Protein Analysis Date:

Project:

Description / Objective:

Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Concentrations / Dilutions / Calculations

Western Blot Staining Protocol

Objective:

Test of protein gel electroblots with specific antibody probes. Achived by incubating the nitrocelloulose blot as specified.

Protocol

- Block overnight with blot block @ room temp.

- Primary antibody,
  Incubate in 1:1000 AB:Blotblock for 2 hr @ RT.

- Wash 3X 5 min in TBS/Tween

- Secondary antibody,
  Incubate in Biotinylated anti producer 1:250 in blotblock

- Wash 3X 5 min in TBS/Tween

- Third antibody,
  Incubate in Streptadvidin alkaline phosphatase for 1 hr @ RT
  Mixed 1:5000 in blotblock (2ul:10 ml)

- Wash 3X 5 min in TBS/Tween

- Wash 1X5 min in AP buffer

-React, 5 min or till well developed
  60µl NBT
  30µl BCIP in 30ml AP Buffer.
  Mix within 30 min of use.

- Quench with dd H2O

Western Blot Staining Planning Sheet **Date:**

Blot Date:

Description:

Objective:

Antibodies / Blots:

Check List

30 Min ±	___	Overnight	**- Block in Blotblock**
===	___	2 Hrs	**- Primary**
|	___	15 Min	**- Wash 3 @ 5 min TBS/Tween**
|	___	1 Hr	**- Secondary**
|	___	15 Min	**- Wash 3 @ 5 min TBS/Tween**
5 Hr.	___	1 Hr	**- 3°AB : Streptavidin**
|	___	15 Min	**- Wash 3 @ 5 min TBS/Tween**
|	___	5 Min	**- Wash 1 @ 5 min AP Buffer**
|	___	5 Min	**- Reaction; NBT & BCIP in AP Buffer**
===	___	1 Min	**- Quench in dd H2O**

Results, Analysis, & Next Steps:

SOLUTION RECIPES

Amido Black Staining Solution (Naphthol Blue Black)

0.1% (w/v) Amido Black	0.1g
10 % Methanol	10 ml
2 % Acetic Acid	20 ml (10% Stock)
Up to 100 ml with ddH2O	

Amido Black Staining Solution (Naphthol Blue Black)

0.1% (w/v) Amido Black	0.1g
45 % Methanol	45 ml
10 % Glacial Acetic Acid	10 ml
Up to 100 ml with ddH2O	

AP Buffer

100 mM Tris Base	12.1 g
100 mM NaCl	5.8 g
5 mM MgCl2 * 6H2O	1.0g
Fill to 1L with dd H2O	

AP Buffer 10X

1M Tris base	121.1g
1M NaCl	58g
50mM MgCl2*6H2O	10g
Fill to 1L with dd H2O	

Blocking Solution With Azide

50 % 0.2 M Phosphate Buffer
10 % Horse Serum
0.5 % Triton X-100 (10% Stock)
0.2 % Na Azide (10% Stock)
Fill to Volume dd H2O

Blocking Solution Without Azide

50 % 0.2 M Phosphate Buffer
10 % Horse Serum
0.5 % Triton X-100 (10% Stock)
Fill To Volume dd H2O

Blot Block

10 % Horse Serum
0.1 % Tween
0.1 % Azide
Fill to Volume with 1xTBS

Blot Transfer Buffer

39 mM Glycine	2.930g
48 mM Tris Base	5.81g
0.0375% w/v SDS	0.375g
20% Methanol	200ml

Fill to 1.0 liter with ddH2O

Blot Transfer Buffer 10x

390 mM Glycine	29.3g
480 mM Tris Base	58.1g
0.375% SDS	3.75 ml 10% Stock

Fill to 1 Liter

Label with the reminder that 1X has 20% Methanol

Coomassie Destaining Solution

20 % Ethanol
10 % Acetic Acid
Fill to volume ddH2O

Coomassie Staining Solution

0.25 % Coomassie R250
50 % Methanol
10 % Acetic Acid
Fill to volume ddH2O

Indian Ink Stain

497 ml PBS
3 ml Tween 20
500µl Indian Ink (Pelican Fount Recommended)

PBS 10x

200 ml 0.2 M Na2HPO4
50 ml 0.2 M NaH2PO4
45 g NaCl
Fill to 1 L dd H2O

PBS 1x

6.52g Na2HPO4.12H2O (91ml .2M)
0.117g NaH2PO4.2H2O (15ml .2M)
17.5g NaCl
fill to 1 liter w ddH20

Peroxidase Anti Peroxidase

1 % PAP From same species as primary AB.
5 % Serum form same species as tissue.
Fill to volume

Bolcking solution without Azide Peroxidase Block (5%)
30 % Ethanol
5 % Peroxide (30% Stock)
Fill to Volume with 0.1 M Tris pH 7.3

Phosphate Buffer 0.2 M pH 7.2
22.72 g Na_2HPO_4 (mw 142 g/mol) di Basic
5.52 g NaH_2PO_4 (mw 138 g/mol) monobasic
Fill to 1 L dd H_2O,
Adjust to pH or 4 : 1 dibasic / monobasic mix

Resolving (Lower) Buffer
36.3g Tris Base
fill to 200 ml
Adjust to pH 8.8 w/ HCl

Running Buffer
12.0 g Tris Base
57.6 g Glycine
80.0ml 10% SDS
Fill to 4l w/ ddH_2O

Sample buffer with BME, 2x
4 ml glycerol
1 ml Stock BME (14.2M)
0.01% Bromphenol blue
Bring upto 10.0 ml with stacking buffer

Sample Buffer with BME, 5x

0.312 M Tris HCl	4.92 g
10% SDS	10.0 g
50% Glycerol	50 ml
25 % BME	25 ml (14.2 M)
.25mg/ml Bromophenol Blue	25 mg

Fill to 100 ml ddH_2O

SSC 10x
1.5 M NaCl
0.15 M Sodium Citrate
Adjust for pH

Subbing solution
5.00 g Gelatin (JT Baker)
dissolve in 500 ml heated dd H_2O
When cooled add 0.5g Chromium potassium Sulfate (Chrom Alum)

TBS 10x

Tris HCl	68.6 g
Tris Base	8.0 g
NaCl	88.0 g

Fill to 1L dd H_2O

TBS / Tween Wash

0.1% Tween

Fill to volume with 1x TBS

Thionin Counter Stain (0.1%)

0.5 g Thionin

10 drops Glacial acetic acid

Fill to 500 ml dd H_2O

Tris 0.1 M pH 7.3

101 ml 1.0 M Tris HCL

10.3 ml 1.0 M Tris Base

1001.33 ml dd H_2O

Adjust to pH

Tris 1.0 M pH 7.7

100 ml 1.0 M Tris HCl

26.0 ml 1.0 M Tris Base

Adjust for pH

Predictors of Nursing Care Quality Outcomes Related to Pain Relief

By

Daniel S. Laferriere

A Master's Research Project

Presented to
Oregon Health Sciences University
School of Nursing
in partial fulfillment of
the requirements for the degree of
Master of Science

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Abstract

TITLE: Predictors of Nursing Care Quality Outcomes Related to Pain Relief

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APPROVED: Christine A. Tanner, PhD, RN, FAAN

The relationships between selected structural characteristics, processes of care and outcomes of care-related pain relief in hospitalized patients were explored. Data were gathered from a sample of 40, of 65 eligible, nurses and a sample of 109 of the 328 patients they cared for during the study period. Methods used included written surveys, interviews, review of the patient records, and queries of two hospital databases to describe three interconnected dimensions of care; structures of care, nursing care processes and outcomes of care. *Structural variables* included: nurse characteristics of knowledge and attitudes related to pain management, and the nurses' typical pain management approach; patient characteristics of functional status, engagement in care, and psychosocial status, measured on admission to the hospital. *Processes of care* included assessment and treatment of pain. Data were gathered on use of both pharmacological and non-pharmacological pain treatment(s). *Outcomes of care* included adequacy of pain relief reported by the patient during hospitalization and pain relief experiences reported by the patient after discharge. Several statistically significant correlations were found: The structure variable of nurses knowledge of pain management was positively correlated with the outcome of patients' rating treatment choices as acceptable. The process variable of the difference between a patient's worst pain and best pain relief correlated positively with a positive response to the outcome of patients' rating treatment choices as acceptable. The larger difference between worst and best pain was

and best pain was created by a statistically significant difference in best pain relief scores. Additionally, patients having had a surgical procedure were more likely to have pain documented using the 0 to 10 pain scale and to have medication efficacy evaluation documented by the nurse. The patients' pain experiences in this study are consistent with other reports including best pain relief, worst pain, and pain at interview except for a lower number of patients receiving maximum amount of pain medication allowed. Implications for clinical practice include; the need to improve use of the 0-10 scale in documentation, documentation of medication efficacy, use of the 0-10 pain intensity scale to rate several dimensions of pain, and annual education in pain management.

Table of Contents

List of Tables

List of Figures

Predictors of Nursing Care Quality Outcomes Related to Pain Relief

Background and Significance

Quality of care has long been a concern in health care. Donabedian's (Donabedian, 1980) framework of structure, process, and outcome was a major influence in this work. In the 1970s much of the work in nursing quality assurance was devoted to development and use of process monitors. Examples include Phaneuf's Nursing Audit, Wandelt's Quality Patient Care Scale, Goldstone's Monitor (Sparrow & Robinson, 1992). More recently quality assurance and assessment have emphasized outcomes, (Donabedian, 1992; Rubenstein, Chang, Keeler, & Kahn, 1992) following the lead of accrediting agencies such as Joint Commission on Accreditation of Health Organizations (JCAHO), and National Committee for Quality Assurance (NCQA) through the Health Plan Employer Data and Information (HEDIS).

The cost of health care has come under increasing scrutiny in recent years as it consumes more of the gross national product. Payers, both public and private, have demanded and created cost control systems as evidenced by such strategies as diagnosis related group payment in the early 80's and the rise of managed care in the last decade. This has lead to many changes in health care delivery. Decreasing length of stay (LOS) for patients and restructuring of nursing services to use less costly health care workers are two strategies being used by hospitals to cope with the pressures of cost control.

In an effort to decrease costs, hospitals have been changing nursing skill mix to include more Certified Nursing Assistants (CNA) and Licensed Practice Nurses (LPN). Decreases in staffing levels are also occurring. As a result of the decreasing LOS and moving more care to outpatient settings only the sickest patients remain in acute care

settings. Nurses are feeling the increased workload and are expressing concern and dissatisfaction with the quality of care they are now able to give. The decreasing LOS and changes in staffing are believed to be affecting the nurse's ability to care for patients(Shindul-Rothschild & Long-Middleton, 1996). The Institute of Medicine (IOM) summary report on nursing staff (Wunderlich, Sloan, & Davis, 1996, p105) completed an in-depth investigation on nurse staff and concluded:

> "...the committee was unable to find evidence of a decline in the quality of hospital care because of any changes in staffing. Lacking reliable measures and data, no one is in a position to draw valid conclusions. The amount of testimony provided, however, and the depth of concern cited, was sufficient to lead the committee to believe that this is an area that requires on-going monitoring and research in order to ensure that the responsibility for providing safe, effective, quality and cost effective care is fulfilled within a health care system."

Recent research has demonstrated a relationship between nursing hours per day and several patient outcomes that should be sensitive to professional nursing care. The findings included differences, attributable to hours of professional nursing care, in the rate of pneumonia, urinary tract infection, venous thrombosis, pulmonary congestion and other pulmonary related problems following major surgery. (Kovner & Gergen, 1998)

The American Nurses Association (ANA) has responded to this concern by creating a set of quality indicators (American Nurses Association, 1995). The structure-process-outcome framework is used to organize recommended care indicators. Pain

management is included as a Process of Care indictor and patient and family satisfaction with nursing care as an outcome indicator. Survey research by the Picker Institute has shown that pain management is an important dimension of patient satisfaction. (Edgman-Levitan, 1998)

Purpose of the Study

The long-term goal of this project is to examine the relationship among professional nurse staffing levels and other structural variables, nursing care processes, and outcomes of care for hospitalized patients specifically focusing on pain management. Figure 1 displays the conceptual model for the larger project. The conceptual connections between dimensions are represented by the over-lapping ovals. Care related structures in this model include organizational variables, nurse characteristics, and patient characteristics. Measures of nursing care are included in the process dimension. Finally, outcomes of care include the patient reported experience related to pain relief. Individual measures related to each major construct are listed in figure 1.

The specific aims of this study are:

1. To examine the relationships among nursing characteristics including knowledge and pain management practice patterns, process variables of nursing care , including pain assessment and management, and outcomes of patient reported experiences with pain relief.
2. To examine relationships among selected patient characteristics on admission, processes of care and outcomes of pain relief.

See figure 2 for a graphical representation of these dimensions and variables.

Review of the literature

As this study seeks to examine relationships among pain related structures, processes, and outcomes of care, the literature related to each concept will be explored.

The role of nursing in pain management

The expectation that nurses will provide comfort and relief from pain is a long held societal belief (Davis, 1998). Assessment of the quality of pain management is accepted by the American Nurses Association as one appropriate measure of nursing care quality (American Nurses Association, 1995, p. 63). Cleary et al., (1991) using focus group methodology identified physical comfort as one of seven dimensions important to patients. Timely response to requests for medication, hospital staff doing everything they could to help control pain, and receiving enough medication are part of this care dimension.

The prevalence of pain in the elderly is two fold higher than those under 60. Among the institutionalized elderly, the prevalence of pain including acute and chronic may be as high as eighty percent. Many of the elderly undergo medical procedures resulting in pain, including treatment for orthopedic conditions and cancer. Medical conditions such as herpes zoster and peripheral vascular disease contribute to their pain. Chronic pain from arthritis is prevalent in this age group (Acute Pain Management Guideline Panel, 1992). In summary, society, the professional nursing organization, research with patients, and medical science all support the importance of pain management as a nursing care quality indicator.

Factors influencing pain management

The relationship of a variety of patient and nurse characteristics to pain and its management have been studied. A review of the literature shows that nurses make judgements about patients' pain which may not be accurate (Allcock, 1996). Some evidence exists showing that the practice of nursing desensitizes nurses to patient's pain, although a more liberal attitude and more current knowledge has been found in nurses regularly providing care to cancer patients (O'Brien, Dalton, Konsler, & Carlson 1996). Personal experience with pain or having a family relationship with the person in pain are factors which increase nurses' sensitivity to patients' pain (McCaffery & Ferrell, 1997). Practicing from a current knowledge base is expected. Pierce has reported on numerous nursing interventions, such as improving nurses' knowledge, and could not find linkages to improved outcomes (Pierce, 1997). An example is the work of Barnason, Merboth, Pozehl, & Tietjen, (1998) which reported improved nursing knowledge of pain management following the development of a standard of care and pain management educational program. The lack of baseline patient data in this report prevents the measurement of care improvement.

Staff knowledge and/or values related to pain management have been studied by several researchers. Researchers in North Carolina studied knowledge and attitudes of oncology nurses in that state (O'Brien et al., 1996). Using a modified form of the Wisconsin Cancer Pain Initiative survey, among their findings were that nurses regularly working with cancer patients had more knowledge about pain management and more liberal attitudes about medication use. Barriers to effective pain management as perceived by the nurses were patients' reluctance to report pain and inadequate

nursing assessment of patients' pain. McCaffery and Ferrell (1997) explored the influence of personal vs. professional role in pain assessment and treatment with opioids. A vignette describing a patient and the same vignette with the patient identified as a sibling was used to assess personal vs. professional role. The survey findings suggest a family relationship increases the nurse's sensitivity to the patient's pain, increasing the belief in the patient's rating of pain, but also increasing concern for respiratory side effects of opioid medications.

In studies of the quality of pain management several researchers have reported problems with patient recruitment due to nurses' failure to identify patients in pain. This further supports the findings of lack nurses' identification of patient pain (Ferrell, Whedon, & Rollins, 1995; Ward & Gordon, 1994).

An important structural aspect of this investigators larger project relates to nursing resource allocation. Susan Pierce discusses the lack of research linking these areas (Pierce, 1997). She concludes that even when accurate measurement of a nursing intervention is described through a study and a positive impact on a patient outcome is validated, the study rarely includes a description of the nursing delivery structural components needed to consistently provide the intervention.

> "There is ongoing evidence in the clinical research literature that counting the number and types of nurses and describing how they are deployed (.i.e., structural indicators) may not be the key correlate to quality outcomes. Rather, the literature implies that what a nurse actually does plays a key role in both health outcomes and client satisfaction." (Pierce, 1997, p. 63)

She suggests integrating clinical studies with studies of nursing care delivery systems in a multifaceted, longitudinal examination as the next important step.

Nursing Processes

As pain is multidimensional, and for many an ongoing part of patients' disease, systematic communication of patient assessment and response to intervention(s) is fundamental to high quality pain management. Use of standardized assessment scales by nurses is the standard of care (Acute Pain Management Guideline Panel, 1992; American Pain Society Quality of Care Committee, 1995; Ward & Gordon, 1994). The ability to trend patient responses to treatments over time is also fundamental (American Pain Society Quality of Care Committee, 1995).

The Agency for Health Care Policy and Research standards of care for pain assessment include use of patient self-report and documentation of pain using a valid and reliable scale. Self-report is used as it is the single most reliable indicator of the presence of pain and it's intensity (Acute Pain Management Guideline Panel, 1992). Use of the patient preferred pain assessment tool is recommended, though the need to provide organizational standards which ensure consistent access to assessment information is recognized. Problems with use of "as needed" medication administration have lead to the recommendation of regularly scheduled administration of analgesics in selected patient populations.

Outcomes of Care

An outcome measure monitored by JCAHO and NCQA that is included in the ANA report card of recommended monitors is patient satisfaction with care. This dimension has been problem prone and difficult to measure (Lin, 1996). Cleary et al.

(1991) in a report on patients' rating of their care, discusses some of the problems related to measurement of patient satisfaction and report on a tool which seems to address these issues. The use of Picker Commonwealth Survey, the tool developed by Cleary et al., has been supported by the American Hospital Association and many state hospital associations (Picker Institute, 1996).

Methods

Study Design

Data were gathered from a sample of 40 nurses of 65 eligible nurses working on the study unit and a sample of 109 of the 328 patients they cared for during the study period. Multiple sources and methods were used including surveys, interviews, review of the patient record, and queries of two hospital databases to describe three interconnected dimensions of care; the structure of care, nursing care processes and outcomes of care, see figure 2. *Structural variables* included: nurse characteristics of knowledge and attitudes related to pain management, measured by a survey, and the nurses' typical pain management approach, a calculation of a ratio of pain medication doses administered by nurses to hours worked; patient characteristics of functional status, engagement in care, and psychosocial status, measured by a 15 item assessment completed on admission to the hospital. *Processes of care* included assessment and treatment of pain. Assessment and treatment of pain was measured by patient report and chart audit. Data were gathered on use of both pharmacological and non-pharmacological pain treatment(s). Patient-reported processes were gathered by interview. Nurse reported processes were gathered from chart audits. Medication administration data was gathered by chart audit. *Outcomes of care* included adequacy of

pain relief reported by the patient on unit during hospitalization and pain relief experiences reported by the patient after discharge, collected by a written, mailed survey. Measures of association include Chi Square, Pearson Product Moment correlation coefficient, Kendall's tua-b, Fisher's exact t test. A p value of .05 was set as the value for statistically significance.

Setting

The study was conducted on a 36 bed adult medical unit which is part of an approximately 200 bed medical center. The medical center is located near Portland, Oregon. The unit provides services to a general medical population and the specialty populations of patients with chronic renal failure and patients with HIV infection. Additionally, some general surgery patients are cared for on the unit. The unit daily census averages about 30 patients. The unit is staffed with RNs, CNAs, unit secretaries, and a combined CNA/ unit secretary role. Day shift nurses' assignments average four to five patients. Evening shift assignments average five to six patients. Night shift assignments average eight to ten patients. Each shift has a charge nurse who carries a patient assignment in addition to facilitating unit flow. The nursing skill mix is nearly 70 % RN.

Sample

Patient Participants

The investigator recruited patients on 37 days during two study periods. Recruiting started September 21st though November 30th, 1998, resumed January 4th and ended January 30th, 1999. This resulted in 50 and 53 patients, respectively, being recruited, interviewed, and mailed surveys at discharge in each period. The patient

selection process started with the investigator identifying recently admitted patients from a copy of the unit daily census. Initial selection criteria included admission to the unit from the clinic or emergency room, English speaking, cognitively intact as judged by the nurse assigned to give care, expected discharge not within one day. The investigator met patients meeting these criteria introduced himself, explained the study and obtained consent.

A total of 109 patients were included in the study, 328 patients were admitted during the 37 days the investigator recruited patients. Seventy-three patients (22 percent) were not available when the investigator could recruit them. Of the 255 available patients 24 percent were not eligible. The principal reason for exclusion was confusion, followed by an expectation of discharge in less than one day, and two were non-English speaking. A total of 165 patients (89 percent of those eligible) agreed to be interviewed. Of the 11 percent declining to participate, about two-thirds declined when recruited, the remainder declined when approached for the interview. Most eligible patients not interviewed were discharged before the investigator could complete their interview. A few were transferred to other units.

To determine if there were systematic differences between those who agreed to participate and those who declined to participate, the groups were compared on age, gender and hospital length of stay (LOS). Table 1 shows age in years, LOS in days, and percentage of female patients participating and not participating in the study. Patients declining to participate were, on average, eleven years older than those agreeing to participate. No statistically significant difference existed for LOS. Fifty-six percent of the participants were female. No gender-related statistically significant difference

between patient participants and non-participants was present (χ^2 0.813, df = 1, p = 0.397).

Table 1.

Comparison of patient age, LOS and gender between study participants and non-participants.

	Study participant	N	Mean	Standard Deviation	P value
Age in years	No Yes	24 109	70.96 59.62	17.96 18.89	.000
Length of stay	No Yes	24 109	4.62 4.52	5.12 3.67	.398
Percent female	No Yes	24 109	45% 56%		.397

Nurse study participants

Nurse study participants were recruited from the regular unit staff and float pool staff in a series of eight sessions over a ten day period in mid September, 1998. A total of 65 nurses were invited to participate. Of these, 40 nurses participated. There were 33 eligible regular staff, of whom seventy-five percent completed surveys during the initial recruitment (n= 25). An additional six regular staff were recruited later for a total of 31 or 93 percent of the regular staff. Twenty-two percent of the 29 float staff completed surveys during the initial recruiting (n= 7). Two additional float staff were recruited raising this group's participation to 31 percent.

To recruit nurses, invitational posters were prominently displayed in staff break and communication areas. Individual invitational letters were sent to each nurse explaining the purpose of the study, the roles of nursing staff and research team members in data gathering, how they could participate and what was expected of them. The recruitment sessions included an explanation of the study and the opportunity to

complete the informed consent for nurse participants. Individual recruitment of a few nurses not working during the recruitment sessions also occurred. The explanation during group sessions and with individuals included a description of the study and measures planned to protect the clinical staff from the potential for increased practice scrutiny created by the researcher also being the unit manager. In addition, staff completed a self-learning module on one of the measures, the Revised-Health Status Outcome Dimension (R-HSOD). Those who consented to participate signed consents and completed nurse demographic forms. Consents and demographic information were gathered during the recruitment sessions after answering all questions. The Nurse Knowledge and Attitudes Survey Regarding Pain (NKASRP) was distributed and agreements about returning the tool were reached. NKASRP identification (ID) codes were recorded on the survey in the space provided. Numbers were assigned corresponding to ID codes printed on the nurse demographic form. Nurse reasons for not participating were not explored due to the investigator's supervisory relationship with staff.

To determine if there were systematic differences between nurses who agreed and those who declined to participate, the groups were compared on level of nursing education, years of nursing and unit experience. Float staff participants and non-participants were compared on number of scheduled hours. The distribution of educational level among nurse participants and non-participants is shown in table 2. No statistically significant difference was found ($\chi^2 = 4.315$, df = 3, p = .229). Nurse participants were compared with non-participants on years of nursing experience and years of unit experience. Differences between the mean years for both measures is

significant as noted in Table 3. Proportionally more float staff with scheduled hours participated than those without regularly scheduled hours($\chi^2 = 10.343$, df = 3, p = 0.016). This difference probably relates to differences in recruiting availability between the groups of float staff. Differences could also be related to the lack of value for participating created by the infrequency with which non-participant float staff work on the study unit.

Table 2.

Comparison of nursing education level between participants and non-participants.

Participated in study	ADN	BSN	Diploma	MSN	Total
No	12	7	5	1	25
Yes	26	11	3		40
Total	38	18	8	1	65

Table 3.

Comparison of nursing experience between participants and non-participants.

	Participated in study	N	Mean	Standard Deviation	P value
Years of nursing experience	Yes	38	9.13	9.27	.024
	No	25	14.36	7.88	
Years of unit experience	Yes	38	2.71	4.01	.001
	No	25	6.52	4.99	

Instruments

Structural variables include nurse or patient characteristics that each brings to the process of care. Processes of care are those variables representing methods of care. Outcomes are the results the interactions between structures and processes of care.

Structural variables

Patient characteristics. To identify if variations among patients accounted for differences in pain management processes or outcomes of pain treatment the R-HSOD instrument was used as the patient predictor variable. As the R-HSOD measures the

patients' health status at a point in time it could be considered a patient variable (Crawford, Talyor, Seipert & Lush, 1996). The R-HSOD tool was developed by Mary Lush, RN PhD, to monitor changes in patient health status over time (Lush, 1997). It is a 15 item instrument used by professional staff to assess patients and their families. Each item has four categories numbered one to four, except ambulation which has a fifth category. The categories describe the performance of the patient or patient's family on that item. Numerical values are assigned to each category with a lower number representing less function. The instrument has three patient related subscales: functional status, engagement in care, and psychosocial status. Internal consistency using Cronbach's alpha for the subscales is .91, .83, and .75 respectively. The fifteen items on this instrument total to a maximum 61 points. Each sub-scale total produces a maximum score for each dimension. Maximum score for functional status, knowledge/degree of engagement and psychosocial well-being are 29, eight, and 24, respectively. Higher scores show more optimal health. Interrater reliability using percent agreement ranged between 89.6 and 100 percent (Outcomes Taskforce, n. d.). As pain management was of major importance to this study a pain symptom status item was added to the instrument, modeled after the symptom status item, see appendix A. Considering this change the tool was titled Modified Revised-Health Status Outcome Dimension (MR-HSOD).

Nurse characteristics. Nursing practice is based on a body of knowledge. Measurement of nurses knowledge and attitudes regarding pain is important to understanding their use of pain related processes and treatments. The NKASRP was used to measure nurses' knowledge and attitudes about pain and its management

The NKASRP is a paper and pencil survey consisting of 22 true/false questions, 13 multiple choice questions and two case studies with two questions each for a total of 39 questions. Ferrell et al. (1995) report on the use of NKARPS, a general knowledge and attitude survey related to pain, as part of a larger institutional assessment of pain management quality assessment and improvement. This survey provides a numeric score. Higher scores represent knowledge and attitudes consistent with current pain management standards. The tool has demonstrated discriminant and construct validity. Content validity is rated at 90% for most of the 23 survey items (Ferrell, McGuire, & Donovan, 1993).

Nurses typical pain management practice pattern is influenced by their knowledge, experience, and the patient population(s) they serve. Overtime this typical practice pattern should be reflected in the amount of medication they dispense and would be modified by how much they practice. Doses per hour worked (DPHW) is a measure that should capture this practice pattern.

The calculation of DPHW was derived from two hospital databases, the automated medication dispensing system and the nurse staffing database. Nurses were excluded if they had not worked at least 40 hours during each half of November. The 40 hour minimum ensured that the nurses included in the measure had worked at least half time. Recognizing that differences in the number of patients assigned to each nurse would affect this variable, an attempt was made to adjust the variable for number of patients assigned to each nurse. Retrospectively, total the number of assigned patients was counted for each nurse working the study unit during November. Due to missing assignment record information (25 to 33%) a reliable total could not be generated.

Therefore the attempt to adjust DPHW for variation in patient assignment volume was abandoned. The reason assignment information was missing was that documentation of this information was a new requirement to the unit staff.

Process of Care

Two processes of care were evaluated, assessment of pain and treatment of pain. These processes were evaluated using both patient report via interview and nurses' report via chart audit.

Assessment. Assessment data were gathered using both a patient interview questionnaire (Appendix C) and a chart audit tool (Appendix D). These were developed after a review of the literature (American Nurses Association, 1995; Acute Pain Management Guideline Panel, 1992; Sindhu, 1996) and a review of several published interview tools (Ferrell et al., 1995; Ward & Gordon, 1994). Findings from these studies guided choice of non-pharmacological items; heat, cold, position change, relaxation, imagery, massage and provision of information. The patient interview tool, requiring less than 5 minutes to complete, included patient demographic information, and in the 13 items; kind of pain, adequacy of pain management measures, and perceived frequency of assessment. The chart audit tool included the same patient demographic information, plus medical diagnoses, assessment frequency, and use of the 0-10 pain intensity scale. The interview and chart audit tools focused on care provided in the 24 hours preceding the interview.

Treatment. Pain treatment questions and chart audit data were gathered using the same tools used for assessment information. This included patient reported non-pharmacological treatments offered by the nurse and usual non-pharmacological

treatments for pain used by the patient. These were gathered during interview. Chart audits were used to gather data related to pain medications ordered and administered, and non-pharmacological pain treatments documented by the nurse.

The amount of medication given was converted to the variable percent maximum dose given (PMDG). Data were gathered on the first three drugs with current orders for the 24 hour period under study resulting in PMDG 1, 2 and 3. PMDG was calculated by summing the total dose given in the 24 hours proceeding the interview. This was used for the numerator. The denominator was calculated by multiplying the dosage ordered by the maximum number of allowed doses. When orders included dosage ranges, the highest dose was used. Where frequency ranges were present the shortest frequency was used. This ratio into a percentage. The drug with the highest percentage was placed in the PMDG1 variable, the drug with the next largest percent was placed in PMDG2, and for patients with three drugs ordered the last drug was placed in PMDG3. The data were exported from the Pendragon Forms® Access® tables into Excel® for sorting and completion of the calculations.

Outcomes of Care

Patient Reported Outcomes During Hospitalization

Several patient interview questions focused on outcomes of care. Adequacy of pain relief was measured by asking the patient to rate their worst pain, best pain relief for the preceding 24 hours, and to rate their pain at interview. The 0-10 pain intensity scale was used. Additionally, on the same scale, patients were asked to identify the level of pain, with which they could continue to enjoy their usual activities. A final question,

asked at interview, was if the pain treatments offered by the staff were acceptable? The response choices were yes or no.

Patient Reported Outcomes by Post-Discharge Survey

Patient's satisfaction with pain management is an important measure of nursing care. As pain is a uniquely personal experience patient reports of pain are the best measures of this outcome. Use of satisfaction as a response set in pain management research and quality assurance has proven problematic (Ferrell et al., 1995). Reported experience on questions related to patient perceptions of care has shown promise in overcoming limitations of satisfaction measurement (Cleary et al., 1991). The pain problem score was used to evaluate patients experiences related to pain management. This is part of the Picker/Commonwealth survey dimension of physical comfort. Problem scores are calculated by dividing patient responses rated as problems by the total responses for a question or set of questions. This is reported as a percentage.

The Picker/Commonwealth survey is a sixty-item questionnaire. The items combine into sub-scales or dimensions of care, and include: respect for patient preferences, coordination of care, information and education, physical comfort, emotional support, involvement of family and friends, and continuity and transition. The dimensions are designed to measure different aspects of care, therefore a traditional measure of internal consistency for the total instrument would be low. The six item satisfaction scale has a Cronbach's alpha of 0.90 (personal communication, Dr. Michael Massagli April 9, 1998). Validity for several areas of the questionnaire have been reported. Discriminate validity has been demonstrated by the range of scores between hospitals and the relationship of these scores with other objective measures of quality.

Face, construct and content validity have been assured through pilot testing with patients and review of the tool by advisory boards including health care professionals and the lay public (Cleary et al., 1991). Patient's experience with pain management was measured using six of the pain related questions in physical comfort sub-scale of the Picker/Commonwealth questionnaire. The sub-scale questions are framed to report the patients' experiences. No response to each question is also reported. Pain-related questions and responses are listed in Table 4. The symbol ρ identifies patient responses used to calculate a problem score and is discussed later under patient experiences with pain.

Table 4.

Picker/Commonwealth Pain Related Questions

Question	Response set
31. Were you ever in any pain?	Yes No(Go to question 38)
32. When you had pain, was it usually;	Severe Moderate Mild
34. Did you ever request pain medicine?	Yes No(Go to question 36)
35. How many minutes after you requested pain medicine did it usually take before you got it.	0 minutes/right away 1-5 minutes 6-10 minutes 11-15 minutes ρ16-30 minutes ρMore than 30 minutes ρNever
36 Do you think that the hospital staff did everything they could to help control your pain?	Yes ρNo
37 Overall, how much pain medicine did you get?	ρNot enough Too much Right amount

Concepts being researched are listed in table 5. The instrument used to gather data for each measure is listed in the second column. Measures included in the variable

Table 5.

Study concepts and measures.

Concept	Instrument	Measurement	Level
Structure of Care			
Patient characteristics	MR-HSOD	Individual functional status	Interval
	MR-HSOD	Individual psychosocial status	Interval
	MR-HSOD	Individual engagement	Interval
	MR-HSOD	Pain status	Ordinal
	Patient interview	Acute vs. chronic pain	Nominal
Nurse characteristics	NKARPS	Average NKARPS	Interval
	Calculated from hospital databases	Doses per hour worked	Interval
Processes of care			
Assessment	Patient interview	Patient reported assessment frequency	Ordinal
Assessment	Patient interview	Patient reported assessment after treatment	Nominal
Assessment	Patient interview	Worst pain in past 24 hours	
Assessment	Patient interview	Best pain relief in past 24 hours	Ordinal
Assessment	Patient interview	Pain at interview	Ordinal
Assessment	Patient interview	Level of pain patient could live with and do usual activities	Ordinal
Assessment	Chart audit	Nurse report assessment frequency	Nominal
Assessment	Chart audit	Nurse report medication evaluation	Nominal
Assessment	Chart audit	Pain assessment by nurse recorded using 0-10 scale	Nominal

table continued

Concept	Instrument	Measurement	Level
Treatment	Patient interview	Patient report non-pharmacological treatment	Nominal
Treatment	Chart audit	Percent maximum dose given, drug one	Ratio
Treatment	Chart audit	Percent maximum dose given, drug two	Ratio
Treatment	Chart audit	Percent maximum dose given, drug three	Ratio
Outcomes of pain management			
Adequacy of pain management	Patient interview	Worst pain in past 24 hours	Ordinal
Adequacy of pain management	Patient interview	Best pain relief in past 24 hours	Ordinal
Adequacy of pain management	Patient interview	Pain at interview	Ordinal
Adequacy of pain management	Calculated	Pain Control Ratio (average of worst, best, and now pain divided by pain could live with)	Ratio
Adequacy of pain management	Calculated	Difference between worst pain and best pain relief	Ratio
Patient report experiences	Patient interview	Treatment choices acceptable	Nominal
Patient report experiences	Picker-Commonwealth	Picker survey pain problem score	Interval

are listed in the third column, measurement. The level of each measure on the nominal, ordinal, interval or ratio scale is indicated in the column labeled, level.

The psychometric characteristics. The R-HSOD instrument has strong discriminate validity with good interrater reliability (Cronbach's alpha of 0.91, 0.83, and 0.75 for the three patient dimensions functional status, engagement in care, psychosocial status, respectively; Lush, 1997). The NKASRP has a strong discriminate, content and construct validity (Ferrell et al., 1993). Picker-Commonwealth questionnaire has strong content, construct and discriminate validity. The Picker-Commonwealth overall satisfaction scale has a Cronbach's alpha of 0.90 (Cleary et al., 1991). Validity and reliability of the patient interview and chart audit tools were unknown.

Procedures

Recruitment

Beginning with the steps outlined under sample patient participant, the recruitment procedures continued. Patients' expressed preferences about an interview time were noted. Most patients were interviewed the following day, though some were interviewed several days later. The delay usually was due to other time commitments of the investigator. In a few cases the delay was at the patient's request. Later, prior to interviewing the patient, demographic information was entered into the PalmPilot® Personal Digital Assistant (PDA) on the Pendragon Forms® main pain study form. The investigator then reintroduced himself and allowed the patient to decline or confirmed their willingness to participate in the study. For those patients agreeing to proceed informed consent was obtained, the consent signed, and interview completed. Following

the interview the chart audit review tool was initiated. A copy of the patient's face sheet was made and attached to the signed informed consent. The investigator monitored the patient's hospital stay and within a day of discharge each patient was sent a Picker Commonwealth survey. Surveys were mailed to the address listed on the face sheet. A follow up survey was mailed to patients not responding to the initial mailing within two to three weeks. Chart audits were completed by two nurses, both unit staff members. Both nurses were on modified duty for the duration of the study and therefore were not simultaneously providing care and auditing care provided . Both nurses had been instructed in use of the audit tools on the PDA. The investigator provided ongoing support in the use of the PDA, including consultation about interpretation of nurse documentation when gathering the needed chart information, and technical troubleshooting for the PDA. Use of the record sort function and instruction on entering new records were the most frequent help needed by auditors. Once during the study the PDA was dropped, resulting in the loss of data entered that day and the need to send it out for repair. During the time required for repair, approximately ten days, the data collection forms were installed on a second PDA. Data was loaded onto the forms, allowing chart auditing to continue for patients with existing records and addition of new patient records.

Missing data

Cases with missing data in the patient characteristics measures were excluded casewise from analysis of the specific measure, as recommended by the tool's author (Lush, 1997).

Given that patients were interviewed without consideration of whether their assigned nurses were also study participants, missing NKARPS values for the three shifts were expected. It was recommended that patients' who did not have at least two NKARPS scores from which to calculate an average NKARPS be excluded from analysis involving this variable (personal communication Jonathon Fields, February 24, 1999). Initial analysis revealed that about 40 percent of the patient sample would be excluded based on this recommendation. Review of the assigned nurse data showed that if six additional nurses could be recruited, a large number of patients would meet the recommended minimum NKARPS score count. Additionally, many more patients' average scores would include all three assigned nurses. I was able to recruit five of the six identified nurses to be study participants. This resulted in 96 patients (88 percent) with usable data. All of the nurses reported that they had intended to participate by completing the NKARPS during the initial recruiting period, but for various reasons had not completed the survey. The sixth nurse was not available to be recruited.

Two nursing care process measures had missing data, nurse reported assessment frequency and nurse reported non-pharmacological treatment. Patients without these data were excluded from analysis of the individual measure.

The high percentage of missing values for the Picker survey pain problem score has two causes. Forty-eight percent of the patients did not return surveys or the returned survey was unusable, a rate consistent with mailed surveys (Picker Institute, 1997 p. 7). Patients who deny having pain during their hospital stay are directed by the survey not to answer the pain-related questions. Twenty-nine percent of the patients returning

surveys reported no pain. This resulted in being able to calculate a pain problem score for only 36 percent of study participants.

Analysis procedures

Data were imported into SPSS for Windows, version 7.5.1 from several Access® tables. Three data sets were created, nurse demographic data, patient demographic data and patient study data. Chi square with 2-tailed significance, and Fisher's exact t test were used to test demographic data for statistically significant differences between participants and non-participants. Pearson Correlation Coefficient was used to calculate correlations between interval level variables and Kendall's tau-b was used to calculate correlations between ordinal level variables in patient study data. Statistical significance was set at $p = .05$. Correlation matrices were generated comparing variables. Scatter plots of the correlated variables were generated and reviewed for outliers and curvilinear relationships. Cronbach's alpha was used to compute internal consistency for the MR-HSOD.

Results

Descriptive Findings

Structural Findings

Patient characteristics. Patient pain was categorized by type and cause. Type of pain was differentiated into two mutually exclusive categories, acute or chronic. Cause of pain was classified into three exclusive categories, related to reason for admission, related to a procedure performed in hospital, or neither. Seventy-three percent of patients interviewed reported having pain ($n = 80$). Acute pain of less than six weeks in duration accounted for 69 percent of pain in the 80 patients, with the remainder having

chronic pain. Pain related to the reason for the patient being admitted accounted for 68 percent of the 80 patients with pain, 22 percent had pain related to a procedure. The remaining 10 percent attributed their pain to other reasons, all of which were chronic.

Patient average NKARPS scores ranged from 60 percent to 91 percent with a mean of 77.7, a Standard Deviation of 7.13, and skew of –0.397.

The patient characteristics as measured by the MR-HSOD are displayed in table 6. The patient scores found in this study were on average higher for the dimensions of functional status and psychosocial status than reported by the instruments developer, though both are within one standard deviation. The mean for the dimension engagement in care is 1.76 points lower than that reported during development and this difference is greater than the standard deviation.

Table 6.

MR-HSOD Statistics and internal consistency.

Dimension	Mean	Median	Std. Deviation	Skew	Chronbach's alpha
Functional Status	21.09	23.00	3.69	-1.258	.81
Engagement in Care	7.05	7.00	1.96	-.438	.62
Psychosocial Status	10.84	11.00	1.35	-1.43	.60

Nurse characteristics. The nurse scores on the NKARPS ranged from 51 percent to 95 percent with a mean of 77.2%, a Standard Deviation of 10.29, and skew of -0.667. Cross-tab tables were developed comparing NKARPS scores with years of unit or nursing experience, personal experience with pain, and educational level. No statistically significant associations were found using χ^2. T tests were performed within the categories of years on unit or nursing experience, personal experience with pain, and educational level. One statistically significant relationship was identified. Nurses who reported attendance at more than three pain related classes in the past three years

averaged 82.9 percent on the NKARPS compared to those attending 3 or less who scored an average of 76.24 percent($t = 1.484$, df 38, $p = .033$). The clinical significance of a 6.5 percent difference is unknown.

The doses per hour worked (DPHW) variable was calculated from drug dispensing data and hours worked providing patient care. The drug dispensing data was exported from a Sure-Med® database in a daily report, text format. These reports were converted into two Excel® spreadsheets, one with data from November 1st through the 15th and the second containing daily reports from the 16th to the 30th. These spreadsheets were then imported into Access®. This resulted in a data base with approximately 25MB of data. Queries were developed that reported the count of pain medications dispensed by nurse for each period. A count of wasted medications by nurse was developed. From these counts the net doses administered per nurse was calculated. This number was divided by the hours worked by each nurse for the respective periods as reported in the hospital staffing computer, ANSOS® resulting in the variable, DPHW. As a test of reliability, the correlation between the half month periods was calculated and found to be $r = 0.201$. Total DPHW per nurse was calculated by totaling doses given and hours worked for each half of the month. Total doses was divided by total hours worked. No relationship was found between NKARPS scores and total DPHW. Because of low stability reliability this variable was excluded from further analysis.

Processes of Care

Patient reported assessment. Most patients reported frequent pain assessment by hospital staff for the 24 hours preceding the interview. Forty-one percent of patients reported being asked about their pain at least every two hours, twenty percent reported

being asked every four hours, eighteen percent reported being asked about their pain every eight hours. Four percent of patients reported being assessed daily for pain and sixteen percent reported never being asked about pain. Patients without pain were less likely to be assessed for pain, although more than half of the patients who reported no assessment by hospital staff did report pain at interview (see Table 7). Eighty percent of patients reported being asked about their pain after treatment. Of the patients who reported being asked about their pain, 94% said their RN asked about their pain, 51% reported the CNA asking about pain, and 45% reported the doctor asking about their pain.

Table 7.

Comparison of patients with and without pain and their reported assessment frequency.

Assessment Frequency	Every 2 hours	Every 4 hours	Every 8 hours	Daily	Never	Total Number	
Had pain in the last 24 hours?							
No	7	3	9	2	8	29	χ^2= 12.229
Yes	38	19	11	2	10	80	P value = 0.016
Total	45	22	20	4	18	109	
Percentage	41	20	18	4	16	100	

Nurse reported assessment. Ninety-one percent of the patient records reflected pain assessment frequency meeting the applicable standard of care. Twelve percent of the patients records reflected an evaluation of medication effectiveness. The 0-10 scale was used to record the report of pain for 8.3 percent of the patients. Chart audits reveal a marked discrepancy in assessment documentation when compared to patient reports of after treatment evaluation in that 80 percent of patients reported assessment after treatment while nurses on the study unit documented findings from this assessment on 12 percent of the patients.

Treatments

Patient reported. Thirty-two percent of patients reported using non-pharmacological treatments for pain. The specific non-pharmacological treatments and frequency of use are shown in table 8. Ten percent of patients reported that nurses offered them non-pharmacological treatments.

Table 8.

Patient reported non-pharmacological treatments.

Non-pharmacological treatment	Percentage of patients receiving treatment
Position change	12.0
Heat	6.7
Relaxation	5.3
Cold	4.0
Imagery	1.3
Information	1.3
Massage	1.3

Nurse reported medication administration. The amount of pain medication given during the 24 hours preceding the interview was gathered during the chart audit. The audit tool had space for three medication orders and three 24 hour totals. Only one patient had more than three medications ordered for pain. Twenty percent (n= 21) of the patients interviewed received no medication for the 24 hours preceding the interview. Six of these patients (28 percent) reported having pain. Of the patients reporting pain in past 24 hours half received one–third or less of the maximum prescribed dose for their primary medication. Only 5.8 percent received the maximum 24 hour dose. The mean for PMDG1 was 39.5, with a standard deviation of 41.38. Ten of the 25 patients reporting no pain during the past 24 hours received pain medication. Fifty-nine of the patients reporting pain had a second pain medication ordered. Forty-six percent of the patients with second drugs ordered did not receive a dose during the 24 hours before the

interview. The mean for PMDG2 was 10.2, standard deviation of 14.21. Sixteen patients had a third medication ordered. Half of the patients reporting pain, who had a third medication ordered, had received at least one dose. The mean for PMDG3 is 16.5, standard deviation of 27.39. The wide range in amount of medication given may be related to variation in patient needs or variation in nursing practice.

Nurse report non-pharmacological treatment. Nurses documented the efficacy of non-pharmacological pain treatment on 2.7 percent of the study patients (n=2). One of these patients reported receiving a non-pharmacological treatment and one did not.

Outcomes

Adequacy of Pain Management

The patients' average best level of pain relief for the past 24 hours was 2.4 on a 0 to 10 scale, with a standard deviation of 2.2, and a range of 0.0 to 8.0. The average reported worst pain was 7.4, standard deviation of 2.3, and a range of 1.0 to 10.0. This finding is consistent with other reports. Pain at the time of the interview averaged 2.8, standard deviation of 2.3, and a range of 0.0 to 10.0. This is lower than other reports (Ward & Gordon, 1994; Acute Pain Management Guideline Panel, 1992). The difference between worst and least pain reported by Ward & Gordon (1994, p. 301) averaged 4.7, standard deviation 2.55 and is similar to the finding of this study, mean 5.0 with standard deviation, 2.45. The average level of pain that patients' believed they could live with and still enjoy their usual activities was 3.7 (standard deviation of 2.5). An interesting finding (see table 9) was that the distribution of best pain relief scores was skewed to the right (0.911) and distribution of worst pain scores was skewed to the left (-0.679).

Table 9.

Distribution of patient reported pain scores

	Mean	Minimum	Maximum	Standard Deviation	Skew
Best pain relief in 24 hour	2.46	0	8.00	2.22	0.911
Worst pain in 24 hour	7.47	1.00	10.00	2.30	-0.679
Pain at interview	2.88	0	10.00	2.55	0.703
Level of pain you could live with and do your usual activities	3.75	1.00	9.00	1.68	0.567
Pain control ratio (PCR)	1.29	0.17	4.00	0.765	1.470
Difference of best relief and worst	5.01	0	10.00	2.45	-0.110

Correlations between the four factors contributing to the Pain Control Ratio (PCR) are shown in table 10. All are moderately strong correlations and are statistically significant.

Table 10.

Pearson Product Moment Correlation Coefficients between Factors Contributing to Pain Control Ratio.

Pearson r (level of significance)	Worst pain in 24 hour	Pain at interview	Level of pain you could live with and do your usual activities
Best pain relief in 24 hour	.415 (.000)	.476 (.000)	.476 (.000)
Worst pain in 24 hour		.477 (.000)	.382 (.000)
Pain at interview			.375 (.001)

Patient Experiences with Pain

Of the patients reporting pain during the 24 hours before the interview (n=80) 88 percent said the treatments offered for pain were acceptable. The Picker survey pain problem score was calculated by summing a count of each patient's problem responses for questions 35-37, and dividing the sum by the count of questions 35-37 that were answered by each patient. Problem responses are identified in Table 4 by the symbol ρ. Most patient's survey responses did not generate a problem score. Table 11 shows the

distribution and percentage of problem scores. Of interest is finding that half of the patients who denied pain during the interview, reported pain during their hospital stay on the survey (n= 18). The study design could clearly create this discrepancy as the Picker survey asks patients to report on their hospital experience, while the study interview focused on one day during that stay. Of importance is that eleven percent of patients who reported pain during the 24 hours preceding their interview denied being in pain during their hospitalization on the discharge survey. This is important as it reflects on a limitation of using patient reported pain experiences to measure outcomes of care.

Table 11.

Frequency of pain problem scores.

Pain Problem Score	Frequency	Percent
0	31	81.6
33	5	13.2
50	1	2.6
67	1	2.6
Total	38	100
Missing	71	

Relationships Among Variables

Structural variables include nurse or patient characteristics that each brings to the process of care. Processes of care are those variables representing methods of care. Outcomes are the results the interactions between structures and processes of care. One relationship between a structure of care and a process of care, one structure to outcome, and a few process of care with outcome relationships were found to have statistically significant correlations. There was a statistically significant relationship between nurses' scores on the NKASRP and the outcome variable, treatment(s) for pain were

acceptable, (see table 12). Scatter plots of other variables were reviewed, no curvilinear relationships were identified.

Table 12.

Statistically significant study correlations.

Dimensions of care	Variables	Correlation	p value	Correlation measure
Structure with outcome	Average NKASRP score vs. treatment(s) for pain acceptable	0.248	0.012	Kendall's tau-b
Process to outcome	PMDG2 vs. best pain relief	-0.305	0.018	Pearson
	Evaluation of medication effectiveness vs. pain at interview	0.224	0.047	Kendall's tau-b
	Difference between worst and best pain vs. treatment choices were acceptable	0.405	>0.000	Kendall's tau-b
	Pain Control Ratio vs. treatment choices acceptable	0.232	.032	Kendall's tau-b

The nurses on the study unit tended to record patient self-assessment using the 0-10 scale more often for surgical patients more than medical patients, $\chi^2 = 5.894$, df = 1, p = .015. The rate of use in surgical patients was 23.5% (N=17), compared to the rate in medical patients of 5.6% (N= 89). Patients' who reported problems with pain management on the Picker questionnaire tended to have the 0-10 scale used in documenting their pain. This finding approaches statistically significance with a correlation of 0.295, and p = 0.067. These findings could be accounted for by assuming that nurses practiced at a higher standard for patients with pain problem scores. Some evidence from this study supports this assumption. Patients whose nurses have documented the efficacy of their medications reported an average pain at interview of 4.25, standard deviation of 2.49, compared to patients whose nurses had not documented medication efficacy, who had a mean of 2.65, standard deviation of 2.52, a statistically significant difference, t = -2.016, df =77, p = 0.047.

The amount of the second medication given as measured by PMDG2 shows a negative correlation to best pain relief in 24 hours, r = -0.305 i.e., more was given to patients who subsequently achieved better pain relief. Another interesting related correlation is that of PMDG1 with PMGD2 at r = 0.437, p > 0.000. The correlation between documentation of medication effectiveness by the nurse and pain at interview was r = 0.204. The final correlation identified was between treatment choices being acceptable and the difference in score between worst pain and best pain relief (r = .333), the bigger the difference, the more likely the treatment choice was acceptable.

Discussion

The study has a number of limitations which could account for some of the findings. First was that some measures were either unreliable or failed to capture the construct of interest. The variables PMDG1 though 3 did not consider the potency of the patients' ordered medications when placing them in order of use. The internal consistency of investigator's modified R-HSOD tool was significantly lower than the original instrument. The DPHW variable was unstable and failed to capture the construct of interest. Infrequent use of two nursing process variables impacted their correlation to other variables. In addition to these issues is instrumentation, generalizability is also limited. The significantly greater years of experience on the unit and in nursing among non-participant nurses could confound the findings from the NKARPS correlations. The effect of the sample bias related to years of unit and nursing experience is unknown. If the more experienced non-participating nurses are significantly more or less skilled in pain management as measured by the NKARPS

then adding their scores into the patient's average NKARPS would strengthen the relationships found in the study. Each of these is discussed in further detail below. In addition the study findings raise interesting theoretical issues

Structure

Limitations of the DPHW may have influenced the findings of a lack of relationship with NKARPS. When developing the DPHW, many factors were unknown. The effect of the range of patient medication needs, and the effect of the volume range of patients assigned to nurses were considered, but efforts to account for the later were unsuccessful. The 40 hour minimum, set to assure exclusion of nurses not working at least half time, assured an assignment of 20 to 36 patient days. This could equal as few as four to eight patients, depending on the nurse's assigned shift and the patient's LOS. However, the lack of reliability, as measured by the spilt half correlation, could well be explained by differences in patient needs.

The addition of the pain status item to the R-HSOD significantly decreased the internal consistency of the tool for the subscales of engagement in care and psychosocial status. Cronbach's alpha for functional status was .89, engagement in care was .62, and psychosocial status was.60. The failure of this study to find correlations between the latter two variables and patients' pain experience could be a result of the lower internal consistency of the modified instrument.

A second issue is one of selection bias. Among the nurses a bias exists in that non-participant nurses were more experienced. The differences in years of experience between nurse participants and non-participants could have several causes. Less experience would argue for more recent education, with more current knowledge related

to pain management, and/or possibly more value for research. The investigator's relationship with unit staff may have influenced nurse choices. Nurses with less experience are more likely to have been hired by the investigator or have more of their work experience under the supervision of the investigator and therefore have a more positive regard for the investigator. The positive regard would dispose less experienced nurses to support the study being conducted by this investigator. This level of inexperience among nurse study participants may have influenced the study findings. An argument for more effective care related to pain management by more experienced nurses could also be made. More experienced staff may be less sensitive to patient pain, resulting in less treatment or they may be more expert in giving medication or offering other treatment choices resulting in better pain relief.

Processes of Care

Another limitation of this study was analysis of PMDG variables without regard for the kind of medication. The PMDG1 was the drug with the largest percentage given for that patient, PMDG2 was the next largest percentage, followed by PMDG3. More potent medications may have been used less frequently and could account the statistically significant correlation of PMDG2 with best pain relief.

A third issue is the poor documentation. Considering the low rate that nurses on the study unit use the 0-10 scale to document patient's reported pain (8.3%) and the low rate of medication efficacy documentation (12.1%) the correlations involving these two variables may be much stronger than is evident by the reported values, r = 0.236 and 0.224, respectively. Nunnally(1994, p. 136) describes the impact of low proportions on correlations. Considering his discussion, This investigator would estimate the maximum

possible correlation to be a little more than 0.50 for these dichotomous variables. This puts the two values near mid-range in correlations, a much stronger position than is evident in the absolute values.

Patient's pain experience. Since the patients declining to participate were older, generalizing results of this study to the very elderly should be done with caution. In this study, the patient's pain experiences are similar to other reports of worst, best (least), and pain at interview. The level of best pain relief is poorer than reported by Ward & Gordon (1994), an average of 1.93, standard deviation 1.88, compared to this study's result of 2.4, standard deviation 2.2. Two relationships were identified with a higher average NKASRP of the patient's assigned nurses, treatment choices being acceptable to the patient and a larger difference between worst and best pain score.

Patients reported pain at a rate consistent with other reports in the literature. As the source of pain was a forced choice, the comparison of acute to chronic pain may not represent medical patients usual state of pain, or all reasons for pain. For this reason and as this study was conducted on hospitalized patients the primary cause of pain may be skewed toward acute pain.

The NKASRP scores show a wide range among nurses. The resulting average score for patients has wide range. The mean score of 77% probably does not represent an expert nursing practice group. Although recent pain management education is related to higher scores, the failure of this measure to correlate with more than a few assessment, intervention, or outcome measures could result from many factors, study design being a primary consideration. As this study focused on one 24 hour period during patients' multi-day hospital the influence of the nurses care for the one day under

investigation many not be sufficient to impact patient perceptions. Use of this measure with a more longitudinal study design comparing care given by high scoring nurses to care given by low scoring nurses over several days may demonstrate a difference. The significant relationship of treatment choices being acceptable with the difference between scores of worst pain and best pain, and the nearly significant relationship of the former with average NKASRP score that nurses with more current knowledge and attitudes are able to more effectively relieve their patient's pain. This effect is more significant considering the strong relationship between patients rating of best pain, worst pain, and pain at interview. This relationship among the three pain measures means that patients tend to cluster their pain ratings and that great differences between best pain and worst pain are not likely.

The correlation between PMDG2 and best pain relief would be expected to be negative, as more medication given results in a higher percentage maximum dose. This should lead to a lower best pain relief value. The correlation with PMDG1 and PMDG2 was $r = .437$, $p > .000$. This relationship with PMDG1 and PMDG2 and PMDG2's relationship with best pain relief might be explained by an expected synergy of using more than one medication for pain. The finding that only 5.8 percent of patients on the study unit received the maximum 24 hour dose raises a concern when compared to the 16 percent reported by Barnason et al. (1998) in a pain management improvement project, where the 16 percent maximum dose given was an outcome following the implementation of an educational program and the establishment of standard of practice based on current research. While Barnason et al.'s study does not suggest their reported maximum dose rate be used as a standard for best practice, the difference between the

finding of this study and theirs suggests nearly a three fold improvement should be possible on the study unit.

Recommendations for Further Research

Structural variables. I would propose several recommendations to researchers interested in developing DPHW as a measure. First, considerable desktop computing power will be needed to process the volume of data. As an example, about an hour and half of processing time was required to import each half of the November data from the Excel® spreadsheet into the Access® database on an IBM PC with 80 MB of RAM and a 166mHz Pentium processor. Next, reliable patient assignment information, preferably from an automated source, would be a valuable addition to account for variations in patient medication need. Conceptually the DPHW ratio should link to PMDG. One strength of the DPHW ratio is that the data is available from automated sources in many institutions and therefore it may be more efficiently gathered than audits of paper charts. If the measure were to prove reliable it could be used as a proxy measure for PMDG. It could be trended over time to monitor the effect of pain management education, or other changes such as changes in resource use, or level of professional vs. support staff.

Factor analysis of the NKASRP tool may prove valuable. Some of the questions clearly relate to cognitive knowledge of pharmacology, other questions relate to values about opioid addiction or *appropriate* behavior for patients in pain. If subscales were found these may correlate more strongly with variables studied here than the overall score.

Process variables. Repeating this study in an environment with more consistent documentation of patients' pain using the 0-10 scale and documentation of the efficacy

of medication may demonstrate a relationship between these two variables with the pain problem score and pain at interview, respectively. Although, this relationship may be unique to the nursing practice on the study unit, reflecting a tendency to document more thoroughly for patients with more severe pain management problems, consistent use of these processes without regard for nurse assumption of worse pain should eliminate any correlation. The somewhat more consistent use of these processes with surgical patients by the study staff would support the argument for nurse assumption of worse pain in this group.

Outcome variables. Considering the large number of patients agreeing that their treatment choices were acceptable I believe this measure is similar to satisfaction with treatment of pain, in that it may be impacted by patient expectations. The correlation between this measure and the difference between scores of best and worse pain adds some value to the measure. Converting this question from a yes or no response to a rating of how acceptable treatment choices were on a poor to excellent scale may prove valuable in the search for pain satisfaction measures that correlate with acceptable clinical outcomes.

Clinical Implications

Understanding that moderate correlations exist between worst pain, best pain, pain now, and level of pain the patient could live with and do their usual activities could be used by nurses in clinical practice to better understand the meaning of the 0-10 pain intensity scale for a specific patient. The correlation between the difference of scores on best pain relief and worst pain with treatment choices being acceptable could also be used by clinical staff to judge adequacy of pain management. The finding of a higher

average NKASRP score for the group of nurses who attended more than three pain related classes in the preceding three years could argue for annual education in pain management.

Summary

The lower number of patients receiving the maximum prescribed dose coupled with the low use of the 0-10 scale and documentation of medication efficacy may be related. These findings along with the difference between patient reported assessment after treatment and nurse documentation of this care process leads to the conclusion that care provided on the study unit does not met national standards. Inspite of this apparent deficit the patients' pain experience in this study is very similar to other reports. This study provides some empirical evidence for the assumed linkage between more current nurse knowledge and attitudes about pain management and the outcome of treatments for pain being acceptable to patients.

Reasons for the lower standards of practice are not clear from the study. Unexplored organizational variables could account for the failure of staff to document care they seem to be providing. The mean score of 77% on the NKASRP would seem to show that significant improvements in staff knowledge and attitudes regarding pain treatment are possible.

Figure 1. Research Model for Pain Management Nursing Care Quality

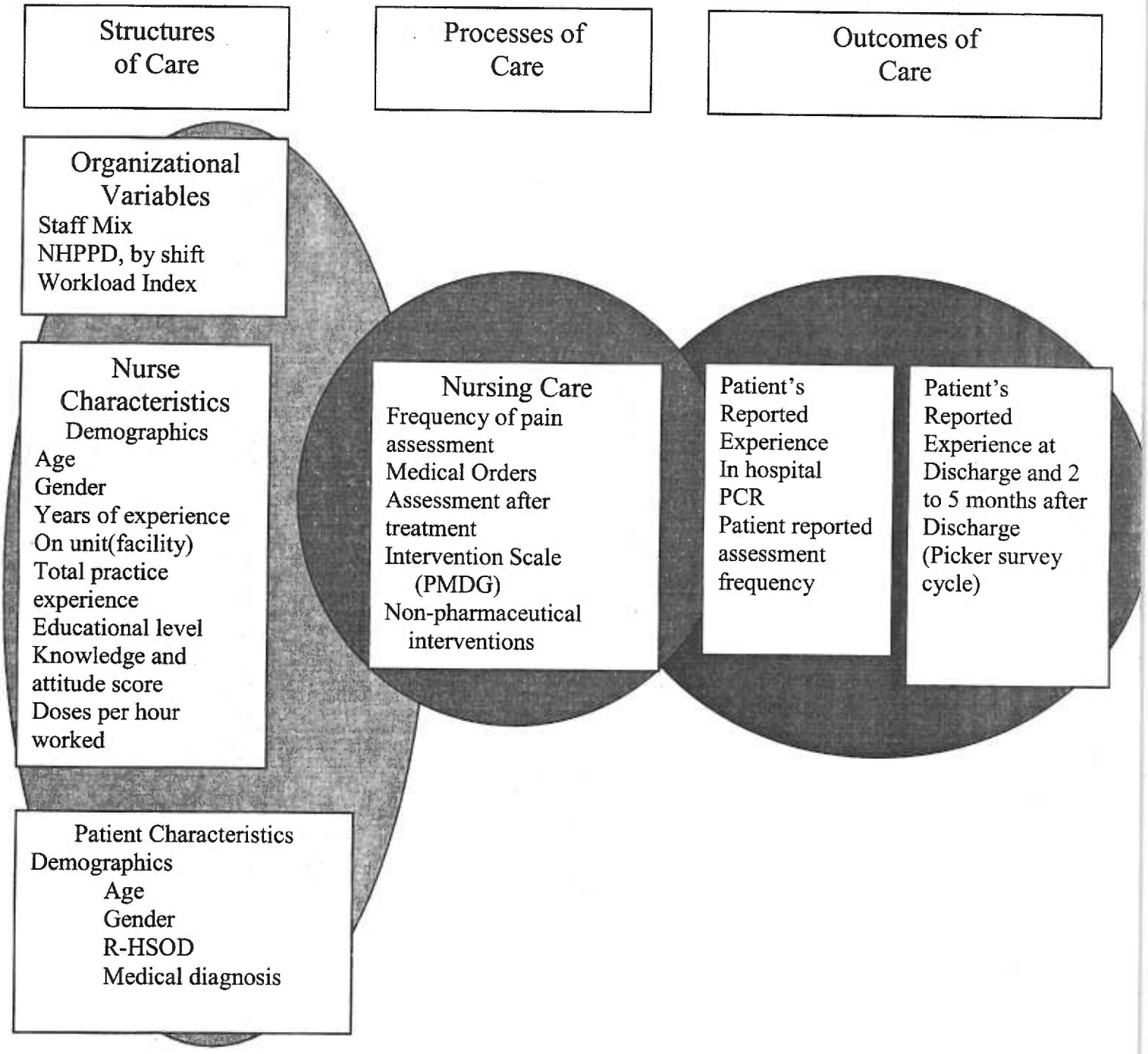
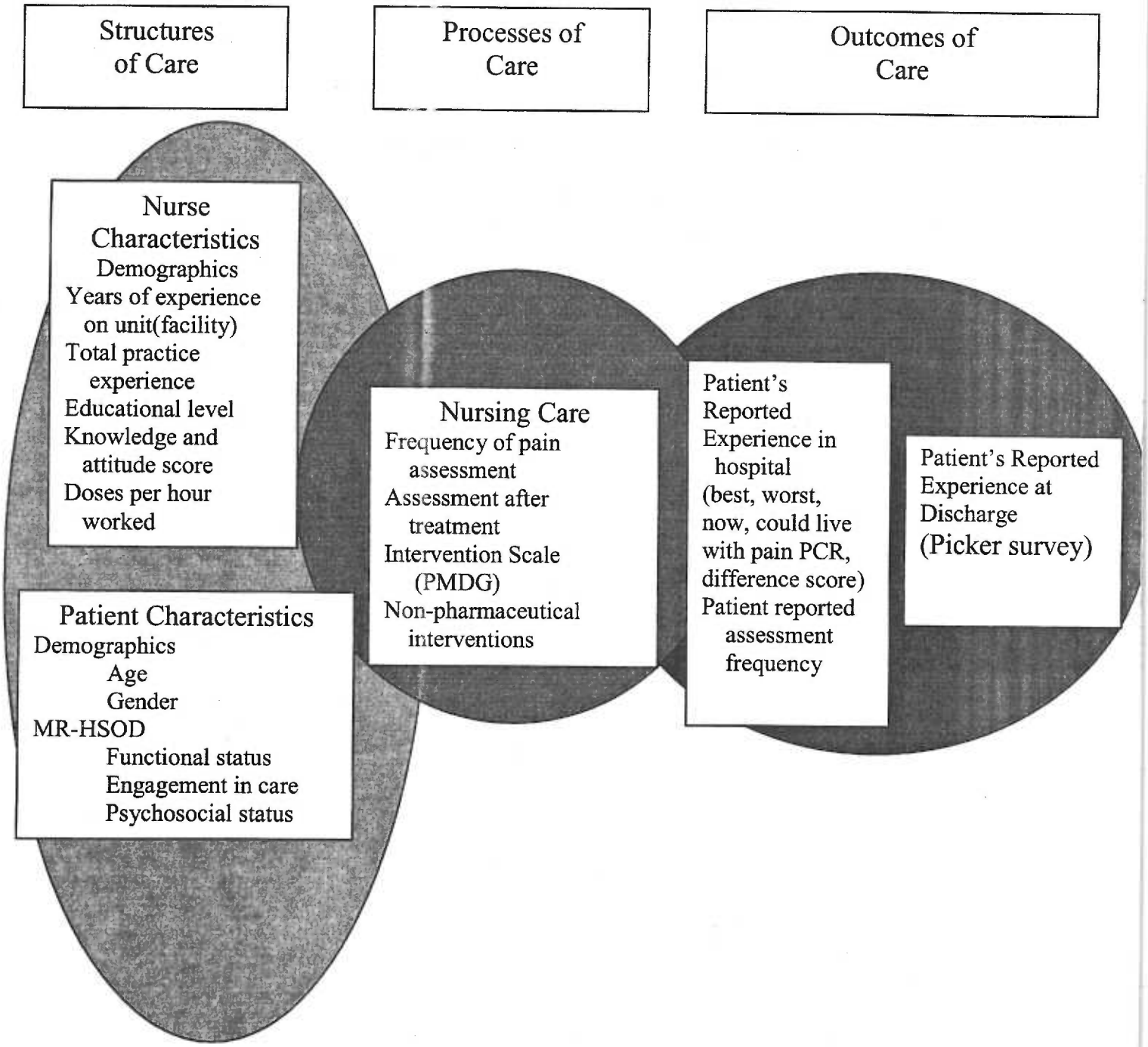


Figure 2. Pain Management Variables Studied.



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Appendix A.

MR-HSOD.

Modified Revised Health Status Outcome Dimension Instrument (MR-HSOD)
Nurse evaluating ADULT or Adolescent (12th Birthday + One Day →)

Completed by_____
Date_____

#1 **Bathing**
Washing and cleaning the body with soap and water.

4. Full self care
3. Requires use of equipment or device
2. Requires assistance or supervision from another person
1. Dependent/does not participate

☐ Unable To Assess

#2 **Grooming**
Combing hair and attending to cleanliness activities – brushing teeth, shaving, etc.

4. Full self care
3. Requires use of equipment or device
2. Requires assistance or supervision from another person.
1. Dependent/does not participate

☐ Unable To Assess

#3 **Dressing**
Applying or putting on clothes, socks, shoes, etc.

4. Full self care
3. Requires use of equipment or device
2. Requires assistance or supervision from another person
1. Dependent/does not participate

☐ Unable To Assess

#4 **Toileting**
Managing the elimination of urine and stool.

4. Full self care
3. Requires use of equipment or device
2. Requires assistance or supervision from another person
1. Dependent/does not participate

☐ Unable To Assess

#5 **Ambulation**
Walking

5. Ambulates independently
4. Ambulates with assist from a device (e.g. cane, walker, etc.)
3. Ambulates with assist from a person
2. Chairbound (includes wheelchair)
1. Bedfast

☐ Unable To Assess

#6 **Patient's Fear**
Anticipated / perceived danger (frightened of something / someone).
☐ Financial ☐ Family ☐ Health
☐ Social ☐ Living Situation
☐ Other_____

4. None
3. Mild
2. Moderate
1. Severe

☐ Unable To Assess

#7 **Patient's Anxiety**
Nervous or restless behavior (patient cannot provide reason).

4. None
3. Mild – sleeplessness; repeats questions; fidgety
2. Moderate - difficulty concentrating; palpitations; tremors; tachypnea; difficulty adapting/analyzing.
1. Severe - unable to concentrate; hyperventilation; tachycardia; headache 'feeling of impending doom'

☐ Unable To Assess

#8 **Patient's Coping**
Ability to deal with problems / stress.

4. Effective - Able to cope with problems/stress.
3. Partially effective ability to cope with problems/stress.
2. Minimally effective attempts to cope with problems/stress.
1. Unable to cope with problems/stress.

☐ Unable To Assess

#9 **Symptom Status**
Presence of symptoms (physical, cognitive, and mental) (SOB, weakness, pain, confusion, etc) during tasks/activities.

4. Asymptomatic
3. Mild symptoms - during tasks/activities.
2. Moderate symptoms - during tasks/activities.
1. Symptoms present - even at rest.

☐ Unable To Assess

#9p **Pain Status**
Presence of pain during tasks/activities.

4. No Pain
3. Mild pain during tasks/activities.
2. Moderate pain during tasks/activities.
1. Pain present even at rest.

☐ Unable To Assess

#10 **Knowledge**
Learning required to understand and provide/direct own care.

4. Well informed - No learning needs identified.
3. Mild learning needs – minimal reinforcement required.
2. Minimally informed – Training on specifics required. Requires basic instruction in plan of care.
1. Uninformed – Extensive knowledge deficits.

☐ Unable To Assess

#11 **Patient Participation**
Implements acute and preventive healthcare recommendations.

4. Doing very well.
3. Mild encouragement needed.
2. Moderate encouragement needed.
1. Does not follow healthcare recommendations.

☐ Unable To Assess

Comments_____
_____
_____
_____
PCG's Name_____
Relationship_____

#12 **Primary Caregiver's Knowledge**
Learning required to understand and Provide/direct patient's care.

4. Well informed – no learning needs identified.
3. Mild learning needs – minimal reinforcement required.
2. Minimally informed – training on specifics required. Requires basic instruction in plan of care.
1. Uniformed – extensive knowledge deficits.

☐ Unable To Assess
☐ N/A (e.g. patient = self-care, in Board & Care, etc.)

#13 **Primary Caregiver's Burden**
Caregiver's perceived physical/emotional strain as a result of patient's current health.

4. None.
3. Mild.
2. Moderate.
1. Severe.

☐ Unable To Assess
☐ N/A (e.g. patient = self-care, in Board & Care, etc.)

#14 **Family Burden**
Physical or emotional strain on family as a result of patient's current health.

4. None.
3. Mild.
2. Moderate.
1. Severe.

☐ Unable To Assess
☐ N/A (e.g. no family/support system, etc.)

#15 **Family Coping**
Family/support system's ability to deal with problems/stress.

4. Doing very well.
3. Mild difficulty.
2. Moderate difficulty.
1. Severe / Unable to cope.

☐ Unable To Assess
☐ N/A (e.g. no family/support system, etc.)

Appendix B.

NKASRP.

Code # _____

Nurses' Knowledge and Attitudes Survey Regarding Pain

True/False - Circle the correct answer.

T	F	1.	Observable changes in vital signs must be relied upon to verify a patient's statement that he has severe pain.
T	F	2.	Because of an underdeveloped neurological system, children under 2 years of age, have decreased pain sensitivity and limited memory of painful experiences.
T	F	3.	If the patient can be distracted from his pain this usually means that he does NOT have high pain intensity.
T	F	4.	Patients may sleep in spite of severe pain.
T	F	5.	Comparable stimuli in different people produce the same intensity of pain.
T	F	6.	Aspirin and other nonsteroidal anti-inflammatory agents are NOT effective analgesics for bone pain caused by metastases.
T	F	7.	Non- drug interventions (e.g. heat, music, imagery, etc.) are very effective for mild-moderate pain control but are rarely helpful for more severe pain.
T	F	8.	Respiratory depression rarely occurs in patients who have been receiving opioids over a period of months.
T	F	9.	Aspirin 650 mg PO is approximately equal in analgesic effect to meperidine (Demerol) 50 mg PO.
T	F	10.	The World Health Organization (WHO) pain ladder suggests using single analgesic agents rather than combining classes of drugs (e.g. combining an opioid with a non-steroidal agent).
T	F	11.	The usual duration of action of meperidine (Demerol) IM is 4-5 hours.
T	F	12.	Research shows that promethazine (Phenergan) is a reliable potentiator of opioid analgesics.

Code # _____ 2

T F 13. Patients with a history of substance abuse should not be given opioids for pain because they are at high risk for repeated addiction.

T F 14. Beyond a certain dosage of strong opioids (e.g. morphine) increases in dosage will NOT increase pain relief.

T F 15. Elderly patients cannot tolerate strong medications such as opioids for pain.

T F 16. The patient with pain should be encouraged to endure as much pain as possible before resorting to a pain relief measure.

T F 17. Children less than 11 years cannot report pain with reliability and therefore, the nurse should rely on the parents' assessment of the child's pain intensity.

T F 18. Based on one's religious beliefs a patient may think that pain and suffering is necessary.

T F 19. After the initial recommended dose of opioid analgesic, subsequent doses are adjusted in accordance with the individual patient's response.

T F 20. In order to evaluate the effectiveness of non-drug interventions, the patient should be advised to use these techniques alone rather than concurrently with pain medications.

T F 21. Giving patients sterile water by injection (placebo) is a often useful test to determine if the pain is real.

T F 22. In order to be effective, heat and cold should only be applied to the painful area.

Code # _____ 3

Multiple Choice - Place a check by the correct answer.

23. The recommended route of administration of opioid analgesics to patients with prolonged cancer-related pain is

_____ a. intravenous
_____ b. intramuscular
_____ c. subcutaneous
_____ d. oral
_____ e. rectal
_____ f. I don't know

24. The recommended route of administration of opioid analgesics to patients with brief, severe pain of sudden onset, e.g. trauma or postoperative pain, is

_____ a. intravenous
_____ b. intramuscular
_____ c. subcutaneous
_____ d. oral
_____ e. rectal
_____ f. I don't know

25. Which of the following analgesic medications is considered the drug of choice for the treatment of prolonged moderate to severe pain for cancer patients?

_____ a. Brompton's cocktail
_____ b. codeine
_____ c. morphine
_____ d. meperidine (Demerol)
_____ e. methadone
_____ f. I don't know

26. Which of the following IV doses of morphine would be equivalent to 30 mg of oral morphine?

_____ a. Morphine 5 mg IV
_____ b. Morphine 10 mg IV
_____ c. Morphine 30 mg IV
_____ d. Morphine 60 mg IV

27. Analgesics for post-operative pain should initially be given

_____ a. around the clock on a fixed schedule
_____ b. only when the patient asks for the medication
_____ c. only when the nurse determines that the patient has moderate or greater discomfort

Code # _____ 4

28. A patient with chronic cancer pain has been receiving daily opioid analgesics for 2 months. The doses increased during this time period. Yesterday the patient was receiving morphine 200 mg/hour intravenously. Today he has been receiving 250 mg/hour intravenously for 3 hours. The likelihood of the patient developing clinically significant respiratory depression is

_____ a. less than 1%
_____ b. 1-10%
_____ c. 11-20%
_____ d. 21-40%
_____ e. > 41%

29. Analgesia for chronic cancer pain should be given

_____ a. around the clock on a fixed schedule
_____ b. only when the patient asks for the medication
_____ c. Only when the nurse determines that the patient has moderate or greater discomfort

30. The <u>most likely</u> explanation for why a patient with pain would request increased doses of pain medication is

_____ a. The patient is experiencing increased pain.
_____ b. The patient is experiencing increased anxiety or depression.
_____ c. The patient is requesting more staff attention.
_____ d. The patient's requests are related to addiction.

31. Which of the following drugs are useful for treatment of cancer pain?

_____ a. Ibuprophen (Motrin)
_____ b. Hydormorphone (Dilaudid)
_____ c. Amitriptyline (Elavil)
_____ d. All of the above

32. The most accurate judge of the intensity of the patient's pain is

_____ a. the treating physician
_____ b. the patient's primary nurse
_____ c. the patient
_____ d. the pharmacist
_____ e. the patient's spouse or family

Code # _____ 5

33. Which of the following describes the best approach for cultural considerations in caring for patients in pain:

_____ a. Because of the diverse and mixed cultures in the United States, there are no longer cultural influences on the pain experience.

_____ b. Nurses should use knowledge that has defined clearly the influence of pain on culture (e.g. Asian patients are generally stoic, Italians are expressive and exaggerate their pain, etc.

_____ c. Patients should be individually assessed to determine cultural influences on pain.

34. What do you think is the percentage of patients who <u>over report</u> the amount of pain they have? **Circle the correct answer.**

0 10 20 30 40 50 60 70 80 90 100%

35. <u>Narcotic/opioid addiction</u> is defined as psychological dependence accompanied by overwhelming concern with obtaining and using narcotics for psychic effect, not for medical reasons. It may occur with or without the physiological changes of tolerance to analgesia and physical dependence (withdrawal).

Using this definition, how likely is it that opioid addiction will occur as a result if treating pain with opioid analgesics? **Circle the number closest to what you consider the correct answer.**

< 1% 5% 25% 50% 75% 100%

Code # _____ 6

Case Studies

Two patient case studies are presented. For each patient you are asked to make decisions about pain and medication.

Patient A (Questions 36 and 37)

Andy is 25 years old and this is his second day following abdominal surgery. As you enter his room to check his vital signs, he smiles at you and continues talking and joking with his visitor. Your assessment yields the following information: B/P = 120/80; HR = 80; RR = 18; on a scale of 0 - 5 (0 = no pain/discomfort to 5 = worst pain/discomfort). Andy rates his pain as "4" at the surgical site.

1. On the patient's record you must mark his pain on the scale below. **Circle the number that represents your assessment of Andy's pain.**

0	1	2	3	4	5
No pain/ discomfort					Worst/pain discomfort

2. Your assessment above, is made four hours after Andy received morphine 10 mg IM. During the three hours following the injection, Andy's pain ratings ranged from 3 to 4 and he had no clinically significant respiratory depression, sedation, or other untoward side effects. His physician's order for analgesia is "Morphine IM 5 to 15 mg Q 3 - 4 hours PRN pain relief". **Check the action you will take at this time.**

_____ a. Administer no morphine at this time.

_____ b. Administer morphine 5 mg IM now.

_____ c. Admi..ister morphine 10 mg IM now.

_____ d. Administer morphine 15 mg IM now.

Code # _____ 7

Patient B (Questions 38 and 39)

Bob is 25 years old and this is his second day following abdominal surgery. As you enter his room to check his vital signs, he is lying quietly in bed and grimaces as he turns in bed. Your assessment yields the following information: B/P = 120/80; HR = 80; RR = 18; on a scale of 0 - 5 (0 = no pain/discomfort to 5 = worst pain/discomfort). Bob rates his pain as "4" at the surgical site.

1. On the patient's record you must mark his pain on the scale below. **Circle the number that represents your assessment of Bob's pain.**

0	1	2	3	4	5
No pain/ discomfort					Worst/pain discomfort

2. Your assessment above, is made four hours after Bob received morphine 10 mg IM. During the three hours following the injection, Bob's pain ratings ranged from 3 to 4 and he had no clinically significant respiratory depression, sedation, or other untoward side effects. His physician's order for analgesia is "Morphine IM 5 to 15 mg Q 3 - 4 hours PRN pain relief". **Check the action you will take at this time.**

_____ a. Administer no morphine at this time.

_____ b. Administer morphine 5 mg IM now.

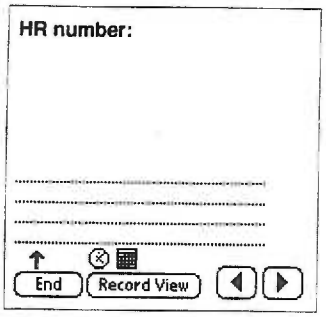
_____ c. Administer morphine 10 mg IM now.

_____ d. Administer morphine 15 mg IM now.

Appendix C.

Form Name: **pain study interview**
Patient interview.

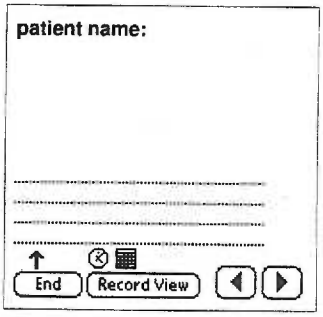
Field: 1



Field Type: **Freeform text**

Parameters:

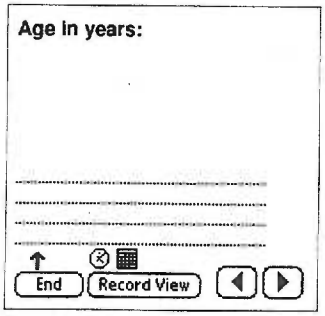
Field: 2



Field Type: **Freeform text**

Parameters:

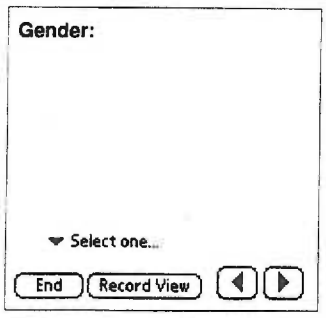
Field: 3



Field Type: **Freeform text**

Parameters:

Field: 4



Field Type: **Popup list**

Parameters: **Male**
Female

Field: 5

Field Type: **Date Only**

Parameters:

Form Name: **pain study interview**

Field: 6

Had pain in last 24 hours?:

Yes | No

End | Record View

Field Type: **Yes or No**

Parameters:

Field: 7

1cause of your pain

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

related to admitting Dx
related to treatment

Field: 8

2cause of your pain

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

acute
chronic

Field: 9

Best pain relief in last 24 hours:

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

0
1
2
3
4
5
6
7
8
9

10

Field: 10

Worst pain you had in last 24 hours

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

0
1
2
3
4
5
6
7
8
9

10

Form Name: **pain study interview**

Field: **11**

What is your pain right now

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

0 1 2 3 4 5 6 7 8 9	10

Field: **12**

What is level of pain you could live better and enjoy normal activities

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

0 1 2 3 4 5 6 7 8 9	10

Field: **13**

How frequently were you asked about your pain in the last 24 hours?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

never daily about every 8 hours about every 4 hours about every 2 hours	

Field: **14**

Were you asked about your pain after treatment?

Yes | No

End | Record View

Field Type: **Yes or No**

Parameters:

Field: **15**

1Who asked you about your pain?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

RN CNA MD PT OT none	

Form Name: **pain study interview**

Field: **16**

2Who asked you about your pain?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

RN CNA MD PT OT none	

Field: **17**

3Who asked you about your pain?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

RN CNA MD PT OT none	

Field: **18**

1Are you using treatments other than medicines for pain treatment?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Field: **19**

2Are you using treatments other than medicines for pain treatment?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Field: **20**

3Are you using treatments other than medicines for pain treatment?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Form Name: **pain study interview**

Field: **21**

4Are you using treatments other than medicines for pain treatment?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Field: **22**

1What was offerer by the nurse?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Field: **23**

2What was offerer by the nurse?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Field: **24**

3What was offerer by the nurse?

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Field: **25**

Where the treatment choices acceptable?

Yes | No

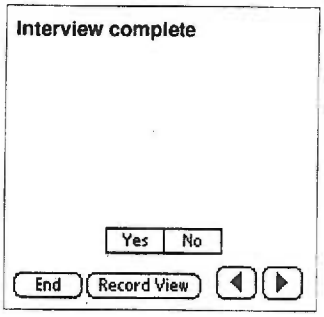
End | Record View

Field Type: **Yes or No**

Parameters:

Form Name: **pain study interview**

Field: **26**



Field Type: **Completion checkbox**

Parameters:

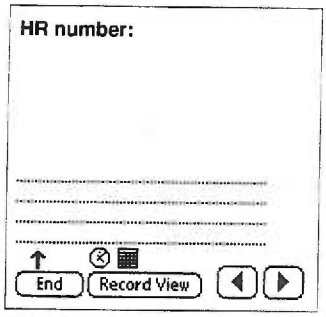
Appendix D.

Form Name: **pain study chart review**

Chart review.

Page 1
Printed 5/1/99 2:56:46 PM

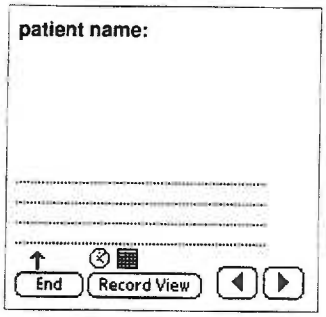
Field: 1



Field Type: **Freeform text**

Parameters:

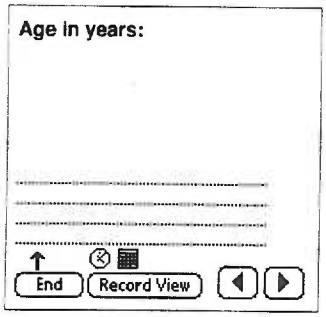
Field: 2



Field Type: **Freeform text**

Parameters:

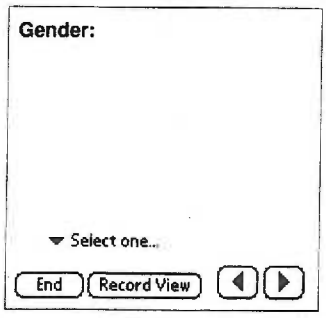
Field: 3



Field Type: **Freeform text**

Parameters:

Field: 4



Field Type: **Popup list**

Parameters:
Male
Female

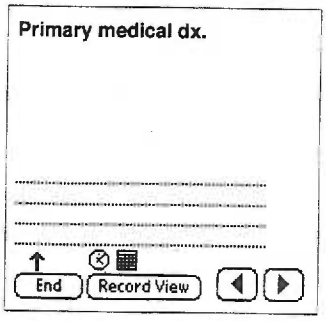
Field: 5

Field Type: **Date Only**

Parameters:

Form Name: **pain study chart review**

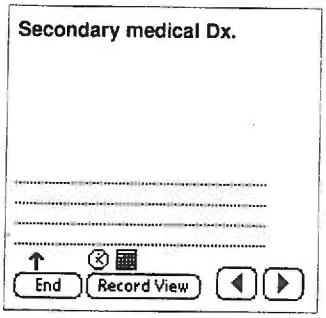
Field: **6**



Field Type: **Freeform text**

Parameters:

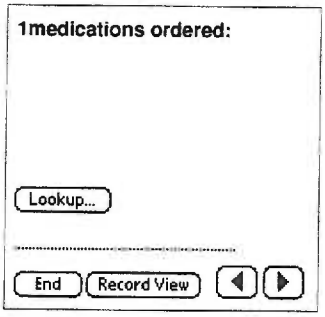
Field: **7**



Field Type: **Freeform text**

Parameters:

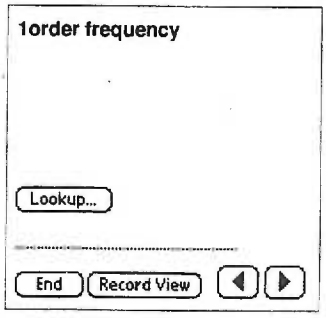
Field: **8**



Field Type: **Lookup list**

Parameters: **drugs**

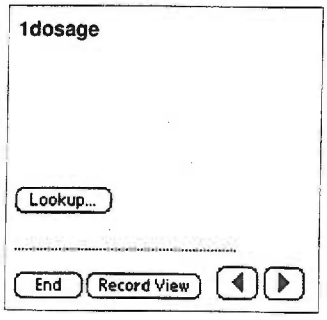
Field: **9**



Field Type: **Lookup list**

Parameters: **order frequency**

Field: **10**

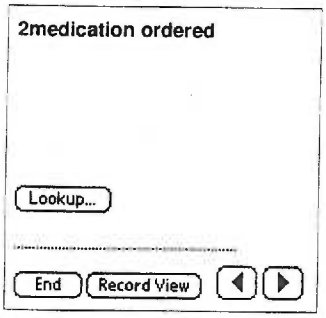


Field Type: **Lookup list**

Parameters: **dosage**

Form Name: **pain study chart review**

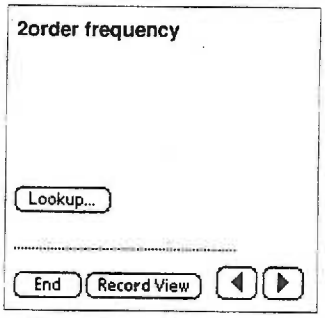
Field: 11



Field Type: **Lookup list**

Parameters: **drugs**

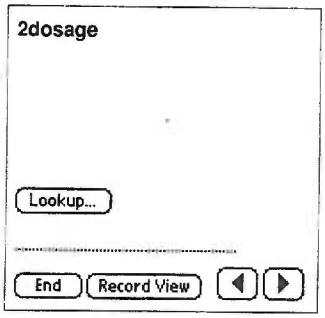
Field: 12



Field Type: **Lookup list**

Parameters: **order frequency**

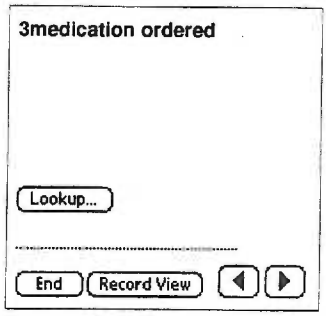
Field: 13



Field Type: **Lookup list**

Parameters: **dosage**

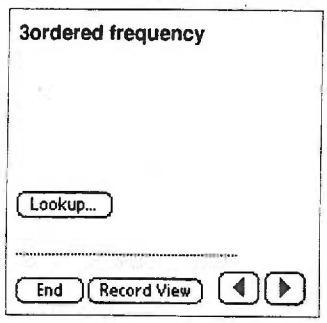
Field: 14



Field Type: **Lookup list**

Parameters: **drugs**

Field: 15

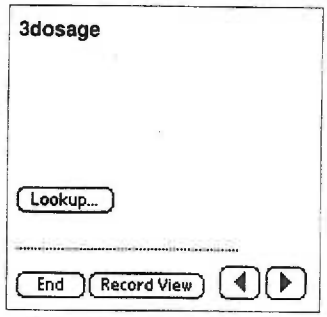


Field Type: **Lookup list**

Parameters: **order frequency**

Form Name: **pain study chart review**

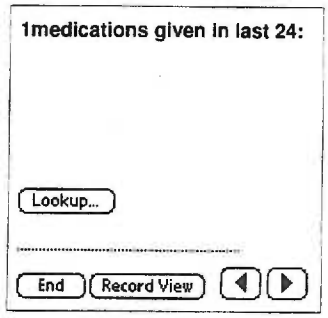
Field: **16**



Field Type: **Lookup list**

Parameters: **dosage**

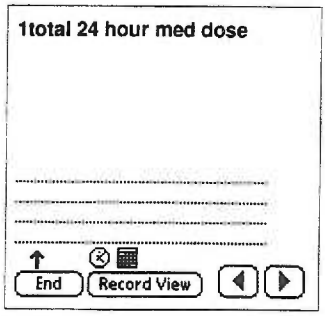
Field: **17**



Field Type: **Lookup list**

Parameters: **drugs**

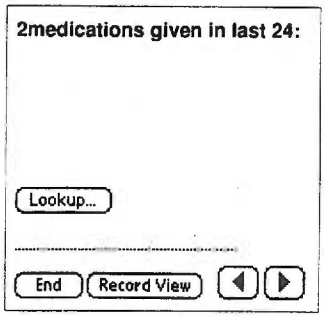
Field: **18**



Field Type: **Freeform text**

Parameters:

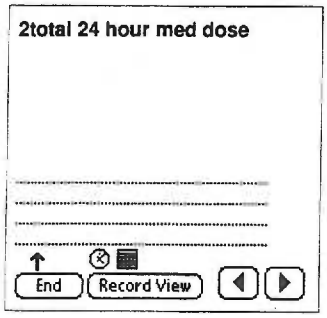
Field: **19**



Field Type: **Lookup list**

Parameters: **drugs**

Field: **20**

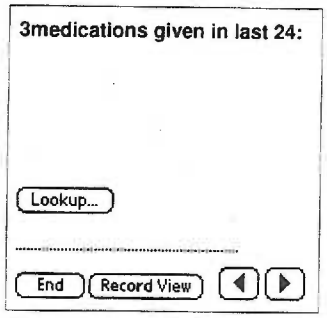


Field Type: **Freeform text**

Parameters:

Form Name: **pain study chart review**

Field: **21**

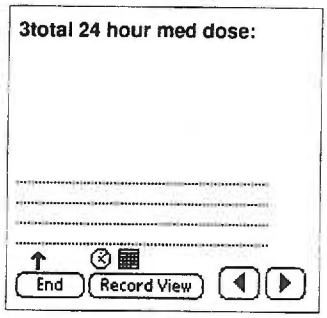


Field Type: **Lookup list**

Parameters:

drugs	

Field: **22**



Field Type: **Freeform text**

Parameters:

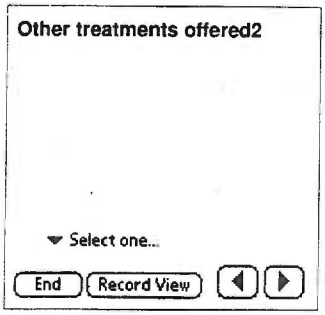
Field: **23**

Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Field: **24**

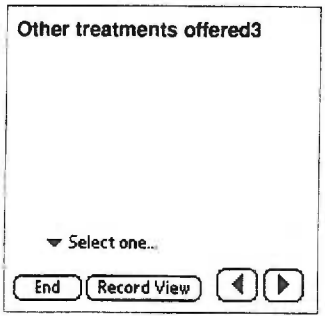


Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Field: **25**



Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Form Name: **pain study chart review**

Field: **26**

Other treatments offered4

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

none Cold Heat position chge TENS unit Imagery Hypnosis Massage Relaxation Music	Information about a procedure

Field: **27**

Pain assessment per care plan

Yes | No

End | Record View

Field Type: **Yes or No**

Parameters:

Field: **28**

Protocol in use:

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

General medical Surgical	

Field: **29**

Assessment used 0-10 scale

Yes | No

End | Record View

Field Type: **Yes or No**

Parameters:

Field: **30**

Assessment frequency per last 24 hours

Select one...

End | Record View

Field Type: **Popup list**

Parameters:

None 1 2 every 8 every 4 every 2	

Form Name: **pain study chart review**

Field: **31**

Evaluation of medication effectiveness

Yes | No

End | Record View

Field Type: **Yes or No**

Parameters:

Field: **32**

Evaluation of other modalities

Yes | No

End | Record View

Field Type: **Yes or No**

Parameters:

Field: **33**

Assigned nurse1

Lookup...

End | Record View

Field Type: **Exclusive lookup list**

Parameters: **Study nurses**

Field: **34**

Assigned nurse 2

Lookup...

End | Record View

Field Type: **Exclusive lookup list**

Parameters: **Study nurses**

Field: **35**

Assigned nurse3

Lookup...

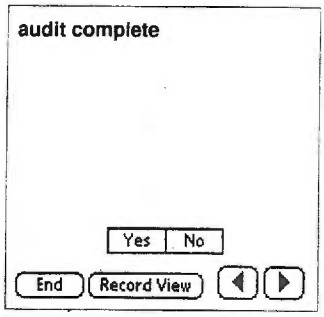
End | Record View

Field Type: **Exclusive lookup list**

Parameters: **Study nurses**

Form Name: **pain study chart review**

Field: **36**



Field Type: **Completion checkbox**

Parameters: