NEURONAL TRANSPLANTS USED TO REPAIR ISCHEMIC LESIONS IN THE RAT CENTRAL NERVOUS SYSTEM: HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND ELECTROPHYSIOLOGICAL CHARACTERIZATION OF DEVELOPMENT AND FUNCTION

BY

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Cerebral ischemia can be caused by many diverse conditions such as cardiac arrest and severe hypotension and is often the cause of secondary brain damage following head injury or infantile birth trauma. The inadequate cerebral blood flow can result in the permanent loss of essential brain circuitries and neurological deficits. Since methods are not currently available for the prevention of neuronal loss following cerebral ischemia, experiments were undertaken to investigate whether transplanted neurons had the potential to afford some measure of repair.

In the present study a model was developed whereby the hippocampal CA1 region of the rat brain could be lesioned by transient forebrain ischemia and subsequently repopulated with suspensions of fetal hippocampal tissue. Histological, immunohistochemical and electrophysiological techniques were used to examine the functional capabilities of the transplanted neurons by assessing their ability to survive when transplanted into the degenerating brain region, to develop intrinsic properties characteristic of the cells they had replaced and, to integrate into the host tissue by reestablishing some of the normal synaptic circuitries.

The ability of the transplanted neurons to remain viable when placed into a degenerating environment was confirmed by the histological demonstration of $^3$H-thymidine labelled neurons in the lesioned region and the demonstration that the transplanted neurons were electrically active.

Histological and immunohistochemical techniques showed that the transplanted neurons developed cytological features that were indistinguishable from their normal counterparts, showed a remarkable degree of organization, and expressed some of the same neuron specific proteins; specifically calbindin-D$_{28K}$ and parvalbumin. Acetylcholinesterase histochemistry and retrograde axonal transport of Fluoro-Gold demonstrated that some afferent and efferent fibre projections could be reinstated.
Electrophysiological recordings confirmed the functional integration of the transplanted neurons in the host tissue. Intracellular and extracellular recordings showed that the restored neural connections formed functional synaptic contacts and that the transplanted neurons developed biophysical properties that were similar to the cells they had replaced.

The data from the present study have demonstrated that transplanted neurons can develop many of the anatomical and electrophysiological properties that are characteristic of the adult cells they have replaced and consequently, have the potential to mediate normal functional capabilities.
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DEDICATION

I would like to dedicate this thesis to my Mom and Dad who have always provided me with their total support and encouragement. Thank you for always being there and reminding me to stop and smell the roses.
CHAPTER 1

GENERAL INTRODUCTION

The aims of this chapter are to describe the rationale for undertaking this investigation, to provide the reader with background information in the field of transplantation research, and to present the objectives of the study.
1. GENERAL INTRODUCTION

I. RATIONALE

Nervous tissue is absolutely dependent upon oxidative metabolism for maintenance of its functional and structural integrity. The oxygen reserves in the brain are extremely low and therefore the brain requires continuous replenishment of its oxygen supply by the circulation. The oxygen content of the arterial blood and the cerebral blood flow determine how much oxygen is available to the brain and if either are compromised, irreversible neuronal damage and neurological deficits can ensue (Sokoloff, 1981). The term cerebral ischemia is strictly defined as a reduction of cerebral blood flow but is now commonly used to describe situations whereby the oxygen supply to the brain is inadequate for its metabolic demands. Therefore, "ischemic brain damage" can result when; the blood contains insufficient oxygen, the brain is unable to use the available oxygen, or the cerebral blood flow is inadequate (Graham, 1985). Cerebral ischemia can occur during many diverse conditions such as cardiac arrest, seizure activity and severe hypotension and, often follows head injury and infantile birth trauma (Dearden, 1985) (Table 1.1). Even in the absence of disease, cerebral ischemia remains a potential hazard of general anesthesia.

While the extent of ischemic damage is largely dependent upon the duration and degree of the particular insult, the location of the cell damage can often be related to the type of ischemia; whether it is focal (localized brain region) or global (the entire brain), complete (no blood flow) or incomplete (greatly reduced blood flow) (Table 1.1). It is known that neurons are much more susceptible to damage than glia (Jacob, 1963) and within the family of neurons, a heirarchy of neuronal vulnerability exists (Table 1.2). The CA1 region of the hippocampal formation has been shown consistently to be the most vulnerable brain region both in rats (Brierly, 1977; Brown, 1977; Diemer & Seimkowitz, 1981; Graham, 1977, 1985; Pulsinelli and Brierly, 1979; Pulsinelli, 1985; Smith et al, 1984a) and humans (Hachinski, 1979; Volpe and Petito, 1985; Zola-Morgan et al, 1986).
Table 1.1

Summary of the 3 major causes of cerebral ischemia (after Dearden, 1985)
Causes of Cerebral Ischemia

REDUCED BLOOD OXYGEN

- Incomplete Global Ischemia
  - REDUCED INSPIRED OXYGEN
    - High Altitude
    - Anesthetic Errors
  - REDUCED OXYHAEMOGLOBIN
    - CO Poisoning
    - Anemia

INADEQUATE CEREBRAL BLOOD FLOW

- Complete Global Ischemia
  - CEREBRAL BLOOD FLOW STOPPED
    - Cardiac arrest

- Incomplete Global Ischemia
  - CEREBRAL BLOOD FLOW REDUCED
    - Cardiac Failure
    - Severe Hypotension

- Incomplete Focal Ischemia
  - INCREASED OXYGEN USE
    - Seizure Activity
  - INCREASED VASCULAR RESISTANCE
    - Vascular Occlusion
    - Compression
  - INCREASED INTRACRANIAL PRESSURE WITH REDUCED CEREBRAL PERFUSION PRESSURE
    - Vascular Engorgement
    - Cerebral Oedema

FAILURE TO USE OXYGEN

- Incomplete Global or Focal Ischemia
  - POSTOCCLUSION STATES
  - POISONING OF OXIDATIVE ENZYMES
    - Cyanide
    - Azide
Table 1.2

Arrows indicate the order of decreasing neuronal vulnerability to damage by cerebral ischemia (after Pulsinelli, 1985)
Hippocampal Formation - CA1, Subiculum pyramids > CA4 > CA3 > granule cells

Cerebellum - Purkinje > stellate and basket > granule > Golgi cells

Striatum - small and medium sized neurons > large neurons

Neocortex - parieto-occipital > temporo-frontal
- layers 3, 5 and 6 > layers 2 and 4
Due to advances in medical care, survival from insults such as myocardial infarction and cardiac arrest has increased dramatically which has made cerebral ischemia one of the leading causes of morbidity (Bedell et al, 1983; Longstreth et al, 1983). Recently, cognitive impairments of learning and memory have been recognized as the major permanent disability (Volpe and Petito, 1985). Many pharmacological attempts have been made to prevent the pathophysiological processes which follow ischemia and while some treatments have been successful in preventing damage in peripheral tissues (Borgers, 1985) none have been very effective in the central nervous system (CNS) (Hinds, 1985; Little et al, 1987; Scheinberg, 1979; Symon, 1985).

Unfortunately, neurons in the adult mammalian brain are unable to undergo mitosis to produce new somata and therefore regeneration is extremely limited. Although events such as collateral axonal sprouting and new synapse formation from undamaged fibres can occur (Cotman, 1985; Cotman and Anderson, 1988; Lui and Chambers, 1958), this "reactive synaptogenesis" does not have the capacity to halt progressive degeneration or to repair lesions resulting from acute injury. Such progressive structural alterations following brain injury may lead to secondary degeneration and/or the formation of abnormal neural circuitries which can be deleterious. For example, retrograde cell death can occur to a population of neurons following loss of their target neurons (Sofroniew et al, 1987). Also, it is well known that epileptiform activity is a clinical sign and symptom of cerebral disorder and the onset of recurring seizures may follow injury to the brain with a delay of weeks, months or years (Gumnit, 1980). The reactive responses to injury may not be confined to the site of damage and may also occur within synaptic circuitries distant to this region (Hoff, 1986). More specifically, the loss of CA1 hippocampal neurons following ischemia can cause CA3 neurons to become hyperexcitable and prone to epileptiform activity (Frank et al, 1988).

During the past decade, evidence has accumulated indicating that transplantation of healthy neuronal tissue is a promising approach for the amelioration of neurological dysfunction (see Bjorklund and Stenevi, 1984; Cotman et al, 1984; Fine, 1986; Fishman,
1986; Gash et al, 1985; Wilberger, 1983; for reviews). Although dramatic advances have been made in the application of neuronal transplantation techniques to problems of some degenerative brain diseases (Bjorklund et al, 1987), surprisingly little research has focused on the use of these techniques to repair brain regions that have been damaged by acute insults such as cerebral ischemia.

In light of this and the fact that ischemic damage is currently almost impossible to prevent, it was of great interest to investigate whether a necrotic region lesioned by ischemia could be repopulated with fetal neurons and subsequently support their survival and integration into the host tissue. It was hoped that the initiation of neurotrophic and neurohumoral mechanisms by the damaged host and the transplant would not only support the survival, development and integration of the transplanted tissue within the host brain but also enhance the regenerative potential of the host by encouraging "appropriate" reactive synaptogenesis. The possibility existed that the transplanted neurons could provide the means for the generation of new efferent projections and prevent the retrograde death of intact afferent projections by providing them with a target. Although, it was likely that the exact reconstruction of the damaged region would not be attained, the transplanted neurons had the potential to afford some measure of repair (Sotelo and Alvarado-Mallart, 1986, 1987a, 1987b). A demonstration of the potential functional capabilities of the transplanted neurons would also have important implications for the clinical management of ischemia induced neurological deficits.

II. TECHNICAL CONSIDERATIONS AND BACKGROUND

A. Factors Critical for Survival

It has now been 100 years since the first documented mammalian brain transplantation experiments were undertaken (Thompson, 1890). The objective of this early study was not to restore function but described the transplantation of mature cat cortex into the cortex of adult dogs. Although it was suggested that the tissue showed some viability after 7 weeks, these data remain controversial due to the lack of observable viable neurons in
the drawings. It is assumed that although a grafted plug was intact, neuronal tissue had subsequently degenerated. Sporadic reports appeared during the next 80 years (reviewed by Bjorklund and Stenevi, 1984; Borges, 1988; Gash, 1984; Gash et al, 1985) which focused on the ability of the transplanted neurons to survive. The fact that fully differentiated neuronal tissue would not survive transplantation procedures was confirmed by Saltykow (1905). Mature cortical tissue which was transplanted into the cortex of young rabbits was shown to degenerate fairly rapidly. In 1917, Dunn reported that cortical tissue from relatively immature rats survived transplantation when placed into a cortical cavity which was in contact with the choroid plexus of the lateral ventricles. The study of Le Gros Clark (1940) was one of the greatest contributions to our understanding of the capabilities of transplanted brain tissue. Fetal cortical tissue was transplanted into young hosts to determine whether the cells could undergo normal differentiation and attain cytological characteristics of the corresponding mature nerve cells. Solid tissue pieces including the neuroepithelium were transplanted deep into the host brain to the level of the lateral ventricles. The neurons were able to undergo remarkably normal differentiation and were mature in appearance. The transplanted pyramidal neurons were oriented correctly and sent out apical dendrites in the same direction. Further, cells of specific types and sizes tended to group together and occasionally form irregular clumps or laminar formations. Le Gros Clark concluded that the differentiation of cell types and the ability to organize into "like" groups was independent of the connections found normally in a developing environment.

The concept of using neuronal transplantation techniques as a therapeutic mechanism rapidly gained momentum in parallel with the gradual acceptance of the fact that the adult CNS was a dynamic rather than a static system and had the ability to sprout collateral fibres and form new synaptic contacts following injury (Lui and Chambers, 1958). By labelling the tissue to be transplanted, in utero with $^3$H-thymidine, Das and Altman (1971) unequivocally demonstrated that donor rat tissue still undergoing neurogenesis could survive, differentiate into cytologically mature neurons and migrate when transplanted to the surface of the cerebellum. It was not until 1976 that a detailed report described the factors that were
essential for the survival and integration of neuronal tissue in the adult mammalian CNS (Stenevi et al, 1976). This study confirmed earlier observations and demonstrated that two factors were critical; the donor tissue had to be at a relatively early stage of development and the transplanted tissue required rapid vascularization and/or contact with the cerebrospinal fluid. Further, by demonstrating that transplanted tissue could survive in adult hosts this study initiated great interest in transplantation research and provided the basis for future studies on the therapeutic potential of neuronal transplantation.

**B. Transplantation Methodologies**

Techniques for the transplantation of nervous tissue into the mammalian brain have been utilized since the turn of the century. The method initially employed for the transplantation of neuronal tissue involved placing pieces of freshly excised donor tissue into a small cavity in the cortex of the host. This technique was used with mixed results to investigate the survivability of the transplanted tissue (Das and Altman, 1971,1972; Saltykow, 1905; Thompson, 1890). A variation of this technique was that described by Stenevi et al (1976) whereby a portion of the cortex was removed to expose the vascular bed of the choroid plexus. Although this technique greatly enhanced the survival of the transplanted tissue the areas acceptable for transplantation were extremely limited.

A two-stage procedure was then developed which involved the aspiration of the cortical tissue above the desired transplantation region followed by closure of the wound for 3 to 6 weeks (Stenevi et al, 1980). This provided a dense vascular bed to form around the edges of the cavity and further enhanced the survival of the transplanted tissue pieces.

A third technique involved injecting small pieces of tissue through a glass syringe to regions deep in the brain parenchyma (Das, 1974; Le Gros Clark, 1940; Lund and Haushka, 1976; Sunde and Zimmer, 1981; Willis, 1935). The attractiveness of this technique was that minimal surgical damage was produced in the regions above the transplantation site. Unfortunately, unless the pieces of tissue were quite small or were in contact with the ventricular space, neuron survival was low.
More recently a technique was described whereby standard tissue culture procedures were utilized to dissociate the tissue into cell suspensions which was subsequently transplanted through a fine injection cannula (Björklund et al, 1983; Schmidt et al, 1981). This procedure allowed tissue to be transplanted into deep structures with minimal observable damage to the overlying cortex. In addition, the number of cells injected could be monitored and injected into multiple precisely located sites.

C. Factors Enhancing Survival and Growth

i) Age of Tissue

Studies have shown that the survival of transplanted tissue is greatly enhanced if the tissue is obtained during the developmental period in which neuron proliferation and migration are occurring (Das et al, 1980; Kromer et al, 1983). The transplantation of solid tissue pieces often leaves the neuroepithelium intact thus allowing neurogenesis and development of the fetal tissue to continue in the host brain in a normal manner. Consequently, good survival of transplanted tissue can occur over a wide range of developmental ages. In contrast, survival following mechanical dissociation occurs only if the tissue is obtained during neurogenesis or migration. Once the neurons have reached the cortical plate they extend axonal and dendritic processes. It is thought that the dissociation procedure damages these processes thereby preventing cell survival due to an inability of the cells to reinitiate this developmental program. These studies demonstrated that the critical period for transplanted neuron survival was dependent upon the timing of neurogenesis for each specific tissue type.

ii) Neurotrophic Factors

One hypothesis states that in the fully innervated undamaged adult CNS there exists a dynamic balance of growth-inhibiting substances and growth-promoting substances (Cotman et al, 1984). Following injury to the brain, astrocytes (primarily type 1) undergo marked proliferation (Lindsay, 1986). These glial cells are thought to be involved in the phagocytosis of neuronal debris and have been shown to increase neurite outgrowth from cultured neurons
Injury to the brain also elicits a delayed release of growth-promoting factors which enhance the survival of transplanted neurons (Collins and Crutcher, 1985; Heacock et al, 1986; Nieto-Sampedro et al, 1982, 1983, 1984; Manthorpe et al, 1983). The time course for this enhanced neurotrophic activity has been correlated with the occurrence of reactive gliogenesis. Over time, neurotrophic quantities may decrease due to a reduction of gliotic levels and/or reinnervation resulting from local collateral sprouting and thereby reinstate the balance of growth inhibiting and growth promoting substances. Therefore, for optimal growth promoting conditions, transplantation procedures should be undertaken at the peak of reactive gliogenesis before extensive collateral sprouting can occur.

iii) Immunological Advantages

The observation that tissue transplanted into the adult brain parenchyma was not aggressively rejected has been coined "immunologic privilege" (Medawar, 1948). Even when neuronal transplantation was undertaken between two immunologically incompatible rat strains (allografts) and between two different species (xenografts) rejection did not occur (Daniloff et al, 1985; Low et al, 1983; Sorensen et al, 1987).

Due to an increasing interest in brain transplantation as a therapeutic tool, the mechanisms underlying this phenomenon are being investigated but remain poorly understood. Initially three factors were thought to influence survival of transplanted tissue in the CNS (reviewed by Head and Billingham, 1985; Fuchs and Bullard, 1988). Firstly, the lymphatic system which mediates the immune response is thought to be absent in the CNS (Yoffey and Courtice, 1970). Secondly, the expression of class II antigens of the major histocompatibility antigen complex (MHC) by antigen presenting cells, which allow the immune system to differentiate between foreign and self substances, may be attenuated greatly (Skoskiewicz et al, 1985; Wong et al, 1985). Lastly the brain parenchyma may possess an extremely low density of antigen presenting cells which are essential for the initiation of the immune response (Hart and Fabre, 1981).
More recent studies have shown that "immunologic privilege" is a relative term. When a comparison was made between isografts (genetically identical) and allografts (genetically dissimilar) it was shown that although isografts were inflammation free, inflammatory cells were found around the allografts. Immunohistochemical techniques detected both major T-cell subsets and antigen presenting cells including macrophages in and around the allogenic grafts. It was suggested that the size of the transplant and the degree of histocompatibility expression may underly the absence of immune attack (Geyer et al, 1985; Nicholas et al, 1987) and confirmed that immunological rejection was not a concern when undertaking experimental studies in genetically similar animals.

D. Neuronal Transplantation as a Therapeutic Tool

During the past decade neuronal transplantation in the mammalian brain has followed primarily three avenues. Firstly, transplanted neurons have been used to study the conditions and factors that influence nerve cell maturation, development of intrinsic properties and the specificity of connections when isolated from the developing environment. Secondly, neuronal transplantation has been used as an investigative tool to understand better the anatomical and physiological interactions between specific neuronal pools. Lastly, and relevant to the present discussion, transplanted neurons have been used as a therapeutic mechanism in an attempt to restore neurological dysfunction.

There have been numerous approaches for applying neuronal transplantation techniques for the purpose of restoring lost neurological function. These have been determined largely by the type of deficit to be restored. By lesioning specific neuronal pools or fibre tracts in the brains of lower mammals or by using animals that genetically lack specific or possess nonfunctional neuronal pools, a number of human chronic and degenerative disorders have been modelled (reviewed by Gash et al, 1985). For example: 1) Brattleboro rats or neurohypophysectomized rats, which lack vasopressin producing neurons, model diabetes insipidus 2) lesions to the acetylcholine producing septal-diagonal band neurons or their projections fibres can produce cognitive deficits which model the learning and memory deficits
characteristic of aging and disease (eg. Alzheimer’s), 3) neurotoxin induced lesions to the dopamine containing cells of the nigrostriatal system, cause specific motor deficits that can be related to the motor dysfunction characteristic of Parkinson’s disease, and 4) excitotoxic lesions to the neostriatum cause behavioural abnormalities similar to those observed in patients with Huntington’s disease.

Marked success in reversing these behavioural deficits has been obtained when neuronal transplantation was undertaken following the experimentally induced lesions. The transplantation of vasopressinergic neurons into the lateral ventricle of neurohypophysectomized rats has been shown to restore the animals’ ability to regulate fluid (Gash et al, 1984). Septal-diagonal band neurons transplanted either close to the denervated or aged hippocampus (Daniloff et al, 1985; Dunnett et al, 1982; Gage et al, 1984; Gage and Bjorklund, 1986; Low et al, 1982; Nilson et al, 1987) or the deafferented neocortex (Dunnett et al, 1985,1987; Fine et al, 1985) have been shown to improve learning deficits in rats. Monoaminergic neurons transplanted into the denervated striatum have been able to correct experimentally induced motor deficits in mice, rats and subhuman primates (Bakay et al, 1985; Bjorklund et al, 1980; Brundin et al, 1986; Dunnett et al, 1981a, 1981b, 1983a, 1983b; Kamo et al, 1986; Redmond et al, 1986) and transplantation of striatal tissue into the lesioned neostriatum can reverse locomotor abnormalities caused by the lesion (Deckel et al, 1983, 1986; Isacson et al, 1985, 1986; Sanberg et al, 1986).

More recently, the ability of transplanted tissue to ameliorate neurological dysfunction has been tested during 5 years of clinical experimentation in Parkinson’s patients. In these patients, loss of the dopamine (a catecholamine) producing neurons in the substantia nigra is thought to be primary in the manifestation of the motor deficits characteristic of this disease (Backlund et al, 1985, 1987; Madrazo et al, 1987; Lindvall et al, 1987). Variable degrees of success have been reported after patients have received catacholamine producing transplants from their own adrenal medulla into the caudate nucleus or putamen.
Although these studies have demonstrated that neurological and behavioural deficits can be partially ameliorated by neuronal transplants, the actual mechanisms underlying these behavioural changes are not well understood. In the aforementioned studies, the tissue has generally been transplanted close to the major terminal field of the lesioned population of neurons or fibre bundles rather than directly into the lesioned area. Although this type of transplantation may have facilitated short neurite projections from the graft to the host, it would restrict greatly normal afferent inputs that would otherwise have impinged upon the grafted cells had they been placed in their appropriate environment: the site of neurodegeneration. It is now evident that a number of factors may work in concert to influence functional recovery (Bjorklund et al., 1987). These may include graft-host synaptic connections, trophic enhancement of regeneration by undamaged host tissue, and/or neurohumoral release of hormones or neurochemicals specifically by the transplant or nonspecifically, due to surgical trauma, by the host.

Whatever the mechanisms, these studies have shown that transplanted brain tissue can mediate some degree of functional recovery in a unidirectional system where reconstruction of neural circuitries may not be essential.

**III. OBJECTIVES**

Although this pioneering work laid the foundation for understanding the influences transplanted tissue could have on the damaged brain, few studies have been initiated for the purpose of investigating the functional capabilities of transplanted neurons to repair acute brain lesions. By focusing on the repair of brain lesions resulting from cerebral ischemia, it was anticipated that the results could have enormous clinical implications for the repair of CNS abnormalities in general, including those resulting from disease, trauma, or birth related abnormalities.

Neuronal transplantation can only be effective if the lesion is repaired functionally (Bartus, 1987; Freed, 1985; Segal, 1987; Sotelo and Alvarado-Mallart, 1987a). This is
dependent upon the ability of the transplanted neurons to develop intrinsic properties (cytological and electrophysiological) that are similar to the cells they have replaced, to integrate into the host tissue (receive normal afferent fibre connections from the host and project fibres along the efferent pathways that have become vacant due to degeneration) and, to mediate a degree of functional recovery (synaptic innervation and restoration of lost behaviours).

The objectives of the present study were therefore;

A. to develop an animal model whereby a brain region lesioned by cerebral ischemia could be repopulated by transplanted neurons.

B. to examine the functional capabilities of the transplanted neurons by assessing their ability to attain the following criteria.

i) to survive when transplanted to the degenerating brain region.

ii) to develop intrinsic properties characteristic of the cells they have replaced.

iii) to integrate into the host tissue by reestablishing some of the normal synaptic circuitries.

The aforementioned objectives were carried out in progressive stages and therefore are described as such in the following dissertation.
CHAPTER 2

THE HIPPOCAMPAL FORMATION

The aims of this chapter are to describe the anatomical and synaptic organization of the hippocampal formation and the transplantation studies that have been carried out in this brain region.
2. THE HIPPOCAMPAL FORMATION

I. INTRODUCTION

The hippocampal formation (HF) provided an ideal locus for this study. Not only is it the region of the human and the rat brain most vulnerable to damage by cerebral ischemia, it has also been investigated extensively in the rat by anatomical, physiological and behavioural procedures. Further, much of the pioneering transplantation research has been carried out in the HF.

In general terms, the phylogenetic appearance of the HF coincides with the transition from an aquatic to a terrestrial existence (Vinogradova, 1975). This evolutionary jump involved the processing of new and complex environmental information. Although present in lower animals the HF attained prominence in the mammalian brain (Angevine, 1975). On an evolutionary scale the mammalian neocortex increased in size progressively and due to limitations in skull volume it underwent a complicated folding pattern which increased greatly its surface area.

The HF is the result of the continuous expansion of the medial edge of the cerebral hemispheres and the subsequent infolding of the cortex upon itself. In this way, the HF came to occupy a position within the lateral ventricles. In lower mammals such as the rat, the HF follows the C-shape of the lateral ventricles from the fronto-parietal region caudally to the temporo-occipital regions and then ventrally to the entorhinal region (Zilles and Wree, 1985).

II. GENERAL ANATOMICAL ORGANIZATION

Detailed anatomical investigations of the rodent HF were first carried out by Cajal (1893) and Lorente de No (1934). In coronal section the dorsal HF appears to be a continuous extension of the retrosplenial cortex which folds back onto itself in a C-shape. The cortex ultimately gets separated from the HF by the fibres of the corpus callosum (Fig. 2.1). Suggesting that this infolding represented two distinct cortical formations, Cajal (1893) separated it into two divisions; Ammon's horn or Cornu Ammonis (the hippocampus proper)
Figure 2.1

Camara lucida drawing of a coronal section through the rat brain illustrating the position of the dorsal hippocampal formation (HF) within the lateral ventricles (LV). The HF is formed by an infolding of the retrosplenial cortex (RC) (indicated by diagonal lines) and separated during development by the corpus callosum (CC).
and the dentate gyrus. The most prominent feature of these two divisions is the distinct laminar arrangement of their principle neurons; the pyramidal cells and dentate granule cells respectively (Fig. 2.2.A). Cajal further divided the hippocampus into superior and inferior regions based on distinct size differences (small and large, respectively) between the pyramidal neurons. As this study is confined to the hippocampus proper, the remainder of the anatomical description will focus on this division of the HF.

A. The Neurons of the Cornu Ammonis

The pyramidal cells of the hippocampus are particularly important because their axons are the primary means by which information exits the HF. There are also many types of neurons whose axons remain primarily within the hippocampus and thereby play an important role in the local processing of information. These interneurons are of variable morphology and location. The most prominent and best characterized are the basket cells due to their uniqueness in being the only interneurons to form synaptic contacts on the soma of the pyramidal cells (Lorente de No, 1934). The axonal projections from these neurons are extensive and their terminal branches form a basket-like plexus around both the pyramidal cell somata and the proximal portion of their dendrites.

B. The Layers of the Cornu Ammonis

Beginning at the edge of the lateral ventricles, Cajal divided the cortex of the Cornu Ammonis into six laminated strata of which the pyramidal cell layer is one (Fig. 2.2.B).

i) Epithelial Layer (EL)

At the edge of the ventricular cavity lie a layer of epithelial cells. During development these cells extend processes which provide an ascending framework for migrating neurons. In the adult most of these processes are lost.

ii) Alveus or White Matter (A)

This layer is composed primarily of pyramidal neuron axons. The axons project either towards the subiculum or the fibres accumulate in the fimbria.
Figure 2.2

A) Coronal section of the hippocampal formation from one hemisphere stained with thionin (38X). This illustrates the laminar arrangement of the pyramidal cells of the hippocampus (H; solid arrows) and the granule cells of the dentate gyrus (DG; open arrows).

B) Schematic of a coronal section of the hippocampal formation illustrating the six layers of the hippocampus and the four CA regions (see arrows).

Abbreviations:

SB - subiculum
DG - dentate gyrus
F - fimbria
EL - epithelial layer
A - alveus
SO - stratum oriens
SP - stratum pyramidale
SR - stratum radiatum
SLM - stratum lacunosum moleculare
iii) *Stratum Oriens* (SO)

The stratum oriens is primarily characterized by the basal dendritic arborizations of the pyramidal cells. A number of polymorphic interneurons, including basket cells, are situated here and project axonal processes in a horizontal or vertical direction.

iv) *Stratum Pyramidale* (SP)

Pyramidal cell somata, three to four cells deep, are situated in this layer. Several types of interneurons also reside in this layer.

v) *Stratum Radiatum* (SR)

The long apical dendritic shafts which extend from the apex of the pyramidal neurons form the widest of the layers. The appearance of this layer delineates the hippocampus from the subiculum. Although not abundant, there are a number of interneuron types in this layer.

vi) *Stratum Lacunosum Moleculare* (SLM)

The apical dendrites of the pyramidal neurons undergo extensive branching and terminate in this layer. Small interneurons are scattered among these dendritic terminals.

C. The Regions of the Cornu Ammonis

Lorente de No divided the superior and inferior hippocampal regions into four CA subfields based on the structural properties of the pyramidal neurons (Fig. 2.2.B)

i) CA1 Region

The somata of the CA1 pyramids are 3 or 4 deep and tightly packed. Their axons descend through the SO, bifurcate in the alveus and project towards the subiculum or fimbria. Their basal dendrites branch extensively in a whorl around the axon in the SO. The apical dendritic shafts ascend through the SR, giving off some branches, and undergo extensive branching in the SLM. While the apical dendritic shafts are spine free, these branches in the SLM possess dendritic spines.

ii) CA3 Region

The CA3 pyramids are characterized by two properties; the apical dendrites do not branch until reaching the SLM and the dendritic extent, including the shaft and terminal
branches, are covered extensively with dendritic spines. The fimbria directed axons of the pyramids give off collaterals which enter the SR and course towards the subiculum. These fibres were first described by Schaffer (1892), hence the name Schaffer collaterals.

iii) CA2 Region

The CA2 pyramids possess properties of both the CA1 and CA3 pyramids. Their apical dendrites are similar to the CA3 pyramids but lack spines on the proximal shaft portion. Their axons do not have Schaffer collaterals. This transition zone is only the extent of 5-10 cell somata in width.

iv) CA4 Region

The CA4 pyramids are not really pyramidal shaped but more polygonal and are therefore called modified pyramids. The cytological characteristics of their dendrites and axons are comparable to those of the CA3 pyramids. In contrast to the CA1-CA3 pyramids, the CA4 pyramids are loosely packed and the plexus of basket cell terminal varicosities is less extensive.

III. CONNECTIONS OF THE CA1 REGION

This section has been confined to the dorsal CA1 region as this region is the focus of the present study. The afferent and efferent connections of the CA1 region are extensive and have been identified using many anatomical and physiological techniques. The details of these connections have become better defined by the introduction of more sophisticated methodologies such as axonal tracing and intracellular electrophysiological recordings.

A. Afferents

Afferent fibres to the CA1 region may be extrinsic, arising from neuronal populations outside of the HF, including projections from somata in the contralateral HF, or intrinsic, arising from neurons within the ipsilateral HF. The major afferents to this region enter medially via the subiculum or laterally via the fimbria-fornix, run perpendicular to the pyramidal neuron dendritic projections and terminate, in a laminar manner, diffusely or at discrete regions along this dendritic axis (Cajal, 1893; Lorente de No, 1934) (Fig. 2.2.B).
i) *Extrinsic Projections*

The afferents arising from extrinsic sources such as those from the raphe nuclei, the locus coeruleus and the medial septal-diagonal band complex, generally form a diffuse pattern of synaptic contacts within the CA1 region (reviewed by Bayer, 1985). In addition, extensive commissural projections from the CA3 pyramidal neurons exit the HF via the fimbria, cross in the ventral hippocampal commissure and enter the SO and to a lesser extent the SR of the contralateral CA1 region via the contralateral fimbria to form synaptic contacts on the CA1 neurons (Blackstad, 1956; Laurberg, 1979; Raisman et al, 1965). By using iontophoretic injections of the axonal tracer horseradish peroxidase, Voneida et al (1981) showed that the commissural projections to the CA1 region arise from both the CA3 and CA1 regions.

ii) *Intrinsic Projections*

The greatest intrinsic input to the CA1 neurons is that arising from the Schaffer collaterals which originate as branches of the CA3 pyramidal neuron axons (Gottleib and Cowan, 1973; Hjorth-Simonsen, 1973; Lorente de No, 1934; Raisman et al, 1965; Schaffer, 1892; Swanson et al, 1978) and form synaptic contacts throughout the SR and to a lesser extent the SO of the ipsilateral CA1 region. Within the CA1 region exist extensive connections between the pyramidal neurons and the interneurons. The synaptic features of the CA1 region will be further discussed later.

**B. Efferents**

The best characterized projection from the hippocampus is that to the septal region and was first described by Cajal (1893). A number of different techniques have now been used to clarify the specificity of this projection. Lesion-degeneration studies suggested that the neurons of the dorsal CA1 region projected by way of the fornix to the medial parts of the septal region including the medial septal, septofimbrial and triangular septal nuclei (Raismen, 1966). A much more specific technique involving anterograde transport of $^3$H-leucine and $^3$H-proline showed that the projection from the dorsal CA1 region was to the lateral not the
medial septal complex and was organized topographically (Miebach and Siegal, 1977; Swanson and Cowan, 1977). The projection fibres travelled via the dorsal fornix and medial part of the fimbria to the dorsal part of the septofimbrial and triangular septal nuclei and the dorsomedial part of the lateral septal complex. In contrast to the CA2 and CA3 pyramidal neurons which project bilaterally, the CA1 neurons projected unilaterally.

The CA1 cells also send an extensive projection caudally to the adjacent subicular cortex (Hjorth-Simonsen, 1973; Raismen et al, 1965; Swanson et al, 1978). Although the ventral CA1 neurons project caudally to the entorhinal cortex (Beckstead, 1978; Swanson et al, 1981) it is not clear if these projections also arise from the dorsal CA1 region.

IV. GENERAL SYNAPTIC ORGANIZATION OF THE CA1 REGION

A. The Hippocampal Slice Preparation

Initial investigations of the synaptic circuitries within the HF were undertaken in vivo (reviewed by Andersen, 1975 and Schwartzkroin, 1986). Although these studies were classical pioneering works they involved extensive experimental preparation and had many problems such as animal mortality due to factors such as anesthetic, mechanical disturbances caused by heart beat and respiration and, difficulty in the precise placement of electrodes due to lack of visual control. In vivo techniques have become more sophisticated and are still used extensively for specific types of investigations such as the study of long-distance synaptic connectivity and synaptic pharmacology.

The technique by which electrophysiological recordings could be made from brain slices was developed by Yamamoto and McIlwain (1966). The brain slice preparation offered many advantages and was implemented rapidly to assess the viability of hippocampal tissue in vitro and to compare the electrophysiological responses with those already observed in vivo (Schwartzkroin, 1975, 1977; Skrede and Westgaard, 1971; Yamamoto, 1972). Due to the uniquely stratified nature of the HF and the lamellar organization of the synaptic pathways (Andersen et al, 1971a) a slice cut in the coronal plane was thought to operate as an
independent functional unit (possess the complete trisynaptic loop). Microscopic viewing of the slice allowed the accurate placement of both stimulating and recording electrodes into regions known to contain specific afferent or efferent fibres and specific neuronal components (e.g. soma, dendrites).

**B. Synaptic Circuitries**

The synaptic events which occur along the dendro-somatic extent of the CA1 neurons modulate the excitability of these neurons and ultimately the extrahippocampal output from the CA1 region. The synaptic projections from the raphe nuclei, the locus coeruleus and the medial septal-diagonal band complex generally play a role in modulating the excitability of the CA1 neurons (reviewed by Dingledine, 1984). The most extensive and best characterized afferents to the CA1 region are those arising from the ipsilateral and contralateral CA3 and CA1 regions. These fibres course through the SO and SR to form *en passage* excitatory synaptic contacts onto the dendrites of the pyramidal neurons and interneurons (Andersen and Lomo, 1966; Cragg and Hamlym, 1957; Kandel et al, 1961). Electrical stimulation of these afferent inputs produce a sequence of excitatory and inhibitory events in the pyramidal cells.

*i) Synaptic Excitation*

The demonstration that the endogenous dicarboxylic acids, glutamate and aspartate could excite cortical neurons was first documented by Okamoto (1951) and Hayashi (1952). Evidence demonstrating that the receptors for these excitatory amino acids (EAA) participate in synaptic excitation at many sites in the CNS is extensive (Foster and Fagg, 1984; Nistri and Constantini, 1979) and has been well documented in the hippocampus (Biscoe and Straughan, 1966; Hablitz and Langmoen, 1982; Spencer et al, 1976). Three distinct receptor types have been characterized by the relatively high affinity of the exogenous agonists N-methyl-D-aspartate (NMDA), quisqualate (Quis) and kainate (Kain) (Watkins and Evans, 1981; McLennan, 1983).

Studies using the potent NMDA competitive antagonist 2-amino-5-phosphonovaleric acid (APV) (Collingridge et al, 1983) and the relatively specific non-NMDA (Quis, Kain)
blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Honore et al, 1987) have shown that in the HF, non-NMDA receptors are primary in mediating the fast excitatory post synaptic potential (EPSP) that results from low frequency stimuli applied in the SO and SR (Andreasen et al, 1988; Collingridge et al, 1983, 1988a; Crunelli et al, 1984; Ganong et al, 1986; Herron, 1986; Koerner and Cotman, 1982; Mody and Heinemann, 1987). Although binding studies have suggested that NMDA receptors are most abundant in the CA1 region of the HF (Monaghan et al, 1984), in the presence of CNQX (5-10 μM) only a small APV sensitive component of the EPSP remains (Andreasen et al, 1988).

The ionophores which are coupled to NMDA and non-NMDA receptors are cation selective. In contrast to the non-NMDA ionophores whose permeability is restricted to monovalent cations (Na⁺, K⁺), the NMDA ionophore is also permeable to Ca²⁺. This has been suggested by the measurement of changes in extracellular Ca²⁺ concentration with ion-sensitive microelectrodes (Burle and Sonnhof, 1983; Lambert and Heinemann, 1986; Pumain and Heinemann, 1985). Ionic conductance through the NMDA ionophore is regulated by Mg²⁺ in a voltage dependent manner (Mayer and Westbrook, 1987; Nowak et al, 1984). At physiological levels of extracellular Mg²⁺ (1-2 mM) and resting membrane potential, ionic conductance through the NMDA ionophore is greatly restricted. The contribution to low-frequency synaptic potentials by ionic conductance through the NMDA-ionophore can be enhanced when the extracellular Mg²⁺ concentration is reduced (Coan and Collingridge, 1987), when feed-back or feed forward inhibition has been blocked by a pharmacological agent such as bicuculline (Wigstrom et al, 1985; Hablitz and Langmoen, 1986), or when the cell is depolarized to levels where the voltage dependent block is decreased (Collingridge et al, 1988b; Herron et al, 1986).

ii) Synaptic Inhibition

The observation that antidromic stimulation following deafferentation resulted in large inhibitory post synaptic potentials (IPSP's) or antidromic activation followed by an IPSP (Spencer and Kandel, 1961) which was maximal at the cell layer coupled with the fact that the
onset of the IPSP was much longer than the antidromic activation led Andersen et al (1964) to suggest that the inhibition was mediated by local interneurons. As the basket cells were the only interneurons to form extensive synaptic contacts onto the pyramidal cell somata, it was suggested that these interneurons were the source of the inhibition and were activated by collaterals from the pyramidal neuron axons.

It is now known that the basket cells contain gamma-aminobutyric acid (GABA) (Freund et al, 1983; Storm-Mathisen, 1983) which is the major inhibitory neurotransmitter in the hippocampus (Andersen et al, 1978, 1980; Curtis et al, 1970). Further, anatomical and electrophysiological evidence have shown that GABA-ergic interneurons in the CA1 region mediate not only recurrent inhibition but also feed-forward inhibition. This is due to the fact that they can receive the same excitatory synaptic inputs as the pyramidal neurons and in turn form inhibitory synaptic contacts onto the pyramidal neurons (Frotscher and Zimmer, 1983; Schwartzkroin and Kunkel, 1985; Schwartzkroin and Mathers, 1978) (Fig 2.3).

When intracellular recordings are made from CA1 pyramidal neurons, subthreshold and suprathreshold activation (for action potential generation) produces a period of excitation followed by a period of inhibition which has a number of components. The EPSP or action potential (AP) is followed immediately by the early IPSP and then the late IPSP. The early IPSP is considered to be evoked by GABA released from inhibitory interneurons (Anderson et al, 1963; Dingledine and Langmoen, 1980; Kandel and Spencer, 1961) in response to collateral stimulation from neighbouring pyramidal cells which have reached threshold and is manifested as an increased Cl⁻ conductance, mediated by bicuculline sensitive GABA_A receptors located primarily on the soma. The late IPSP is now considered to be due to GABA released from interneurons activated by a feed forward pathway (Alger and Nicoll, 1982; Ashwood et al, 1984; Newberry and Nicoll, 1984) and is thought to result from an increased K⁺ conductance mediated by phaclophen sensitive GABA_B receptors (Dutar and Nicoll, 1988), which are located primarily at dendritic sites. Following suprathreshold activation, intrinsic Ca²⁺ activated K⁺-conductances (afterhyperpolarization (AHP)) are thought to overlap with the early and the late IPSP (Fig 2.4).
Figure 2.3

Schematic to illustrate the general synaptic interactions within the CA1 region. The pyramidal neurons (P) receive excitatory input from afferent fibres coursing through the SR (shown), SO and SLM (not shown). Their axons in turn form excitatory synaptic contacts onto the GABA-ergic interneurons (I) which mediate recurrent inhibition by forming a plexus of terminals around the pyramidal neuron soma and initial segment. The GABA-ergic interneurons also receive excitatory input from afferent fibres coursing through the SR, alveus (shown) and SO (not shown) and are thought to subsequently mediate feed-forward inhibition via a synaptic plexus in the dendritic regions (after Schwartzkroin, 1986).
Figure 2.4

A schematic to illustrate the hyperpolarizing events thought to occur following subthreshold (dotted line) and suprathreshold activation (solid line). The EPSP is followed immediately by the early IPSP (recurrent inhibition) which causes a GABA mediated increase in Cl\(^{-}\) conductance at the somatic level. The late IPSP (feedforward inhibition) causes a GABA mediated increase in K\(^{+}\) conductance in the dendritic regions. Following suprathreshold activation the Ca\(^{2+}\)-activated K\(^{+}\) conductance mediates an afterhyperpolarization (intrinsic inhibition) which overlaps with the early and late IPSP.
early IPSP  late IPSP

-50ms -

Ca^{++} activated K^{+} conductance
iii) Evoked Fields

When extracellular recordings are made in the SP following subthreshold or suprathreshold orthodromic stimulation of the afferent fibres in the SO or SR, evoked field potentials (population-EPSP's and population-spike, respectively) can be measured. The field potentials are produced by summation of individual EPSP's or action potentials of many contiguous pyramidal neurons (Andersen et al, 1971b). The population spike is manifested as a series of positive-negative-positive potentials indicative of the population EPSP, cell discharge and the population IPSP's. The field EPSP recorded at the site of transmitter release (SR or SO) is observed as a negative potential resulting from synaptic current flowing into the apical dendrites (current sink) and appears as a positive potential when recorded from the SP (current source). Population spikes can also be evoked antidromically by stimulation of the axonal projection fibres in the alveus and occur with a much shorter latency due to direct rather than synaptic activation. Population spike amplitude is dependent upon synchronous cell discharge, the laminar distribution of the population somata and is directly related to the number of discharging cells.

V. TRANSPLANTATION IN THE HIPPOCAMPAL FORMATION

A. Survival and Growth of Transplanted Hippocampal Neurons

The first study to describe the transplantation and subsequent survival of hippocampal tissue was that documented by Sunde and Zimmer (1981). Almost the entire hippocampal formation from embryonic and early postnatal rats was transplanted to various brain regions contiguous with the ventricular surface of early postnatal and adult hosts. This study showed that survival of the transplanted hippocampal neurons was not dependent upon the age of the host or the region of transplantation. This survival was confirmed by Kromer et al (1983) and shown to be optimal when the solid tissue pieces were obtained from embryonic day 17-19 fetal rats. All principal cell types were shown to be present and a high degree of laminar organization was maintained when the tissue was obtained at a very specific developmental
B. Transplant-Host Interactions

Transplant-host connections have been studied extensively in the hippocampus primarily because of the well characterized laminar organization of the principal neurons and their afferent and efferent fibre projections. The investigations have focused either on the ability of different types of tissue (heterotypic transplants), transplanted adjacent or within the hippocampus, to innervate the host hippocampus or the ability of transplanted hippocampi (homotypic transplants) to receive afferent fibre projections or project efferent fibres in a normal manner.

i) Heterotypic Transplants

By producing synaptic vacancies within the HF by lesioning specific nuclei known to have well characterized afferent projections to the HF the pattern of fibre ingrowth from different types of tissue, transplanted adjacent to the hippocampus, has been investigated (Bjorklund et al, 1976, 1979; Bjorklund and Stenevi, 1977; Gibbs et al, 1985, 1986). These different types of tissue had unique innervation patterns characteristic of their normal counterparts following lesions to corresponding host cells. When non-corresponding host cells were lesioned, the fibre projections from the transplanted neurons generally tended to fill vacant synaptic spaces in a non-specific manner. Furthermore, these studies showed that the degree of fibre ingrowth was dependent upon the degree of denervation produced in the hippocampus.

Other investigations have shown that the fibre outgrowth from the transplants was restricted if the host afferent neuronal population was only partially lesioned. This was thought to be due to competition between collateral sprouts from remaining host fibres and the fibre projections from the transplanted neurons (Lewis and Cotman, 1982a, 1982b; Zhou et al,
1985). More recent reports have shown that fibre projections from the transplanted neurons are capable of forming extensive synaptic contacts with the host hippocampal neurons (Anderson et al, 1986; Clarke et al, 1986a).

**ii) Homotypic Transplants**

When hippocampal tissue was transplanted adjacent to the hippocampus of host rats, extensive cholinergic fibres grew into the transplants which were thought to arise from septal-diagonal band neurons (Sunde and Zimmer, 1983). The transplants received only sparse innervation from a number of other afferent systems even when placed in close proximity to these projections. These afferents were relatively non-specific and seemed to be related to the closeness of the fibres and their ability to sprout collateral fibres which could cross the transplant-host interface. The degree of innervation was, however, enhanced when damage was incurred to the host pathways which further supported the "neurotrophic" hypothesis (section 1.II.C.ii).

Fibre outgrowth from hippocampal transplants was investigated by using immunohistochemical techniques to distinguish between donor and host mouse tissue. Projections from the transplanted hippocampi to the adjacent host HF seemed to be more dependent upon denervated terminal fields than specific fibre pathways.

**C. Functional Restoration**

Since few studies have examined the electrical activity of the transplanted regions or the cell-specific electroresponsive properties of the transplanted cells, the functional capabilities of the transplanted neurons remain poorly understood (Bartus, 1987; Freed, 1985).

The first study investigated the electroresponsiveness of locus coeruleus tissue, transplanted adjacent to the hippocampus in the retrosplenial cortex, following sympathectomy and destruction of the host locus coeruleus neurons and fibre projections (Bjorklund et al, 1979). It was demonstrated that the 7 transplanted neurons recorded from 5 transplants were electrically viable, spontaneously active and responded to glutamate application by firing
at an increased rate. As it had been shown anatomically that the tissue could reinnervate the vacant synaptic sites within the HF, the presence of functional synaptic innervation was assessed. Shocks applied to the transplanted region were able to reduce the firing rate of spontaneously active host hippocampal neurons in a manner similar to that observed following activation of normal noradrenergic inputs from the locus coeruleus. These data demonstrated that transplanted neurons were capable of making viable synaptic connections with the host tissue.

When large pieces of hippocampal tissue (3 x 1mm) were transplanted into a cavity produced by the total removal of the fimbria-fornix and overlying cortical tissue, the transplanted tissue was shown to be synaptically innervated by the adjacent septal tissue (Segal et al, 1981). In addition, small field potentials (approx 0.3mV) could be evoked in the intact pyramidal cell lamina by shocks applied to the septal region. Further, when septal tissue was transplanted directly into the host hippocampus, in vitro analysis showed that the transplanted cells could form functional cholinergic synapses with the adjacent hippocampal cells (Segal et al, 1985, 1987).

Intracellular recordings have been made in raphe, cerebellar and hippocampal neurons transplanted to heterotypic sites. They have been shown to have the ability to develop many cell-type specific biophysical properties, without the influences of a developing environment, when regional organization was maintained by transplanting solid tissue pieces (Hounsgaard and Yarom, 1985; Segal, 1987; Segal and Azmitia, 1986).

VI. TRANSPLANTATION AND CEREBRAL ISCHEMIA

By using a hypoxia model which caused a population of cortical neurons to exist in a reversible dystrophic state (darkly stained, vacuolized) it was shown that solid pieces of embryonic tissue could survive when transplanted into the cortex 91 days post-hypoxia (Polezhaev and Alexandrova, 1984; Alexandrova et al, 1985). Although these studies did not show that transplanted neurons could survive when placed into a region undergoing degeneration, they did provide an optimistic basis for the present study.
CHAPTER 3

INDUCTION OF CEREBRAL ISCHEMIA

The aim of this chapter is to describe the method used for the induction of cerebral ischemia and to confirm that it was consistent in producing selective cell loss in the CA1 region of the HF.
3. INDUCTION OF CEREBRAL ISCHEMIA

I. INTRODUCTION

Numerous methodologies have been developed for the induction of transient forebrain ischemia in small animals (Blomqvist et al, 1984; Blomqvist and Wieloch, 1985; Kameyama et al, 1985; Pulsinelli and Brierly, 1979; Smith et al, 1984b). These models have been utilized for the investigation of the pathophysiological mechanisms underlying ischemic cell death and selective neuronal vulnerability. The most frequently used models involve the cauterization of the vertebral or basilar arteries and the occlusion of the common carotid arteries thereby preventing blood flow to the forebrain. This procedure produces ischemic damage in the CA1 region of the hippocampus and extensive pathology throughout the striatum and neocortex. Due to arterial cauterization and the permanent impairment of cerebral blood flow, long-term survival is rare. An attractive model proposed by Smith et al (1984b) combines bilateral carotid occlusion with haemorrhagic hypotension. Blood flow to the hippocampus is reduced to a trickle during the procedure and following reperfusion of the shed blood, normal cerebral blood flow is reinstated. Unfortunately, to obtain consistent results, the timing of this procedure is critical and differences of seconds can greatly increase or decrease the degree of damage to the selectively vulnerable regions.

For the present study it was critical that the model of choice fulfil two criteria. Firstly, it was important that the model produced consistent highly selective cell pathology that was easily quantifiable. Secondly, it was essential to keep mortality levels low to allow long-term survival. Therefore a model was developed to fulfil these criteria by modifying the aforementioned methodologies.
II. METHODS

A. Ischemia Induction

Male Wistar rats weighing 300-350g (n=30) were allowed food and water *ad libitum* until the time of surgery. The animals were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and treated with atropine (0.2 mg i.p.) to decrease respiratory secretions. Animals were allowed to breathe spontaneously and body temperature was maintained at 35°C with a YSI temperature regulator. The common carotid arteries were isolated and a silk loop placed around each artery for quick access. The femoral artery was cannulated with PE50 tubing which was connected to a Statham pressure transducer and coupled to a saline primed reservoir. The pressure signal was converted to an electrical signal by using a transducer converter (S.E. Labs, type SE 905). The signal was then amplified and led to a Gould chart recorder. In some of the experiments the signal was led to a Columbus Instruments Cardiomax II which also allowed the measurement of heart rate. The system was calibrated with a mercury manometer system. Baseline mean arterial pressure (MAP) and heart rate were recorded. Animals were then treated with heparin and haemorrhaged until the MAP reached 30 mm Hg (approx. 7 minutes). Atraumatic arterial clamps were then quickly placed on both common carotid arteries. The MAP was maintained at 30 mmHg throughout the period of arterial occlusion by further removal or reinfusion of blood. The total blood loss was 7-10 ml. After 20 minutes the clamps were removed and the shed blood reinfused by applying 200 mmHg of backpressure to the reservoir (8-12 minutes). MAP recovered to preischemic levels, or slightly higher, immediately upon reinfusion (Fig. 3.1). The femoral cannula was then removed and the animal left to recover. All treated animals were caged separately with food and water *ad libitum*.

B. Histology

Having attained some consistency with the technical aspects of this procedure, a group of control animals (n=8) and ischemic animals (n=12) were used to quantify the
Figure 3.1

A chart record of the mean arterial pressure (MAP) from one animal during ischemia. A) Baseline MAP and heart rate (HR) were recorded following the infusion of heparin. At t=0:00 minutes haemorrhage was begun. B) At t=8:00 minutes the MAP had reached 30mmHg (5.7 cc of shed blood) and both common carotid arteries were occluded. C) At t=28:00 minutes the clamps were removed and the shed blood reinfused (9.5 cc). Note the quick return of the MAP to baseline levels.
Mean Arterial Pressure (mmHg)

A

HR 344
MAP 95

B

HR 307
MAP 30

C

HR 416
MAP 110

$\text{t}=0:00$
haemorrhage

$\text{t}=8:00$
clamped
5.7 ml

$\text{t}=28:00$
unclamped
blood reinfused
9.5 ml
reproducibility of the pathology produced by using this model. Seven days postischemia animals were perfused transcardially with 10% phosphate buffered formalin following a saline flush. Brains were removed, embedded in paraffin and coronal sections (6μm) were made and stained with thionin (0.5%) (see section 6.II.A).

C. Quantification of Ischemic Damage

Since the degree of cell degeneration following ischemia was consistent throughout the dorsal CA1 region (Smith et al, 1984a), one coronal section was chosen for quantification, from each control and ischemic brain, from the middle of the dorsal hippocampus (Fig. 3.2.A). This was used to represent the degree of ischemic damage. The cells in the stratum pyramidale, throughout the CA1 and CA2-CA4 regions, were counted with the aid of the Kontron videoplan computerized analyzer. This allowed both viewing at 200X magnification and accurate length measurement of the counted cell layer. A Student t-test was used to compare the mean number of cells in the CA1 and CA2-CA4 regions in the control preparations with those in the ischemic preparations. Sections from both hemispheres were counted so that a comparison between hemispheres could be made.

III. RESULTS

A. Animal Behaviour

Hyperactivity and seizure activity, manifested by running fits, reached a maximum 16-24 hours post-ischemia and occurred in 50% of the animals. Seizure activity usually caused respiratory complications which were the cause of death in 70% of these animals. After the critical post-operative period of 36 hours, animals survived for the full 1 week period during which activity was not obviously different from control animals.

B. Pathology

Histological examination revealed that the model used to induce forebrain ischemia was consistent in producing highly selective neuronal degeneration (93%) in the stratum
Figure 3.2

A) A schematic of a coronal view of the hippocampal formation illustrating the CA regions. Cell counts were made in the stratum pyramidale of the CA1 region and in the CA2-CA4 regions. B) The histograms represent the number of neurons / mm counted from coronal sections through the dorsal hippocampal formation. Well defined somata were counted (eg. # of neurons in Fig. 3.3.A = 41) but pyknotic or lysing nuclei were not (eg. # of neurons in Fig. 3.3.B = 0). Error bars represent the standard deviation. Sections from control animals were compared with sections from ischemic animals. Ischemia causes bilateral loss of 93% of the neurons in the CA1 region. In contrast, very little damage (9.5%) is produced in the CA2-CA4 regions. Although the cell loss in the CA1 region was statistically significant (p<<.001) the cell loss in the CA2-CA4 region was not (p>.05). No differences were observed between hemispheres.
A

CA2

CA1

CA3

CA4

B

CONTROL VS ISCHEMIA

300

200

100

0

LEFT

RIGHT

CONTROL

(n=9)

ISCHEMIC

(n=12)

NEURONS/mm

CA1 CELL LAYER

CA2-4 CELL LAYER
pyramidale of the CA1 region (Figs. 3.2.B, 3.3). This degeneration was observed throughout the rostro-caudal extent of the dorsal hippocampal formation. Minimal cell loss (9.5%) was observed in the remainder of the CA regions. No obvious damage could be observed in the striatum, cortex or thalamus. The degree of damage did not differ in seizing and non-seizing animals.

Preliminary experiments showed that body temperature was the most critical variable during the ischemic insult. If maintained below 33.5°, ischemic damage could be prevented and if maintained above 36.5° there was 100% mortality. Consistent cell loss was produced only when body temperature was maintained between 34° and 36°. Cell loss could be observed in other vulnerable brain areas, such as the striatum and cortex, if the duration of ischemia was increased past 20 minutes.

IV. DISCUSSION

Although this model of cerebral ischemia greatly reduced the animal mortality rates one week post-ischemia, these results demonstrated that only 65% of the animals subjected to this surgery would survive the procedure and be healthy enough to undergo further transplantation surgery. More importantly, the anatomical control data clearly demonstrated that, in the surviving animals, this model of cerebral ischemia produced highly selective damage within the brains of adult rats. The fact that the pathology was restricted to the CA1 region of the HF and was equal in both hemispheres allowed the use of intra-animal controls to base the results of neuronal transplantation.

V. SUMMARY

The model chosen for the induction of cerebral ischemia proved to be consistent in producing highly selective bilateral cell loss in the CA1 region of the HF thus providing a locus for the transplantation of new healthy cells.
Figure 3.3

Left Panel: Low power (21X) view of Nissl stained coronal sections comparing a control preparation (A) illustrating the normal cell density and distribution throughout the CA regions with the dramatic cell loss and gliosis in the ischemically damaged CA1 region one week post-ischemia (B). Right Panel: High power (355X) view comparing the appearance of the intact CA1 pyramidal cells (A) with the necrotic CA1 region (B).
The aim of this chapter is to demonstrate immunohistochemically the long term structural changes that occur in the CA1 region of the HF following cerebral ischemia.
4. **LONG-TERM STRUCTURAL CHANGES FOLLOWING CEREBRAL ISCHEMIA**

**I. INTRODUCTION**

Transplantation studies are generally long-term to allow the transplanted neurons time to undergo development in the adult host. Although it is known that a consistent and extensive degree of damage is produced in the CA1 region of the HF following cerebral ischemia, little is known about the long-term events which occur secondary to the insult. The CNS is a dynamic structure and following injury the neurons not affected by the insult have the ability to undergo collateral sprouting and new synapse formation (Raisman, 1969; Cotman, 1985). Therefore, before the transplantation experiments were undertaken, the next phase of this study was to examine the long-term pathological events which followed cerebral ischemia. Antisera against the calcium binding proteins Calbindin-D$_{28K}$ (CaBP) and Parvalbumin (PV) were utilized due to their unique immunoreactive staining patterns in the hippocampal formation.

In the hippocampus, GABA is the major neurotransmitter present in the local circuit neurons such as the basket cells. The antiserum to PV labels a subpopulation of GABA-ergic interneurons throughout the hippocampal formation (Celio, 1986; Kosaka et al, 1987). In the CA1 region PV has been localized in 50-70% of the GABA-ergic neurons in the SO, SP and SR. PV-like immunoreactivity is present in the soma and throughout the network of terminal varicosities which surround the pyramidal neurons. In addition, PV containing dendrites extend throughout the SR and intersect a densely stained band thought to represent a PV-positive fibre bundle coursing through the SLM (Fig. 1C). Antiserum to a second calcium binding protein, CaBP, can be used to label 2 of the 3 principal cell types within the hippocampal formation: the CA1 pyramidal and dentate granule cells but not CA3 pyramidal cells (Baimbridge and Miller, 1982; Jande et al, 1981). Like PV, CaBP is a soluble protein and antiserum to it labels the entire cytoplasmic volume of the superficial CA1 pyramidal neurons (furthest from the epithelial layer) including their long apical dendrites which extend
through the SR. The labelled apical dendrites end at a distinct bundle of fibres which travels through the CA1 region at the SR-SLM border. In addition, the dendrites of granule cells in the molecular layer of the dentate gyrus stain intensely and delineate the position of the hippocampal fissure (Fig. 1E).

II. METHODS

A. Tissue Preparation

One to twelve months following ischemia animals (ischemic, n = 26; controls n = 15) were perfused transcardially with a saline flush followed by either ice cold 4% paraformaldehyde (PFA) in 0.1% phosphate buffer (PB) (500 ml, pH 7.4) or ice cold 2% PFA in 0.1 M acetate buffer (100 ml, pH 6.5) and then ice cold 2% PFA/0.1% glutaraldehyde in 0.1 M sodium borate buffer (BB) (400 mls, pH 8.5) (Berod et al, 1981; Sloviter and Nilaver, 1987). The perfusion rate was controlled using a Cole-Parmer peristaltic pump. The brains were post-fixed for either 2 hours in 4% PFA in 0.1% PB or for 24 hours in 0.1 M BB, respectively, and then taken through a series of 10-30% sucrose over the next three days. The brains were stored at 4°C in 30% sucrose for cryoprotection until cutting. Serial sections (30μm) were cut in a cryostat and stored in phosphate buffered saline (PBS) with .04% sodium azide at 4°C until used for immunohistochemistry. Adjacent sections were either mounted on glass slides and stained with the Nissl stain thionin (0.5%) or stained free-floating with antibodies to CaBP and PV.

B. Immunohistochemistry

In the first half of this study peroxidase-anti peroxidase (PAP) immunohistochemical (IHC) procedures were undertaken as this technique was considered the most sensitive on formaldehyde fixed material (Sternberger, 1979). A more sensitive technique which used the avidin-biotin peroxidase complex (ABC) (Hsu et al, 1981; Sloviter and Nilaver, 1987) had been shown to reduce greatly the background staining and was therefore implemented for the second half of the study. The antisera used in this study were produced and purified in this
laboratory and details of their specificity have been described (Baimbridge and Miller, 1982; Buchan and Baimbridge, 1988; Freund and Antal, 1988; Gerfen et al, 1985; Sloviter, 1989). The anti-CaBP antibody (R202) was raised in rabbit to monkey CaBP and the anti-PV antibody (R301) was raised in rabbit to rat muscle PV.

Sections fixed with 4% PFA were stained using the PAP technique as follows: Sections were washed in 1% H$_2$O$_2$ in 10mM PBS to remove endogenous peroxidase activity. Following 3 washes in PBS, sections were incubated in 50% normal goat serum, pH 7.4 for 30 minutes at room temperature to minimize nonspecific background staining. The sections were then incubated for 24-48 hours in the primary antibody (anti-PV 1:200 dilution or anti-CaBP 1:300 dilution in IHC buffer containing 20 units/ml heparin; 1 mM EDTA; 6% goat serum; .04% sodium azide; 0.5% Triton X-100 in 10mM PBS, pH 7.4). After this and all subsequent exposures to other antibodies, sections were washed with agitation, 3 times for 20 minutes in PBS. The sections were then incubated in the secondary antibody which was goat-anti rabbit IgG (1:120 in PBS, Cappel) for 90 minutes and then the tertiary antibody, rabbit peroxidase-antiperoxidase complex (1:120 in PBS, Cappel) for 60 minutes. Sections were then rinsed in 0.05% Tris, pH 7.6 for 5 minutes and then in the same buffer containing 0.1 mg/ml 3,3' diaminobenzidine (DAB) for 5 minutes. Hydrogen peroxide (0.01%) was then added to visualize the position of the peroxidase antibody complex.

Sections fixed with 2% PFA were stained in a similar manner by following the technique described by Sloviter and Nilaver (1987). Sections were washed in Tris A (0.1 M Tris, pH 7.6 with 0.1% Triton-X) for 5 minutes, Tris B (Tris A with 0.005% BSA) for 15 minutes and then 1% H$_2$O$_2$ in Tris for 30 minutes. The sections were then incubated for 24 hours in primary antisera, anti-PV 1:1000 or anti-CaBP 1:700 in Tris B. Sections were washed in Tris A and B for 15 minutes each and then incubated in the second layer which was biotinylated Protein A (1:400) for 45 minutes (Protein A from Genzyme, Inc., biotinylated in our lab). This was followed by washing in Tris A and B for 15 minutes each and then incubation in ABC (1:1000 in Tris B, Vector Labs) which formed the third layer. After two 5
minute washes in Tris, sections were incubated in 0.05% DAB in Tris containing glucose oxidase (0.3 mg/100ml; Sigma), ammonium chloride (40 mg/100ml) and B-D (+) glucose (200 mg/100ml) to visualize the bound antibody avidin-biotin-peroxidase complex.

Following either of these procedures, sections were washed in Tris buffer, mounted on glass slides, dehydrated in 100 % alcohol, cleared in xylene and coverslipped for viewing.

III. RESULTS

Histological observations, of the thionin stained sections, six months following ischemia, again demonstrated an almost total loss of neurons in the SP of the CA1 region and a faint line of remaining gliosis (Fig. 4.1.B). The most prominent structural alteration was the severe shrinkage of the SR and SO (Fig. 4.1.B,D,F). The PV staining confirmed that some GABA-ergic interneurons remained (Schlandor et al, 1987) but their normally extensive terminal network was reduced greatly (Fig. 4.2.D,J). The dendrites which normally project into the SR seemed to be retracted. Further, PV-immunoreactivity appeared to be depleted in the fibres coursing through the SLM of the CA1 region (Fig. 4.2.D). CaBP-immunoreactivity was almost totally absent in the CA1 region; only scattered somas remained which lacked dendritic processes. Curiously, the fibres coursing through the distal SR remained intact. Although the degeneration observed in the CA1 region was severe, the remainder of the hippocampal formation did not demonstrate aberrant staining with these immunohistochemical markers.

IV. DISCUSSION

Immunohistochemical staining procedures showed that degeneration in the CA1 region 6 months following ischemia had progressed from that observed after 7 days (compare Fig. 3.3.B with Fig. 4.1.B). Not only did the entire region undergo severe shrinkage but the thick band of afferent fibres which course through the SLM was no longer visible. These data indicated that, due to the loss of their normal synaptic targets, the fibres may have undergone retrograde degeneration or aberrant sprouting by forming synaptic contacts elsewhere. The
Figure 4.1

Low power (27X) view of adjacent coronal sections stained with thionin (A,B), PV (C,D) and CaBP (E,F) comparing a control preparation (left panel) illustrating the normal cell density and staining pattern throughout the CA regions of the hippocampal formation with the dramatic cell loss and shrinkage in the ischemically damaged CA1 region (right panel). Arrows indicate the CA1 region. Abbreviation: SO Stratum oriens, SR stratum radiatum, SLM stratum lacunosum moleculare.
Figure 4.2

(A-F): High power view (55X) of the series illustrated in Fig. 1 to demonstrate the cytological features in controls (A,C,E) and the shrinkage of the stratum radiatum and stratum oriens following cerebral ischemia (B,D,F). Upper arrows are directed towards the stratum pyramidale; lower arrows indicate the hippocampal fissure.

(G-L): A greater magnification (110X) shows the degree of pyramidal cell loss following ischemia (G,H). Parvalbumin immunoreactivity in (J) indicates the surviving interneurons in the stratum pyramidale lacking the extensive terminal network observed in (I). CaBP immunoreactivity present in the apical dendrites in the stratum radiatum (K) is absent following ischemia (L).
fact that degeneration remained localized to the CA1 region and the observation that afferent fibres did not travel through the CA1 region to the same degree has important functional implications, especially if these fibres are coursing back to form synaptic contacts elsewhere.

It has been hypothesized that reactive gliosis following injury may play a role in focal epilepsy (Pollen and Trachtenberg, 1970). Collateral fibres that sprout following injury may get deflected from their normal path by the gliosis which surrounds the damaged region and subsequently form inappropriate synaptic contacts (Nieto-Sampedro and Cotman, 1985). A situation may exist that is similar to that resulting from kainic acid destruction of the CA3, CA4 pyramidal neurons; the dentate granule cells sprout collateral fibres back onto their own dendrites causing the dentate granule cells to become hyperexcitable (Tauc and Nadler, 1986). These data suggested that neuronal tissue transplanted directly into the lesioned area may have the capacity not only to reconstruct the damaged area but also to have an important role in preventing progressively deleterious structural alterations in areas not directly affected by the initial insult.

V. SUMMARY

The observations reported in this chapter clearly demonstrate that degeneration following cerebral ischemia continues in a progressive manner until the CA1 region of the HF is extremely shrunken. These chronic alterations may have important implications for the etiology of secondary conditions which follow ischemic brain damage such as epileptiform activity.
The aims of this chapter are to describe the transplantation techniques used for the repopulation of the lesioned CA1 region and the experimental protocol used to carry out the transplantation experiments.
5. NEURONAL TRANSPLANTATION

I. INTRODUCTION

Having established the short-term and long-term pathological structural changes which occur in the CA1 region following cerebral ischemia, repopulation of the damaged region with neuronal tissue was undertaken. In attempting to repair the damaged region it was important to utilize a transplantation technique whereby minimal additional damage was incurred. It was also crucial to optimize the factors which are known to enhance the survival and growth of transplanted neurons. Furthermore, it was important to know how many cells were lost by the ischemic insult and subsequently, how much tissue should be used to repopulate this damaged region. A discussion of these technical considerations follow.

II. EXPERIMENTAL TRANSPLANTATION PROCEDURES

A. Cell Loss

In attempting to repair the lesion that resulted from the induction of cerebral ischemia the objective of the next experimental procedure was to repopulate the damaged CA1 region with transplanted fetal hippocampal tissue. It was important therefore, to consider the number of cells lost in the CA1 region so that a favourable number of neurons could be transplanted.

Cell counts indicated that there were greater than 320,000 neurons in the entire CA1 stratum pyramidale (Schlessinger et al, 1978). Since the cell loss resulting from cerebral ischemia was restricted to the dorsal part of the CA1 region, the cell number was estimated for this fraction. This was done by calculating the area of the CA1 region, multiplying this value by the cell density and then dividing by the cell diameter (Fig. 5.1). These calculations provided a cell number estimate of 136,000 cells.
Figure 5.1

Estimation of the total number of cells in the stratum pyramidale (SP) of the dorsal CA1 region (A) The cell density was obtained from cell counts (Fig. 3.2) (B) The mean diameter of cells in the CA1 SP has been measured previously (Schlessinger et al, 1978) (C) The geometrical formula for a trapezoid was used to calculate the area of the dorsal CA1 region. The dimensions were taken from the rat stereotaxic atlas of Paxinos and Watson (1986).
Cell Density = 250 cells/mm

Cell Diameter = .011 mm

Area of Dorsal CA1 = Area of Trapezoid

\[ \frac{A + B}{2} \times h \]

\[ = \frac{2 + 4}{2} \times 2 \]

\[ = 6 \text{mm}^2 \]

\[
\text{Cell Density} \div \text{Cell Diameter} \times \text{Area of Dorsal CA1} = \text{Total # of Cells}
\]

\[ = 136,364 \text{ cells} \]
B. Cell Preparation

The hippocampus has a precise intrastructural gradient of neurogenesis (Bayer 1980a, 1980b; Schlessinger et al, 1978). The majority of cells in each field are born in a 24-48 hour period and the CA1 cells are generated 24-36 hours later than the CA3 cells. To optimize the selective survival of CA1-like pyramidal neurons, embryonic day 18.5 (E18.5) fetal hippocampal tissue was used which is at a developmental stage where the majority of the CA1 pyramidal neurons have just become post-mitotic or are in the process of migration. Cells at this stage of development are more likely to survive the process of mechanical dissociation and transplantation (see I.II.C.i).

At the time the present study was initiated, a methodological description for the preparation of hippocampal cell suspensions was not available in the literature. Since standard tissue culture procedures had been utilized to dissociate other types of tissue into cell suspensions, in the present study hippocampal cell culture techniques were used for the preparation of hippocampal cell suspensions (Banker and Cowan, 1977). The uterus was removed from a pregnant female Wistar rat (E18.5) and placed in a sterile petri dish. The brains (8-15) were removed sequentially from each fetal rat and the hippocampi dissected free. A Leitz dissecting microscope was used for visual aid during the procedure. During the dissection, hippocampi were stored in a sterile petri dish containing ice cold Ca\(^{2+}\)-Mg\(^{2+}\) free Hanks’ buffered salt solution containing 15mM HEPES buffer and 0.6% glucose (HBSS; pH 7.4). The hippocampi (16-30) were transferred in a Pasteur pipette to a sterile vial. The HBSS was replaced with 0.6% glucose-saline (25ul/hippocampus) and the tissue was mechanically dissociated by trituration which involved drawing the suspension into 3 Pasteur pipettes (10 strokes each) of decreasing bore size (Fig. 5.2). Trypsinization was not used as it had been shown to attenuate greatly the survival of some neurons (Bjorklund et al, 1986). An aliquot of suspension was added to a trypan blue solution (0.4%) and the viable cells were counted after 5 minutes, using a haemocytometer (average yield: 15,000 cells/ul). The
suspension was stored in a cooled closed vial during the period of transplantation which began immediately and lasted up to 8 hours. Cell viability had been shown to decrease approximately 15% during this time (Brundin et al, 1985).

C. Transplantation

After careful consideration of the factors known to enhance transplanted neuron survival and integration in adult host tissue, transplantation techniques were chosen to optimize these factors. As discussed earlier, a delayed release of neurotrophic factors has been correlated with the period of reactive gliogenesis which occurs following injury. This proliferation begins within hours following an ischemic insult (Petito, 1986) and reaches a maximum approximately 7 days later (du Bois, 1985a, 1985b). Therefore, the dorsal CA1 region was repopulated with 18.5 day old fetal hippocampal tissue one week following the ischemic insult. To allow for some cell death following transplantation a total of 15ul of dissociated cell suspension (150,000-300,000 cells) was injected, unilaterally or bilaterally, into 3-5 stereotaxically determined sites (Fig. 5.2) in a manner similar to that described by Schmidt et al (1981).

The animals were anesthetized with sodium pentobarbital (50mg/kg) and placed into a standard Kopf stereotaxic apparatus. Due to the high mortality rates initially encountered for this second surgical procedure a dose of anesthetic lower than that used for the ischemia surgery was used. This dose was able to produce the same degree of anesthesia as the dose used one week prior. A skin incision was made to expose the surface of the skull. Injection sites were located by taking coordinates from Paxinos and Watson (1986) (Fig. 5.2) and burr holes were drilled. A sterile 30 gauge cannula was attached with PE 100 tubing to a 5ul Hamilton syringe and housed on a Sage Instruments syringe pump. This allowed visualization of the suspension before the cannula was lowered to the appropriate depth and during injection at a controlled rate of 0.5 ul/min.
Hippocampi were excised from embryonic day 18.5 fetal rat brains and transferred to a sterile vial containing 0.6% glucose saline. The tissue was mechanically dissociated by trituration to produce a cell suspension. This was done by drawing the tissue up and down into 3 Pasteur pipettes of decreasing bore size. Aliquots of cell suspension were drawn through a fine cannulae (30 gauge) into PE 100 tubing by suction from a 5ul Hamilton syringe. The cells were injected into 5 stereotaxically determined sites (Paxinos and Watson, 1986) at a rate of 5ul/min controlled by a syringe pump. (Coordinates in mm: posterior to Bregma -0.25, -0.37, -0.49; lateral to Bregma +0.15, +0.18 and +0.30, +0.25 and +0.40; ventral to cortex -0.24, -0.21, -0.20)
Embryonic Brain

Hippocampi

Mechanical Dissociation

Cell Suspension

Cell Injections
III. EXPERIMENTAL DESIGN

Experimental groups usually consisted of 1 age matched control, 1 control with hippocampal saline injections, 1 control with hippocampal transplants, 1 ischemia only, 1 ischemia with hippocampal saline injections and 7 ischemia with hippocampal transplants. The transplantation procedures generally lasted 45-90 minutes per animal. Since good cell viability could be obtained up to 8 hours following cell dissociation (Brundin et al, 1985) and to utilize maximally the tissue obtained from each pregnant female rat, up to 8 animals were transplanted on the same day.

One week prior to transplantation, 12 animals were subjected to cerebral ischemia allowing for mortality due to the insult. The animals were closely observed for approximately 36 hours and respiratory secretions cleared when necessary. Diazepam (1mg/kg) was administered if seizure activity ensued. Five days after this critical period, transplantation procedures were undertaken in 1 control animal and up to 7 ischemic animals. This was followed by saline injections into the hippocampus in one control and one ischemic animal. After recovery the animals were housed individually with food and water ad libitum with a normal 12:12 light dark cycle.

The above protocol was repeated 24 times but on 3 occasions the females were not pregnant. In one instance the supplied embryonic tissue was embryonic day 19.5. A total of 280 animals were used for this study. Mortality due to ischemia, transplantation surgery, anatomical surgery and disease related complications was 37%.

In some experiments the cells used for transplantation were labelled in utero with \(^3\)H-thymidine (New England Nuclear, specific activity 20Ci/mmol). This was done by administering \(^3\)H-thymidine to the pregnant females by daily intraperitoneal injections (0.5ml, 1mCi/ml), for 4 days, beginning at embryonic day 15; a total of 5uCi/gm body weight was administered (Schlessinger et al, 1978; Bayer, 1980a). This was to allow unequivocal identification of transplanted neurons in the host tissue. Due to expense, this procedure was
undertaken in 4 groups only. Other methods to label the donor cells were not used due to reports of inconsistent results, lack of long-term labelling or inability to label a large percentage of the cells.

The remainder of this thesis describes the experiments that were undertaken to investigate the survival, development, and integration of the transplanted hippocampal neurons and their potential to repair the CA1 region which had been lesioned by the ischemic insult. Animals were chosen from different groups for each specific study.
CHAPTER 6

SURVIVAL DEVELOPMENT AND INTEGRATION

The aim of this chapter is to describe how a number of anatomical techniques were used to investigate the survival, development, and integration of fetal hippocampal neurons transplanted into the degenerating environment of the CA1 region of the HF.
6. SURVIVAL, DEVELOPMENT AND INTEGRATION

I. INTRODUCTION

Many events occur in the CA1 region of the adult hosts following ischemia, including cell death, astrocytic proliferation, phagocytosis of debris, angiogenesis and blood brain barrier repair (Lindsay, 1986). To assess whether the transplanted cells, when placed into this extracellular milieu, could survive, mature and develop characteristics that were similar to their normal counterparts, anatomical procedures were undertaken in three phases. Histological staining was undertaken to observe the general properties of the transplanted neurons including morphology, size, organization, and effects on the host tissue. To characterize some of the biochemical properties of these neurons, immunohistochemical (IHC) procedures were undertaken to determine whether CaBP- and PV-like immunoreactivity could be localized in the grafted neurons and whether the transplanted tissue could influence the long-term structural changes which occurred following ischemia. Histochemical procedures were used to assess whether the transplanted neurons received host fibres from one of the major extrinsic afferent systems which normally has extensive projections to the hippocampal formation. The last phase of anatomical investigation was undertaken to ascertain the capacity of the transplanted neurons to project efferent fibres that mimicked the axonal projections of normal CA1 pyramidal neurons to the contralateral hippocampus and the lateral septal nucleus.

II. METHODS

The techniques used for fixation and cutting the brain tissue were as described in sections 2.II.B. and 4.II.B. Animals 2-14 months post transplantation were used from all experimental groups. The anatomical procedures described below were undertaken on approximately 130 animals including transplanted and controls. In approximately 15 transplanted animals transplanted neurons were absent or small in number. This was always correlated with blocking of the injection needle.
Anatomical observations were recorded on film by using an Axiophot fluorescent microscope (Carl Zeiss). For photomicrography, Kodak Tmax (100 ASA) was used to produce black and white negative images and Kodak Ektachrome (160 ASA) was used for colour positives. Fujichrome P1600 D (800 ASA) was used for fluorescence photomicrography.

A. Histology

The most frequently used dyes for general microscopic observation of nervous tissue are Nissl stains (Bancroft and Cook, 1984). This group of dyes has a high affinity for Nissl substance or those parts of the cell containing nucleic acids (RNA and DNA). Therefore colour is conferred in the nucleus, nucleolus, and the granular endoplasmic reticulum. In the present study the Nissl stain thionin was used.

Sections cut on a microtome from paraffin blocks and those cut in a cryostat were mounted onto glass slides. When dry, the paraffin and/or lipids were removed from the tissue by xylene. The xylene was removed by absolute ethanol, the tissue was rehydrated with decreasing concentrations of ethanol in dH$_2$O and then the sections were placed into the dye solution until the appropriate colour was obtained. After staining the tissue was dehydrated in increasing concentrations of ethanol and placed in xylene. The sections were then coverslipped with permount and allowed to dry before viewing.

When the transplanted neurons had been labelled in utero by $^3$H-thymidine, the slides were dipped in Kodak NTB-2 emulsion, exposed for six weeks and developed in Kodak D-19. The sections were then stained with thionin and coverslipped (Bayer, 1980).

B. Immunohistochemistry

Peroxidase anti-peroxidase (PAP), avidin-biotin peroxidase (ABC) (see section 4.II.B.), and occasionally indirect fluorescence IHC procedures were undertaken. In the fluorescent methods the second antibody layers were conjugated to either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (Rhodamine) which emit apple green or red fluorescence respectively (Sternberger, 1979).
C. Histochemistry

Many of the afferent projections to the HF from the medial septal-diagonal band nuclei contain acetylcholine (ACh) (Sofroniew et al., 1982). The catabolic enzyme acetylcholinesterase (AChE) is present in all cholinergic neurons and histochemical techniques have identified the distribution of cholinergic terminals in the HF (Storm-Mathisen and Blackstad, 1964). The presence of AChE terminals in the HF are assumed to arise primarily from the cholinergic nuclei in the medial septal-diagonal band nuclei (Lewis et al., 1967).

In the present study a modification (Hedreen et al., 1985) of the methods of Karnovsky and Roots (1964) was used for the demonstration of axons and terminals in the control, ischemic and ischemic/transplanted CA1 region of the HF. Sections which had been stored in PBS were mounted onto glass slides and, when dry, were rinsed twice in 0.1M acetate buffer (pH 6.0). Sections were then incubated for 15 minutes in 250 ml of medium containing: 162.5 ml of 0.1M acetate buffer, 10 ml of 0.1M sodium citrate, 25 ml of 0.03M cupric sulfate, 42.5 ml of distilled water and 2.5 ml of 0.005M potassium ferricyanide. This was followed by a 30-45 minute incubation in the same medium containing 125mg of acetylthiocholine iodide which acted as a substrate for the enzyme to produce copper thiocholine. The sections were rinsed with 5 changes of acetate buffer and then treated with 1% ammonium sulfide for 2 minutes to form a brown precipitate at the site of enzyme activity. The sections were then rinsed with 5 changes of 0.1M sodium nitrate followed by exposure to 0.1% silver nitrate for 2 minutes. Finally, the sections were well rinsed with 5 changes of acetate buffer, dehydrated, cleared and coverslipped. One half of the slides were counterstained with thionin prior to dehydration.

D. Retrograde Tracing

Six to fourteen months following transplantation, animals were anesthetized with sodium pentobarbital (50mg/kg) and placed into a standard Kopf stereotaxic apparatus. The fluorescent retrograde tracer Fluoro-Gold (FG) (Schmued and Fallon, 1986) was pressure injected into either the contralateral hippocampus (CH) or the dorsomedial lateral septal nucleus (LSN) by using a Kopf micromanipulator and a 1 ul Hamilton syringe. FG (4%, 0.2
ul) was injected into the CH of controls (n = 2) and ischemics with transplants (n = 3) and into the LSN of controls (n = 3), controls with transplants (n = 3) and ischemics with transplants (n = 10). Stereotaxic coordinates were taken from Paxinos and Watson (1986) [LSN in mm: AP = 1.2, -0.4; ML = 0.3; DV = 4.5, 3.5 / CH: AP = -3.0, -4.0; ML = -1.5, -2.5; DV = -2.5, -2.4]. FG was also injected into 5 animals which died during or shortly after the stereotaxic surgery.

Following an 8-10 day survival period, the brain was fixed with the pH shift fixation procedure described earlier (section 4.II.B). Cryostat sections (30μm) were made and stored in the dark in PBS. Sections were then counterstained with ethidium bromide (0.0001%) (Schmued et al, 1982) and mounted onto glass slides for fluorescent photomicrography.

III. RESULTS

A. Histology

i) Physical Effects of the Injection Procedure

The injection of saline into control animals did not cause any observable damage to the overlying cortex or within the hippocampus. When injected into ischemic animals, the degree of damage was not different from ischemic animals without injections. Occasionally in the cortex, the cannula track could be located by a fine line of gliosis.

When fetal neurons were injected into the ischemic CA1 region the transplanted tissue became incorporated into the host parenchyma. A glial border was rarely observed (approx. 3%) in the sections that were Nissl stained. Interestingly, the gliosis in the CA1 region observed after ischemia was substantially reduced. This observation has been confirmed recently by the IHC localization of the astrocytic marker glial fibrillary acidic protein (GFAP) (Tonder et al, 1989). Only twice were transplanted neurons obvious around the needle tract in the overlying cortex and this probably resulted from too rapid withdrawal of the injection cannula.
ii) Gross Anatomical Properties of the Transplants

Transplants were recovered in every cell injected brain with the exception of those correlated with cannulae blockage and those prepared from ED 19.5 fetal tissue. The 2.5 $\mu l$ injections of transplanted neurons tended to disperse into a volume approximately 0.5-2.0mm in diameter; the size being dependent upon the cell density of the suspension. This resulted in an almost continuous (approx. 4.5mm) transplant in the dorsal CA1 region. As the depth of the CA1 SP extended only 50 um it was extremely difficult to place accurately the dissociated cells in the SP. Initially a number of transplants were located in the lateral ventricle and dentate gyrus. Later the transplants were always located in the CA1 region directly into the SP, the SO or occasionally the SR. The transplanted cells did not seem to migrate from the site of injection which was in agreement with the data reported by Wells et al (1987).

iii) Cytological Features of the Transplanted Neurons

The histology demonstrated that the grafted cells appeared morphologically similar to adult hippocampal neurons and approximately 70% appeared to be the size of CA1 pyramidal neurons (nuclear measurements were not undertaken but the size was compared to normal CA1 (mean diameter 11 um) and CA3 (m.d. 19 um) pyramidal neurons by using photomicrographs of the same magnification (Schlessinger et al, 1978)). The dispersed cells often demonstrated the ability to segregate into aggregates that had a stacked arrangement characteristic of the hippocampal CA1 pyramidal cell layer (Fig. 6.1). It was quite obvious from the photomicrographs that only small CA1-like neurons formed these aggregates. Larger neurons, although not abundant, tended to remain scattered.

iv) Organization of the Transplanted Neurons

When the cells were transplanted accurately into the SO or SP of the CA1 region a remarkable degree of laminar-like organization by the aggregates was observed (Fig. 6.1). $^3$H-Thymidine labelling confirmed that the neuronal aggregates in the SO and SP of the CA1 region were transplanted neurons (Fig. 6.2). This pattern of organization did not usually occur when the suspension cell density was high and in these cases the transplants often caused
Figure 6.1

Left Panel: Low power (21X) view of Nissl stained coronal sections comparing a control preparation (A) with 2-month old transplanted neurons segregating into laminar neuronal aggregates (B). Right Panel: High Power (355X) view comparing the appearance of CA1 pyramidal cells (A) with a transplanted aggregate (B). Note the similarity in morphology and density.
Transplanted neurons were labelled in utero with $^3$H-thymidine to confirm that the cells observed in the SP after ischemia were transplanted neurons. (A) dark field photomicrograph (135X) illustrating the position of the labelled neurons. (B) light field photomicrograph of the same Nissl stained section. Large arrows indicate the ischemic SP. Small arrows indicate the position of the transplanted neurons.
distortion of the CA1 region. A laminar organization of transplanted neuronal aggregates was also not observed when the neurons were placed into the SR or the ventricle where they tended to remain dispersed or form unorganized clumps (Fig. 6.3).

When transplantation procedures were carried out in control animals, where the CA1 SP was still intact, the organization of the transplanted neurons appeared to be similar to the organization observed in the ischemic animals. An important observation was that demonstrated by the transplantation of neurons labelled with $^3$H-thymidine into the SP of control animals. In the immediate area of the injections host neurons were absent and had been replaced by the transplanted neurons; this did not cause death or incur damage to host cells adjacent to the cell injection (Fig. 6.4). The transplanted neurons were able to "relaminate" the SP but not with the same density as that observed in the normal CA1 SP.

This pattern of neuronal replacement and relamination was also observed when neurons were transplanted too deeply and invaded the dentate gyrus ($n=12$). The ability of the transplanted neurons to form well organized laminar arrangements in the dentate gyrus was marked (Fig. 6.5). Transplanted neurons could be identified by the obvious size difference between the transplanted neurons and the dentate granule cells (Fig. 6.6).

**B. Immunohistochemistry**

Immunohistochemically two of the prominent cell types of the CA1 region were identified within the transplants. PV-like immunoreactivity, which is localized in a subpopulation of GABA-ergic interneurons, was observed within the transplants. The PV-positive neurons were intensely stained and were scattered among extensive networks of terminal varicosities which were usually located around aggregates of the transplanted neurons (Fig. 6.7). These interneurons were noticeably larger than those observed normally in the HF and they did not send processes through the SR in a manner similar to their normal counterparts.

CaBP-like immunoreactivity, which is normally localized within a subpopulation of CA1 pyramidal neurons, was also detected in many of the transplanted neurons (Fig. 6.8).
**Figure 6.3**

Examples of control (B,C) and ischemic (A,D) preparations to illustrate the appearance of the transplanted neurons (indicated by arrows) when placed in the stratum oriens (A) (33X), the stratum pyramidale (B) (44X), the stratum radiatum (C) (47X), and the lateral ventricle (D) (48X). Note the aggregates which form laminar-like arrangements in (A) and (B) but not (C) and D).
Figure 6.4

The transplanted neurons were able to replace the host neurons when placed into non-ischemic animals with the pyramidal cell layer intact. (A) dark field photomicrograph (128X) indicates the labelled transplanted neurons (between arrows). Label was not present in the host pyramidal neurons. (B) corresponding light field photomicrograph to show the laminar arrangement of the transplanted neurons in the SP.
Figure 6.5

Examples to illustrate the ability of the transplanted neurons (25X) (indicated by arrows) to replace the dentate granule cells and "relaminate" when placed within the dentate gyrus. Although distorted by freezing damage, (E) shows extensive lamination by the transplanted neurons.
Figure 6.6

(A) illustrates a low power view (85X) of the dentate gyrus from (C) in the previous figure. Curved arrows indicate the ability of the transplanted neuronal aggregates to form laminar arrangements even in the dentate gyrus after having displaced the host dentate granule cells. (B) is a high power view (215X) of remaining host dentate granule cells indicated by the large arrow. (C) is a high power view (215X) of the adjacent transplanted aggregate. Note the size difference and the CA1-like appearance of the transplanted neurons.
Figure 6.7

PV-immunoreactivity (peroxidase anti-peroxidase labelled) within the GABA-ergic interneurons and terminal varicosities of the control CA1 SP (A) (252X) and PV-positive (fluorescein labelled) neurons scattered among networks of terminal varicosities within the transplanted region (B) (200X). Note the difference in magnification in (A) and (B). PV-positive transplanted neurons are larger than normal CA1 PV-positive interneurons.
Figure 6.8

The immunohistochemical localization (peroxidase anti-peroxidase labelled) of CaBP throughout the entire cytoplasm of CA1 pyramidal cells (A) and the localization within the CA1-like pyramidal cells of a grafted aggregate (B). Note the apical dendritic processes which are projected with correct polarity. (420X).
Since the antiserum to CaBP conveniently labels the entire cytoplasmic volume of the somata and apical dendrites of neurons containing this protein, this property allowed the gross morphological characteristics of the transplanted neurons to be studied in detail. What was most striking was that the laminar-like transplanted neurons extended dendritic processes reminiscent of the apical dendrites of normal CA1 cells. The dendritic processes projected through the SR demonstrating the correct polarity. When the neurons were transplanted accurately into the lesioned pyramidal cell layer, they were able to obtain a considerable degree of organization. The projection of apical dendrites in a normal manner provided a new matrix within the CA1 region and was able to prevent the progressive shrinkage observed in the CA1 region six months following ischemia (Fig. 6.9).

C. Histochemistry

In the control sections AChE containing terminals could be observed throughout the CA1 region but a denser plexus was present around the pyramidal cell somata. The axons appeared to be irregularly shaped with darkly stained knob like terminals (Fig. 6.10.A). In the shrunken CA1 SP of ischemic sections, a diffuse pattern of AChE activity could be observed but the axons were not as well defined and seemed to have fewer of the darkly stained terminals (Fig. 6.10.B). The AChE-positive terminals which surrounded the transplanted neuronal aggregates did not appear to be different from those observed around the CA1 pyramidal neurons (Fig. 6.10.C).

D. Retrograde Tracing

Retrogradely transported FG was rarely observed in the control CA1 neurons and never observed in the transplanted neurons when FG was injected into the CH. In contrast, following FG injection into the LSN (Fig 6.11), retrogradely transported FG was always observed in the control CA1 neurons and the transplanted neurons (n=7). Transport up to 4.0 mm caudal to the LSN FG injection was observed in the transplants but the number of labelled cells decreased in a rostro-caudal manner and was dependent upon the accuracy of the
**Figure 6.9**

Low power (42X) view of CaBP-like immunoreactivity in the HF comparing (A) a control preparation illustrating the densely stained somata and apical dendrites in the SR of the CA1 region (arrows) with (B) illustrating the dramatic cell loss and shrinkage in the ischemically damaged CA1 region and (C) the transplanted CA1-like hippocampal neurons (arrows) which prevent this shrinkage and extend apical dendrites with normal polarity.
Figure 6.10

Histochemical visualization of acetylcholinesterase (375X) containing axons and terminals in the CA1 pyramidal cell layer of a control preparation (A), following ischemia (B), and a transplanted aggregate (C). Note the well defined terminals in (A) and (C) but not in (B).
Figure 6.11

(A) camera lucida drawing of a coronal section through the lateral septum to indicate the size and location of a representative Fluoro-Gold (FG) injection site. Some tissue necrosis occurred during a 10 day survival period (B) low power (42X) view of the region within the rectangle in (A).
FG injection. Up to 80% of the transplanted neurons in the rostral CA1 region contained retrogradely transported FG (Fig. 6.12, 6.13, 6.14). When the injection had been placed too lateral, outside the septal region, FG labelled cells were not observed (n = 3). Interestingly, in animals where neurons had been transplanted into the region of the dentate gyrus, few FG positive neurons were observed. Further, in the control transplanted group, FG was found in only those neurons transplanted dorsal to the intact CA1 region where damage to axons or somata had been incurred by the volume of transplanted tissue.

IV. DISCUSSION

The control injections of saline demonstrated that mechanical disturbance by the injection cannula was minimal and did not affect the degree of necrosis in the CA1 region. Although the CA1 region was undergoing degeneration, it provided a favorable milieu for the maturation of the transplanted neurons. The neurons were able to attain cytological properties which were remarkably similar to those of their mature counterparts and the dispersed cells had the ability to aggregate into stacked-like arrangements. These observations suggested that these developmental processes were intrinsically controlled or that the trophic substances that are normally present during development are also present during degeneration. The glial cells from the transplant and/or the host may have been able to provide a matrix which was able to support neuronal aggregation in a manner similar to that which occurs during normal migration of the HF pyramidal neurons from the ventricular surface to the cortical plate.

The ability of the transplanted neuronal aggregates to obtain laminar-like organization was most often observed when they were placed close to the principal cell layers in the hippocampus and the dentate gyrus. Recently, it has been shown that "cleavage planes" are present in the HF along the SO, the hippocampal fissure and the dentate granule cell layer (Vietje et al, 1987) and when fluorescent microspheres are injected into these regions they tend to spread along these planes. The degree of organization by the transplanted neurons that was observed, especially in the dentate gyrus, may have been influenced by this unique
Figure 6.12

(A) coronal section (48X) stained with ethidium bromide to illustrate the entire population of cells in the hippocampal formation (B) same coronal section showing the retrograde transport of FG from the septum in the transplanted CA1-like neurons (large arrows). Note that the CA3 neurons which also project to the septum are labelled with FG but the dentate granule cells (small arrows), which do not have septal projections, are not labelled.
Figure 6.13

High power (192X) views comparing the appearance of the FG labelled transplanted neurons in the ischemic CA1 region (A) and the FG labelled neurons in the intact CA1 SP (B).
Figure 6.14

(A) is another example of an aggregate of transplanted neurons (192X) which contain retrogradely transported FG. (B) is a Nissl stained section (162X) to show the position of the FG containing aggregate observed in (A) slightly above the necrotic CA1 SP.
morphological feature. From an anatomical viewpoint, what was quite suprising was that the general organization, density and distribution of the transplanted neurons did not seem to change from 2-14 months.

An important observation was that the transplanted neurons had toxic effects when transplanted to intact regions and could replace host neurons in the immediate area. The possibility exists that this could be due to a neurotoxic substance released by the transplanted neurons or glia that would enhance their own survival. This observation also questions the results of Polezhaev et al (1986) where they suggested that transplanted neurons could normalize dystrophic neurons after hypoxia, based on their absence after transplantation. It is quite possible that the transplanted neurons simply replaced the dystrophic neurons.

A degree of selectivity for the survival of CA1-like neurons was observed following the transplantation of E18.5 tissue. The IHC techniques showed that subpopulations of the transplanted neurons contained the neuron specific proteins CaBP and PV. The observation that the transplanted region contained both excitatory (CaBP-containing) and inhibitory (PV-containing) neurons suggested an anatomical organization reminscent of the normal CA1 region.

The histochemical localization of AChE showed that the transplants were well innervated by host cholinergic afferents in a manner that was characteristic of the pattern of AChE terminals normally observed around hippocampal pyramidal neurons. Since it is unlikely that the cholinergic fibres from the medial septal-diagonal band nuclei would have begun retrograde degeneration after only a week and more likely that they had undergone reactive collateral sprouting, it was assumed that the majority of the cholinergic activity around the transplanted neurons originated from the normal source in the host basal forebrain.

The data from the tracing study suggest that fibre outgrowth from the transplanted neurons is dependent upon their accurate transplantation into the lesioned area and/or their close proximity to the normal CA1 efferent pathways. This was demonstrated by the
extensive fibre projections to the LSN from neurons transplanted to the SO and SP, which are regions where degeneration of the normal axonal projection pathways had occurred, and the lack of projections to the LSN from neurons transplanted into the dentate gyrus. Since the dentate gyrus has extensive commissural projections, the transplanted neurons may have mimicked these projections when transplanted here but this possibility was not investigated. The fact that FG transport was not observed following injections into the CH may have been due to the lack of targets in the CA1 region. Conversely, it has been reported that commissural CA1 efferent fibres do not transport FG well (Swanson et al, 1981). From these data it could not be determined whether or not a commissural projection had been made. Recently, Tonder et al (1988a) have shown, by the use of FG, that the transplanted neurons also project ipsilaterally to the adjacent subiculum.

Long-distance projections by transplanted neurons have been demonstrated in newborn or young hosts (Castro et al, 1987; Chang et al, 1986; Floeter and Jones, 1985; Stanfield and O'leary, 1985; Tonder et al, 1988b) but in adult hosts fibre projections tend to be restricted to closely adjacent regions (Clarke et al, 1986b; Gibbs et al, 1985; Pritzel et al, 1986; Raisman and Ebner, 1983; Sotelo and Alvarado-Mallart, 1987). Most of these observations have been made from solid tissue transplants. It has been well documented that solid tissue transplants often remain "ectopic" rather than integrating into the host parenchyma such as that observed following the transplantation of dissociated cells. The data from the present study demonstrate that fibre projections can be made through the adult host parenchyma to a region located at some distance from the transplanted tissue but may be dependent upon a suitable anatomical and neurochemical environment. Firstly, dissociated tissue becomes well integrated into the host tissue with no obvious glial "barriers". Secondly, the time course of neurotrophic activity in this environment has been correlated with the period of reactive gliogenesis which is enhanced greatly throughout the spatial extent (soma, dendrites, axonal projections) of the CA1 neurons including their efferent projections. In contrast to some forms of mechanical brain injury where retrograde cell death occurs from axons to somata,
ischemia, fibre degeneration follows the loss of somata. The glial cells may not only provide a trophic influence along the degenerating pathway but also may clear a passage by phagocytosing the necrotic debris. Lastly, fibre outgrowth from transplanted neurons is enhanced greatly when the corresponding host cells are destroyed. The fact that the CA1 projection cells are totally absent following ischemia may prevent competition between host and transplant fibres to reinnervate the synaptic vacancies.

V. SUMMARY

These anatomical data suggest that a brain lesion resulting from cerebral ischemia provides an extracellular milieu that allows mechanically dissociated transplanted neurons to survive, attain normal cytological features and integrate extensively into the host tissue. What is surprising is that these developmental processes can occur even after the transplanted neurons have been deprived of the well organized stratified matrix normally present in the developing hippocampus. The fact that the transplanted neurons can project afferent fibres, through the neuropil of the adult host brain, to appropriate extrahippocampal targets may have important implications in the restoration of lost synaptic circuitries. From a functional perspective, by providing synaptic space for normal afferent projections not damaged by the primary insult, and by decreasing the degree of reactive gliosis the transplanted neurons may play a critical role in the prevention of progressive deleterious structural alterations. These data strongly suggest that the transplanted neurons could influence the ischemically lesioned brain in a positive manner.
Chapter 7

Electroresponsiveness and Reestablishment of Circuitries

The aim of this chapter is to describe how a number of electrophysiological techniques were used to assess the functional capabilities of the transplanted neurons including their biophysical characteristics and their ability to reestablish lost synaptic connections.
7. ELECTRORESPONSIVENESS AND REESTABLISHMENT OF CIRCUITRIES

I. INTRODUCTION

To assess the maturational and integrative capabilities of the transplanted neurons, electrophysiological studies were undertaken in three phases. Extracellular recordings were made during the initial experiments to assess the viability of the transplanted neurons. When it was clear that they were electrically active, a preliminary study of their synaptic connectivity was undertaken. Since a degree of selectivity for the survival of CA1-like pyramidal neurons had been demonstrated by anatomical investigation, the cellular and synaptic features of the transplanted neurons were then investigated by intracellular recording techniques and compared to those characteristic of the neurons normally present in the CA1 region. Finally, preliminary experiments using Ca\(^{2+}\)-sensitive microelectrodes were undertaken to determine whether the excitatory amino acids (EAA), N-methyl-D-aspartate (NMDA), glutamate (Glu) and quisqualate (Quis), could induce changes in the extracellular Ca\(^{2+}\)-concentration within the transplanted region. These studies were carried out by using the hippocampal slice preparation for a number of reasons. Firstly, information regarding the biophysical and synaptic properties of the CA1 neurons, recorded in vitro has been extensively documented. Secondly, the location of the transplanted neurons could be easily visualized in vitro but not in vivo. Lastly, considering the long-term nature of transplant investigations, animal mortality due to age and anesthetic was a concern in in vivo studies which dictated use of the slice preparation.

II. METHODS

A. Slice Preparation

Hippocampal slices were prepared from 35 transplanted animals and 8 age matched controls, over the two to fourteen month period following transplantation in a manner similar to that described previously (Dingledine et al, 1980; Schwartzkroin, 1981). Animals were decapitated and the skin and connective tissue overlying the skull removed. The occipital
bones were removed and the parietal plates carefully lifted off the surface of the brain. The tissue was bathed with cold oxygenated Ringer's medium containing (mM) NaCl, 125; KCl, 5; NaHCO₃, 22; CaCl₂, 2; MgSO₄, 2; NaH₂PO₄, 1.25; D-glucose, 10. The dura was removed and complete coronal cuts made caudally, between the occipital cortex and cerebellum and rostrally, at the level of the coronal suture. The blocked brain was carefully removed from the skull and immersed in a beaker of cold oxygenated medium. The block was then placed on filter paper in a Ringer filled petri dish. The two hemispheres were separated and the nontransplanted hemisphere was put back into the Ringer for later dissection. Using paint brushes, the cortex was separated from the diencephalon and the exposed hippocampus carefully rolled away from the cortex. Upon dissection, the transplants could be observed as elevations on the dorsal surface of the hippocampal formation beneath the alvear fibres. Cortical adhesions were cut and the hippocampus placed on a McIlwain tissue chopper. Slices nominally 400 um thick were made from these regions and transferred to the surface of a nylon net within a recording chamber and supported at the gas-liquid interface. Slices were perfused at a rate of 1 ml min⁻¹ with Ringer which was continuously gassed with 95% O₂-5% CO₂ to give a pH of 7.3-7.4. Humidified gas formed the atmosphere in the chamber containing the slices. Chamber temperature was maintained at 34°C and slices were allowed 2 hours equilibration time, prior to electrophysiological recordings, to recover from dissection trauma.

Three different recording chambers were used for these studies which were similar in principle to that described by Schwartzkroin (1981). The third varied slightly from the others in that the net space was considerably larger.

In order to obtain healthy slices we found that great care had to be taken during slice preparation. Difficulty was occasionally encountered with older animals due to increased skull thickness and meningeal adhesions. In addition, the position of the transplants dorsal to the CA1 region predisposed them to dissection trauma. In healthy slice preparations, a clear cell line could be observed in the dentate gyrus and CA3 region, the CA1 region was shrunken with only a dark line remaining and the transplants could be identified microscopically as
opaque foci dorsal to the degenerated CA1 stratum pyramidale. Although the dispersed transplanted neurons often aggregated into laminar-like arrangements (see chapter 6), they did not attain the packing density or degree of laminar homogeneity present in the normal hippocampal formation.

**B. Recording Techniques**

*i) Extracellular*

Recording electrode tips of 2-10 Mohm impedance (Omega dot glass tubing, 1.5mm O.D.) were filled with either 2M NaCl or Pontamine Sky Blue (PSB) (2% in 0.5M sodium acetate) and the barrels with 2M NaCl. Extracellular recording electrodes were connected to a silver-silver chloride half cell, mounted on a Burleigh Inchworm Controller (PZ-550) and connected to the preamplifier headstage of a microprobe systems amplifier (WPI, Model M707) to record potentials in reference to a silver-silver chloride bath ground. Extracellular unit potentials were filtered using a 0.1Hz-10kHz bandpass. Recorded electrical activity was displayed on a dual beam storage oscilloscope (Tektronix) and recorded on film. The position of the extracellular recording electrodes was marked with PSB by passing 2-10μA currents for 1-2 minutes.

The viability of the ischemic and transplanted slices was assessed by recording evoked field potentials in the dentate granule cell layer. The loss of CA1 neurons due to ischemia was confirmed by lack of evoked potentials in the CA1 region even following high intensity stimuli (Fig. 7.2.A). The recording electrode was then used to probe the grafted area until units were located and adjusted to isolate single units. When it was possible to remove the slices from the netting at the end of the recording sessions, they were immersion fixed in 10% formalin containing 1% Ca^{2+}-acetate, sectioned (30 μm) in a cryostat or with a vibratome, and stained with thionin (0.5%).

*ii) Intracellular*

Recording microelectrode tips (Omega-dot glass tubing, 1.2mm O.D.) were filled with either 1 M K^+ acetate (40-80 Mohms) or 5% LY (Lucifer Yellow-CH; dilithium salt; Sigma) in
H$_2$O (80-120 Mohms) and the barrels with 1 M K$^+$ acetate. The intracellular electrodes were connected to a silver-silver chloride half cell and mounted on a Unity-Gain headstage amplifier (HS-2L) of an Axoclamp-2 microelectrode clamp (Axon Instruments) to record potentials which were filtered using a DC-10KHz bandpass. Command pulses to the microelectrodes were provided by a custom-made "probe-driver". Electrodes were placed in media and their impedances balanced by using the "bridge" circuitry of the Axoclamp.

Once a stable recovery had been obtained following impalement, the neurons were activated by shocks applied to anti- or orthodromic pathways or by current injection through the recording electrode utilizing conventional current clamp techniques. The evoked responses were recorded on film from the oscilloscope screen during the experiments or stored on magnetic tape for later analysis. The synaptic potentials and electrical properties of the transplanted neurons were then compared to those characteristic of normal CA1 hippocampal neurons (Schwartzkroin, 1981).

Some impaled transplanted neurons (n = 11) were identified by intracellular injections of LY to permit post-experimental assessment of morphological characteristics. This was achieved by passing hyperpolarizing current pulses (0.5-1.5 nA; 700 ms; 1 Hz) through the recording electrode for 3-8 minutes. Although the firing pattern and membrane properties were measured, it was not always possible to maintain stable recordings for long periods when LY was used to fill the tip of the recording electrode. Therefore, in some slices LY was used to mark the position of transplanted neurons close to the damaged CA1 region. Electrophysiological analysis was then carried out on transplanted neurons slightly dorsal to the LY filled neuron. At the end of the recording sessions (6-8 hrs) the slices were immersion-fixed in 10% formalin containing 1% Ca$^{2+}$-acetate and 20 um cryostat sections were made for histological analysis. Following fluorescence photography of the LY injected cells the sections were counterstained with 0.5% thionin to confirm the position of the neurons within the transplant.
iii) Ion-Sensitive

Triple barrelled iontophoretic electrodes were made by glueing three Omega dot glass pipettes (2 barrels 1.2mm O.D., 1 barrel 1.5mm O.D). A two step pulling procedure was used first to fuse the three barrels and second to pull the fused barrels to a fine tip (1μm). Tips were broken back to give a diameter of 2-3μm, barrels were filled with NMDA, Glut (1.2mm barrels) or Quis (1.5mm barrel) (100mM in 150mM NaCl, pH 7.4), and air bubbles were removed with a heating element. A Silver wire was inserted into each barrel and the complex was sealed with wax.

Ion-sensitive/reference electrodes were pulled from theta glass and the reference barrel was filled with 150mM NaCl. The ion-sensitive barrel was filled with 100mM CaCl₂, silanized to minimize electrical shunting, and the tip was filled with Ca²⁺-sensitive resin (Fluka cocktail 20148), by using a pressure pump (tip diameter 1-3μm; reference barrel 1-10 Mohms; calcium sensitive barrel 5-40 Gohms) (see Heinemann et al, 1977; Heinemann and Pumain, 1980; Mody and Heinemann, 1986). Calcium selective electrode sensitivities (slope) equalled a 26-30mV change in potential in response to a 10-fold change in extracellular calcium concentration [Ca²⁺]₀ (0.3-3.0 mM in the presence of 150 mM NaCl).

Calcium sensitive electrodes were then glued to the iontophoretic electrodes with a separation of 5-10μm between the two tips to permit recordings in the immediate vicinity of amino acid application. Electrical contact to the calcium sensitive/reference microelectrode was made with Ag/AgCl wires. Extracellular potentials detected by the reference electrode and the sum of the extracellular and ionic potentials detected by the Ca²⁺-sensitive electrode were led to a Meyer and Renz 1D-AZ triple channel, high impedance (10¹⁴ ohms) differential amplifier which included a negative capacitance circuit. Reference potentials were subtracted from the ion-sensitive potentials to determine the ionic signals.

Iontophoretic barrels were connected to a standard iontophoretic unit (Medical Systems Corporation). Laminar profiles have shown the changes in [Ca²⁺]₀, in response to NMDA and Quis application, to be maximal in the SP of the CA1 region (Hamon and Heinemann, 1986). Therefore, the electrode complex was placed in the CA1 SP of a control slice and the
retaining and ejection currents were adjusted such that the Ca\(^{2+}\)-sensitive electrode gave a stable measurement, the population spike did not attenuate due to EAA leakage, and so that EAA application produced a large clear decrease in \([\text{Ca}^{2+}]_0\). The microelectrode complex was then placed in the SP of another control slice to record the changes in \([\text{Ca}^{2+}]_0\) and associated negative field potentials following iontophoretic application (15 seconds) of NMDA, Glut, and Quis. Five minute intervals were allowed between each application or stimulus.

After 3-5 measurements along the somato-dendritic extent (100µm increments), the electrode complex was positioned within the shrunken CA1 region of a slice prepared from the non-transplanted hemisphere of experimental animals and measurements made. Finally, the microelectrodes were positioned within the slices prepared from ischemic/transplanted animals and measurements were made from the opaque transplanted regions. After recording in the transplanted slices, the electrode complex was returned to the control slice and measurements were made to confirm that the sensitivity of the electrodes had not been altered during the experiments.

C. Stimulating techniques

Bipolar stimulating electrodes (Nichrome wire, diameter 62µm) were mounted on a micromanipulator (Narishige) and then placed in the SR or SO/AL to investigate the presence of normal Schaffer collateral-commissural afferents to the transplanted neurons and efferent fibre projections from the transplanted neurons. Square wave pulses (0.1ms, 0.2Hz, 5-40V) were delivered from Isolated Stimulators (Digitimer, Model DS2). The general positions of recording and stimulating electrodes are indicated in Figure 7.1.

III. RESULTS

A. Extracellular Recordings

During the initial stages of the electrophysiological experiments extracellular recordings were made from hippocampal slices prepared from 12 animals containing 2-3 month old transplants. Field potentials, evoked by single stimuli to the SR, were observed in
Figure 7.1

The histological section (36X) is a representative of the slices used for recording purposes. Aggregates of cells were identified microscopically as opaque areas dorsal to the necrotic CA1 cell layer (arrows). A recording electrode (R) was used to probe the transplanted region (T) and stimulating electrodes were placed in stratum oriens towards subiculum (SO1), stratum oriens towards fimbria (SO2) and the stratum radiatum (SR). Stimulation of the fibres in these regions normally activates antidromic spikes and action potentials in CA1 neurons.
only one slice. Although the waveform was similar to that observed in the intact CA1 SP, the field was extremely small (1mV compared with 5-15mV) (Fig. 7.2.B). Histological examination revealed that in this one case the electrode had been placed directly within a small transplanted aggregate. The inability to evoke field potentials more frequently was probably dependent upon an inability to visualize the cell distribution within the transplanted areas when placing the recording electrodes. Laminar-like aggregates were often observed microscopically after the slices used for recording purposes were stained.

Activity recorded from the remaining transplants indicated large numbers of electrically active neurons. Recordings revealed that many of the neurons within the grafted area were spontaneously active and typically fired single spikes or 2-6 spike trains (Fig. 7.2.C,D). Single and multiple spikes could be evoked from up to 1mm distance (Fig. 7.2.E,F). The latency and latency jitter suggested that the potentials were synaptically generated (Fig. 7.2.G). This was confirmed by lack of collision extinction when the neurons were spontaneously active. Antidromic-like potentials were difficult to evoke from distances greater than 0.5mm but were identified by their constant, short latency and their ability to follow high frequency stimuli (Fig. 7.2.J). Collision extinctions were performed when the cells were spontaneously active. When stimuli were applied to spontaneously firing neurons, the evoked activity was followed by a period of inhibition before spontaneous firing resumed (Fig. 7.2.I). The presence of pyramidal-like activation-inhibition sequences indicated the presence of some form of intrinsic inhibitory mechanism (Fig. 7.2.H).

B. Intracellular Recordings

Intracellular recordings were made from hippocampal slices prepared from 28 animals containing 3-14 month old transplants and 8 age-matched controls. Immediately upon impalement a small amount of hyperpolarizing current (0.1-0.5nA) was used to stabilize the neurons, some of which were spontaneously active. Following the gradual removal of this current, passive and active membrane properties were assessed by the injection of current pulses. Due to the low packing density of the transplanted neurons and an inability to identify
Figure 7.2

Examples of extracellular recordings to illustrate the spontaneous and evoked responses recorded from hippocampal slices prepared from transplanted animals. (A) viability of the slices was assessed by evoking field potentials in the dentate gyrus (upper trace); the inability to evoke potentials in the nontransplanted contralateral hemisphere confirmed the lack of viable neurons in the ischemic CA1 region (lower trace). (B) six traces to illustrate the evoked field potential in a transplanted aggregate (SR stimulation). A field EPSP can be observed as the positive potential following the stimulus. A number of units fire independently or synchronously causing a small negative deflection at the peak of the EPSP. Spontaneously active transplanted neurons fired single spikes (C) or 2-6 spike trains (D). Single (E) and multiple spikes (F) could be evoked from all stimulating electrode positions (see Fig. 7.1). (G) orthodromic activation was confirmed by the latency to spike and the latency jitter. (H) pyramidal-like activation-inhibition sequence from five superimposed sweeps; the cell was activated from the SR, was inhibited for 200 ms and subsequently resumed firing (I) collision extinction by a spontaneously occurring spike is observed by the lack of evoked activity (upper trace) when compared with the evoked spikes following SO or SR stimulation (lower trace). (J) illustrates a spike evoked antidromically which demonstrates a constant short latency (<2ms).
regions of the transplant containing aggregates in the unstained slice, it was exceedingly
difficult to impale large numbers of neurons in a given slice but both pyramidal-like (n=42)
and interneuron-like (n=10) (see Fig. 7.3) cells could be identified within the transplants
based on characteristics described previously (Schwartzkroin 1975, 1977; Schwartzkroin and
Mathers, 1978; Lacaille, 1987). Electrophysiological analysis was undertaken on the
pyramidal-like neurons.

i) General Membrane Properties

In 40 transplanted pyramidal-like neurons good cell penetrations were obtained in
which membrane properties were stable for at least one hour. The cells had resting
membrane potentials of -55mV or more with a mean of -64.0 ± 6.2 mV (SD).
Hyperpolarizing current pulses were used to determine the passive neuronal input resistance
which was calculated from the slope of the current/voltage plot (mean 24.3 ± 6.0 Mohms).
The relaxation phase of the membrane potential during a constant hyperpolarizing current
pulse was exponential and cell time constants, calculated from the charging curve of
hyperpolarizing current induced voltage deflections, ranged from 9.0 to 15.5 ms (Fig. 7.4).
The mean action potential amplitude measured at the RMP, during the injection of depolarizing
current to bring the cell to threshold, was 74.4 ± 13.9 mV. These values were similar to
those reported for normal CA1 pyramidal cells (Schwartzkroin 1975, 1977; Langmoen and
Andersen, 1981) and were not obviously different from control CA1 pyramidal neurons
measured in the same bath (Table 7.1).

ii) Response to Depolarizing Current Injection

Pyramidal-like neurons within the transplant responded in a normal manner to
depolarizing current pulses (0.3-2.0 nA; 125-225 ms) which could progressively evoke single or
multiple action potentials. As the number of spikes increased with increased current injection,
the early spike intervals were greatly reduced. Although the firing pattern closely resembled
that described for normal CA1 pyramidal cells (Schwartzkroin, 1977, Langmoen and
Andersen, 1981), the current necessary to produce sustained cell firing (1.0-2.0 nA) was
Figure 7.3

Intracellular recordings comparing the firing characteristics of the transplanted hippocampal neurons (B) with those of adult CA1 hippocampal neurons (A). During the injection of a depolarizing current pulse (1) pyramidal-like neurons responded by firing a series of action potentials followed by a large and long-lasting (500-800 ms) AHP. Note the accommodation of cell firing during the depolarizing command. When the current pulse was increased (2) pyramidal-like neurons fired a spike train which was continuous throughout the pulse. Recordings in (1) and (2) are from different cells. (3) Interneuron-like cells responded by firing a train of high frequency spikes.
Figure 7.4

The voltage changes produced by passing a family of rectangular wave current pulses (A). The steady-state (rather than the peak) hyperpolarizing voltage deflection was measured and the passive (resting) neuronal input resistance was calculated from the slope of the current voltage plot (B).

Figure 7.5

Responses following subthreshold and suprathreshold SR stimulation in a CA1-pyramidal neuron (A) and transplanted pyramidal-like neuron (4 months post-transplant) (B). In the transplanted neuron, note the prolonged EPSP (top) and the DAP (prolonged depolarization) which follows an evoked spike (bottom). Compare this response with the synaptically evoked hyperpolarization observed in (A).
Table 7.1

Electrophysiological characteristics of transplanted pyramidal-like neurons and control CA1 pyramidal neurons.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Transplanted (n=20)</th>
<th>Control (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane Potential (mV)</td>
<td>-64.0 ± 6.2</td>
<td>-64.7 ± 4.1</td>
</tr>
<tr>
<td>Input Resistance (Mohms)</td>
<td>24.3 ± 6.0</td>
<td>21.3 ± 3.4</td>
</tr>
<tr>
<td>Spike Amplitude (mV)</td>
<td>74.4 ± 13.9</td>
<td>76.5 ± 4.6</td>
</tr>
<tr>
<td>EPSP Amplitude (mV)</td>
<td>12.6 ± 4.5</td>
<td>10.8 ± 2.3</td>
</tr>
<tr>
<td>EPSP Duration (ms)</td>
<td>72.0 ± 63.9 (25-200)</td>
<td>13.2 ± 2.1</td>
</tr>
</tbody>
</table>
greater (Fig. 7.3). A long-lasting afterhyperpolarization (AHP) could be observed as a voltage deflection (2-10 mV) below pre-stimulus baseline following the termination of the pulse and was probably due to the calcium activated potassium conductance (Ca2+ activated gK) associated with current evoked spike activation (Hotson and Prince, 1980; Schwartzkroin and Stafstrom, 1980).

**iii) Response to Electrical Stimulation: younger transplants (2-7 months)**

Synaptic potentials could be evoked in the transplanted neurons (2-7 months) by SR and SO stimulation (Fig. 7.1) (0.5-1.0 mm from recording electrode) and were followed by small amplitude inhibitory responses. The EPSP (measured just subthreshold for spike activation) rose sharply to a mean amplitude of 12.6 ± 4.5 mV followed by a very prolonged decay to baseline (>25 ms; mean 72 ± 63.9 ms) when compared to control values (Table 7.1). The strong hyperpolarization which normally follows the EPSP in control pyramidal neurons was not observed in the transplanted neurons (Fig. 7.5). When the membrane potential was hyperpolarized at different levels by current injection, the EPSP increased in amplitude in a normal manner (Fig. 7.6.A). The slight hyperpolarization reversed at approximately -80 mV which suggested the presence of some form of K+ conductance.

An action potential could be triggered from the EPSP by increasing the stimulation intensity (Fig. 7.5). In some cells the spike discharge seemed to be initiated sharply from baseline even though subthreshold stimulation produced large EPSP's. A depolarizing afterpotential (DAP) was always observed following spike discharge. When the membrane potential was depolarized, a hyperpolarizing event, which might have been synaptically mediated, could be observed (Fig. 7.6.B). Hyperpolarizing the cell tended to increase the DAP and the injection of depolarizing current decreased the DAP.

Although synaptic inhibitory mechanisms, mediating the early bicuculline sensitive IPSP, were not strong in the younger transplanted neurons, a long lasting afterhyperpolarization followed suprathreshold activation (Fig. 7.7). More than one action potential and occasionally repetitive spikes could be evoked in some cells within young
Figure 7.6

(A) A subthreshold stimulus to the Schaffer collateral-commissural pathway (SR) evokes a prolonged EPSP in the transplanted pyramidal-like neurons (5 months). By hyperpolarizing the cell, the amplitude of the EPSP is increased and the AHP reverses at approximately -80 mV suggesting some form of $K^+$ conductance. (B) Spike profile observed in the transplanted neuron with the greatest repolarization duration (RMP -60mV). A small amplitude inhibitory response was observed when the cell was depolarized.
Figure 7.7

Although the presence of the early IPSP was not obvious in the transplanted neurons (A), suprathreshold activation was often followed by a long lasting afterhyperpolarization (B).

Figure 7.8

Synaptically evoked burst in a transplanted pyramidal-like neuron (3 months) (B) appears similar to the burst evoked in a normal CA1 pyramidal neuron (A) following pharmacological blockade of both IPSP's and the AHP (from Peet and McLennan, 1986).
transplants (2-3 months). These bursts appeared very similar to bursts that could only be evoked in normal CA1 pyramidal neurons following the pharmacological blockade of both IPSP's and the AHP except that the spikes did not arise from the same degree of EPSP depolarization (Fig. 7.8). These neurons also tended to be spontaneously active.

Antidromic spikes could be evoked by shocks applied to the SO (Fig. 7.1) but not the alveus. The antidromic spike was usually followed by a depolarizing synaptic potential from which a spike could be generated at higher stimulation intensities (Fig. 7.9).

iv) Active vs Passive Membrane Conductance

Since the synaptic inhibitory mechanisms which normally control the excitability of CA1 pyramidal neurons were not obvious in the younger transplanted neurons, an attempt was made to uncover the presence of an intrinsic membrane conductance which could possibly mediate some form of inhibition in the transplanted neurons (4-7 months). A hyperpolarizing current pulse (1nA; 200 ms) was passed between 125 and 200 ms, at the peak of the late IPSP, following subthreshold and suprathreshold synaptic stimulation and the input resistance was compared with that observed without synaptic excitation. In control CA1 pyramidal neurons, input resistance decreased by $18.45 \pm 7.3 \%$, following subthreshold stimulation (from age matched controls and see Peet and McLennan, 1986) and decreased $23.9 \pm 7.5 \%$, following suprathreshold stimulation (Fig. 7.10.A). The increase in membrane conductance following suprathreshold stimulation has been attributed to the overlap of the IPSP and the Ca$^{2+}$-activated gK (AHP) resulting from spike activation (Alger and Nicoll, 1980; Nicoll and Alger, 1981). In the transplanted neurons, the active membrane conductance was no different from that observed in the passive membrane (Fig. 7.10.A). However, when more than one spike was evoked, a $20.0 \% (n=4)$ increase in membrane conductance was observed which may have been due to the AHP (Fig. 7.10.B).

v) Response to Electrical Stimulation: older transplants (7-14 months)

Although some of the older transplanted neurons demonstrated electrical properties which were similar to their younger counterparts ($n=8$), inhibitory synaptic events were
Figure 7.9

Stimulation of the fibres in the stratum oriens produced antidromic spikes which were always followed by a synaptic potential (A). A slight increase in stimulation intensity produced both an antidromic and an orthodromic spike (B). A slight adjustment in the position of the stimulating electrode allowed us to isolate the orthodromic component (C).
Figure 7.10

(A) A comparison of the active (right) and passive (left) membrane properties in a control CA1 pyramidal cell (top) with a pyramidal-like transplanted neuron (bottom) following suprathreshold SR stimulation. The CA1 pyramidal neuron demonstrates a 23% change in input resistance measured during the peak of the late IPSP. No change in membrane conductance was observed in the transplanted neuron. Note the absence of any hyperpolarization in the transplanted neuron immediately following the spike (arrow). (B) When the stimulation intensity was increased from subthreshold (top left) to suprathreshold levels in another transplanted neuron, an increase in membrane conductance was evident, by the decrease in voltage deflection during the hyperpolarizing pulse, when more than one spike was evoked. (active and passive traces in (B) superimposed)
clearly present in others (n=25). Some pharmacological studies were undertaken to
investigate the mechanisms underlying these observations. Bath application of APV (DL-
APV; 30μM; Cambridge Research Biochemicals) reduced the EPSP amplitude and duration in
3 of 5 neurons demonstrating "younger" properties (prolonged EPSP) (Fig. 7.11.A). The EPSP
duration (> 60ms) was prolonged in these neurons whereas the EPSP duration was much
shorter (< 30ms) in the neurons not demonstrating an obvious APV sensitive component (Fig.
7.11.B).

In the neurons demonstrating "mature" properties (EPSP-IPSP sequences) the early
IPSP was blocked by bicuculline (2.5-5μM) in a reversible manner resulting in an enhancement
of the EPSP (Fig. 7.11.C).

vi) Characteristics of Transplanted Neurons filled with LY

The intracellular injection of LY demonstrated that the transplanted neurons were
morphologically similar to hippocampal pyramidal neurons (Fig. 7.12.A,B). Basal dendritic
arborizations could be observed in addition to the apical dendritic processes observed
immunohistochemically. In some preparations where the transplant was situated in the SO,
elongated processes reminiscent of apical dendrites could be followed through the damaged
CA1 cell layer to the stratum radiatum. Figure 7.12.B shows a typical example which was
representative of the majority of the LY cells. In addition, dye coupling was observed in three
LY injected preparations (Fig. 7.12.C,D).

C. Extracellular Ca\(^{2+}\) Recordings

Experiments were carried out to record changes in \([\text{Ca}^{2+}]_0\) and associated field
potentials from hippocampal slices prepared from 6 ischemic/transplanted animals (9-14
month old transplants) and 6 age matched control animals.

In control slices NMDA and Glut application (20-60nA) produced concentration
dependent decreases in \([\text{Ca}^{2+}]_0\) with similar kinetics (Fig. 7.13.A, 7.14.A). Control
experiments have shown this decrease to be due to activation of the NMDA receptor and not
Figure 7.11

(A and B) are examples to illustrate the effects of bath applied DL-APV (30μM) on the EPSP recorded from transplanted neurons (10 months). The amplitude and duration could be reduced by APV (5min) in neurons with a very prolonged EPSP (A). APV did not affect the EPSP when the duration was less prolonged (B). (RMP -76mV, -73mV respectively). (C) illustrates an EPSP followed by a strong IPSP. The IPSP was blocked by bicuculline (2.5μM; 15min) which increased the amplitude and duration of the EPSP. The IPSP recovered after washout (25 min) of bicuculline.
Figure 7.12

The intracellular injection of Lucifer Yellow demonstrates that the transplanted neurons (A) are morphologically similar to normal CA1 pyramidal neurons (B). Apical and basal dendritic arborizations have formed in the normal orientation. Dye coupling was observed in some of the transplanted neurons (C). Following fluorescent photography of the LY filled cells the slices were counterstained with thionin (D) to confirm the position of the neurons within the transplant. (all 340X).
voltage dependent calcium channels (Pumain et al, 1987). Decreases in $[Ca^{2+}]_o$ could be observed at the onset of iontophoresis and continued to decrease until the current was discontinued. The $[Ca^{2+}]_o$ then returned rapidly to baseline. Quis (45-100nA) evoked changes which varied with concentration; a net increase in $[Ca^{2+}]_o$ corresponded with small applications and larger doses evoked a net decrease. An increase was sometimes observed at the onset of Quis application which is thought to result from shrinkage of the extracellular space during depolarization (Dietzel et al, 1980). Decreases in $[Ca^{2+}]_o$ were observed after this short increase or at the onset of application if the neurons were depolarized sufficiently to activate voltage dependent $Ca^{2+}$ channels (Lambert and Heinemann, 1988) (Fig. 7.13.B). $Ca^{2+}$ levels recovered to baseline and usually increased from baseline after termination of Quis application which has been attributed to an active extrusion mechanism (Pumain et al, 1987). All responses to EAA application were accompanied by a slow negative field potential. Repetitive stimuli produced decreases in $[Ca^{2+}]_o$ the size of which was dependent upon the stimulus intensity. When similar recordings were made in ischemic slices, changes in $[Ca^{2+}]_o$ were not observed, even at high ejection currents. This was due to the lack of viable neurons in the CA1 region of these slices (Fig. 7.13).

Within the transplants, Quis, Glut and NMDA induced concentration dependent changes in $[Ca^{2+}]_o$ and corresponding negative potential shifts, the kinetics of which were comparable to those observed in the intact CA1 (Fig. 7.13, 7.14). After NMDA and Glut application, decreases in $[Ca^{2+}]_o$ were similar or greater in amplitude (7 of 8 slices) to those recorded in the normal CA1 region. In contrast, changes in $[Ca^{2+}]_o$ after Quis application usually (6 of 8 slices) required very high ejection currents to produce a similar response. Repetitive stimuli from the SR produced changes in $[Ca^{2+}]_o$ only when very high stimulus intensities were used (>40V).
A comparison of the changes in $[\text{Ca}^{2+}]_o$, and corresponding extracellular field potentials evoked by the iontophoretic application (15 seconds) of Glut (A) and Quis (B) in control, ischemic, and transplanted slices. The response to Glut in the transplant is similar to that evoked in the control. In contrast, the response to Quis application is much smaller in the transplanted slice. The responses are minimal in the ischemic slice.
Figure 7.14

The action of NMDA (A) on the extracellular potential and $[Ca^{2+}]_o$ is similar to that evoked by Glut. Evoked responses are strong in response to NMDA but minimal after Quis application even at extremely high ejection currents (B).
A

Control

Transplant

150nA
NMDA

-25nA
NMDA

-35nA
NMDA

B

Quis

-60nA
Quis

-150nA
Quis

[Ca^{2+}]_o

1.60

1.30

1.05

0.85

0
FP

-3
mV

-6

2.0

[Ca^{2+}]_o

1.60

1.30

1.05

0
FP

-6
mV

-12

20s
IV. DISCUSSION

A. Firing Characteristics

The extracellular recordings confirmed the viability of the transplanted neurons by demonstrating that they were electrically active. Two types of firing patterns were demonstrated by neurons within the transplants; those firing single spikes and those firing multiple spikes. Although detailed investigation was not undertaken to characterize the firing properties of these two neuronal types in detail, they may correspond to the simple and complex spike cells described by Fox and Ranck (1975, 1981) thought to represent interneurons and pyramidal neurons respectively.

By using intracellular electrophysiological criteria, two populations of neurons were clearly identified within the transplants; those that showed interneuron-like firing patterns and those demonstrating pyramidal-like characteristics. Many of the intrinsic membrane properties of the pyramidal-like neurons were characterized and the observations demonstrated that even after dissociation, transplantation and development in an adult environment, the transplanted neurons had the ability to attain biophysical properties that were very similar to those demonstrated by mature CA1 pyramidal neurons.

B. Synaptic Inhibition

The presence of IPSPs arising from local inhibitory circuits were not obvious in young transplants, following subthreshold or suprathreshold activation, as demonstrated by the prolonged EPSP and absence of an early or late hyperpolarization. This pattern has been observed previously in heterotypic transplants (Hounsgaard and Yarom, 1985). This was suprising since immunohistochemical observations had demonstrated the presence of PV-positive interneurons which, in vivo, have been associated with GABA containing interneurons in the hippocampus. The fact that many of the older neurons clearly demonstrated bicuculline sensitive IPSP's indicated that synaptic inhibition due to collateral activation was intact and functional in these neurons.
i) Inhibition

The delay of observable inhibition may simply relate to an alteration in the balance of synaptic inhibition and excitation. A number of factors could play a role in attenuating the inhibitory influences. Firstly, the formation of inhibitory synaptic contacts may be delayed. Secondly, the inhibitory synapses may be intact but the interneurons may not receive sufficient feed-forward and/or feed-back excitatory input. Although the interneurons form a terminal plexus around the pyramidal-like neurons they do not seem to send processes towards the SR in a manner similar to interneurons in the intact CA1 region. Thirdly, the inhibitory synapses may be intact but may be acting at a different post-synaptic receptor. It has been suggested that in immature neurons GABA_B receptors normally present in the dendritic but not somatic membranes are also present in the somatic membranes. With maturity these receptors which mediate depolarizing events are replaced by GABA_A receptors which mediate hyperpolarizing events (Schwartzkroin, 1982, 1986). This could underlie the prolonged DAP observed in the transplanted neurons.

ii) Excitation

The present results could also be explained if the inhibitory synapses were intact but the hyperpolarizing events were masked by enhanced excitatory events. It is probable that the extensive neurite networks, observed anatomically, receive an irregular density and distribution of afferents which may eventually 'normalize'. Early in development, neurons usually receive an extensive afferent component which is attenuated greatly over time. This results from "programmed" cell death and removal of excess synaptic contacts. Afferent fibre distribution to the transplanted neurons may occur in a similar manner, by extensive sprouting by the adult host, but the programmed cell death is not likely to occur. Therefore it may take much longer for the density and distribution of afferent fibres to approach normal levels. The ability of the transplanted neurons to obtain a totally mature membrane composition may be dependent upon a normal complement of afferent input.

Although speculative, it is possible that during development in the host environment the transplanted cells may undergo a delayed transition from a "Ca^{2+} dependent" system to
a "Na\(^+\) dependent" system. In young transplanted neurons excitatory events may result in an excessive increase in Ca\(^{2+}\) influx which could mask the inward Cl\(^-\) current mediating the early IPSP (Inoue, 1986). Some evidence for this contention has been obtained from immature neurons in which NMDA-evoked currents are less voltage dependent and can be activated at quite negative membrane potentials (Ben-Ari et al, 1988). One result of an enhanced role of NMDA receptor mediated Ca\(^{2+}\) influx in low frequency synaptic transmission in the transplanted neurons, could be a prolongation of the EPSP. The data from the present study are certainly consistent with this hypothesis.

Although no definitive conclusions could be drawn from pharmacological experiments that were carried out while making intracellular recordings, the decrease in EPSP amplitude following APV application was also suggestive of an enhanced NMDA component. Furthermore, the extracellular calcium measurements suggest that receptors for the excitatory amino acids are abundant in the transplanted neurons. The large decreases in \([\text{Ca}^{2+}]_o\) in response to NMDA when compared to Quis may be due to a difference in receptor density and/or an attenuated voltage dependency. Ben-Ari et al (1988) have suggested that, in immature neurons, an enhanced NMDA-evoked Ca\(^{2+}\) influx may have an important role in promoting neuronal growth and differentiation. The same may be true for the transplanted neurons but it may be necessary that this growth enhancing property to remain "turned on" for a longer time.

**C. Intrinsic Inhibition**

Whatever the dominant receptor mediated events may be, the excitability of the transplanted neurons seemed to be controlled. This may be due to intrinsic inhibitory mechanisms such as voltage- and/or Ca\(^{2+}\)-activated \(K^+\) conductances whose presence are indicated by the normal activation-inhibition sequences, and by the AHP following current evoked spike trains and synaptically evoked multiple spikes. These intrinsic inhibitory events could be the primary method by which the transplanted neurons control their excitability until
reaching a totally mature state. Further, although *in vivo* investigations were not undertaken, the possibility exists that input from extrinsic sources may have an enhanced role in modulating the excitability of the transplanted neurons.

**D. Afferent and Efferent Fibre Projections**

In addition to the aforementioned biophysical properties, the data from the present study have also demonstrated that the transplanted neurons have the ability to become integrated into the synaptic circuitries of the host tissue. This may have been enhanced by the fact that the cells were transplanted into the adult region which is equivalent to the region from which they were taken; the CA1 region of the hippocampal formation. Orthodromic activation of these neurons by shocks applied to the SO and SR, which contain afferents from a number of sources, always evoked synaptic responses in the transplanted neurons even when they were quite dorsal to the necrotic CA1 cell layer. Examination of the LY filled pyramidal-like cells confirmed the projection of elongated apical processes which extended into the SR, perhaps to receive these afferents. The observation that small extracellular fields could occasionally be generated showed that the transplants had the capacity to obtain a high degree of organization. These observations showed that the transplanted neurons could provide a new synaptic target for the host afferent fibres that were not initially affected by the ischemic insult. The degree to which each specific afferent system contributes to the synaptic innervation of the transplanted neurons is not known.

The transplanted neurons could also be stimulated antidromically as demonstrated by the constant latency of the evoked response and collision extinction. The fact that antidromic spikes could be evoked from the SO and not the SR suggested that the transplanted neurons projected basal neurites with the same polarity as the axonal projections of normal CA1 pyramidal neurons. It is not known by what route these fibres exit the hippocampus but the fact that these projections reach the lateral septal nucleus is quite remarkable. The axons may run in the SO until reaching the fimbria and then follow degenerating fibres to this target.
V. SUMMARY

These electrophysiological observations suggest that the transplantation of homotypic fetal neuronal tissue may be a promising approach for the restoration of lost synaptic circuitries following acute trauma to the CNS. The data demonstrate that the transplanted neurons can develop many of the electroresponsive properties that are characteristic of the adult cells they have replaced and consequently, have the potential to mediate normal functional capabilities. In addition, the fact that the transplanted neurons can attain a degree of synaptic integration suggests that the transplanted tissue could conceivably provide the relay necessary to carry information across the acute lesion.
CHAPTER 8

GENERAL SUMMARY

The aims of this chapter are to summarize the observations made in the present study and to put forth suggestions for the future direction of investigations of the "repair potential" of transplanted neurons.
8. GENERAL SUMMARY

The present study describes the development of a model whereby degeneration and repair could be investigated in the adult rat CNS. Investigations were carried out using this model to examine both the long-term degeneration that occurs following an insult of cerebral ischemia, and the functional capabilities of fetal neurons subsequently transplanted into the region of degeneration. The data have shown that transplanted neurons can obtain a remarkable degree of functional integration in the host tissue and have great potential in the reconstruction of lost synaptic circuitries, in the prevention of secondary degeneration and optimistically, the reduction of neurological deficits.

I. SUMMARY OF RESULTS

1) A method was established whereby the induction of cerebral ischemia consistently produced a region specific lesion in the adult rat brain which showed similar pathology to that observed in the human brain following a similar insult. This method produced highly selective bilateral damage to 93% of the dorsal CA1 hippocampal cells.

2) Neuronal transplantation techniques were then developed for the repopulation of this lesion in the CA1 region with suspensions of fetal hippocampal tissue. Optimal conditions for the survival and growth of the transplanted tissue were taken into account and these included the correct developmental stage of the fetal tissue, an appropriate quantity of tissue, the period of enhanced neurotrophic activity and the most accurate and least traumatic method of transplantation.

3) The ability of the transplanted neurons to remain viable when placed into a degenerating environment was confirmed by the anatomical demonstration of $^3$H-thymidine labelled neurons in the lesioned region and the demonstration that the transplanted neurons were electrically active.
4) Histological and immunohistochemical techniques showed that the transplanted neurons had the ability to develop intrinsic properties that were similar to the cells they had replaced. The transplanted neurons developed cytological features that were indistinguishable from their normal counterparts, they showed a remarkable degree of organization, and they expressed some of the same neuron specific proteins.

5) Anatomical techniques also demonstrated that the transplanted neurons had the capacity to become well integrated into the host tissue. Histological observations showed that the transplanted tissue did not remain "ectopic" but became continuous with the host parenchyma with no visible gliotic host-transplant barrier. Histochemical and axonal tracing techniques showed that some afferent and efferent fibre projections could be reinstated (Fig. 8.1).

6) Electrophysiological recordings confirmed the functional integration of the transplanted neurons in the host tissue. Calcium measurements and intracellular recordings showed that although maturation seemed to be delayed, the transplanted neurons developed electrophysiological properties that were similar to the cells they had replaced. Intracellular and extracellular recordings demonstrated that the restored neural connections formed functional synaptic contacts (Fig. 8.2).

II. DISCUSSION AND FUTURE DIRECTION

Cerebral ischemia and other acute brain insults can result in the permanent loss of essential brain circuitries and neurological deficits. Presently, methods are not available for the prevention of neuronal loss and therefore the possibility that transplanted neurons possess the potential to repair such damage has immense clinical implications.

Alterations following injury to the brain may cause progressive and widespread cerebral atrophy due to a "domino" effect of fibre degeneration and subsequent loss of neuronal pools. The observations from the present study showed that neuronal transplants
Figure 8.1

A schematic of a horizontal section through the rat brain to summarize anatomical observations. The hippocampal neurons that were transplanted to the ischemically damaged CA1 region of the adult hippocampus can project fibres to the lateral septum.
Figure 8.2

A camera lucida drawing of an LY filled transplanted neuron to illustrate the dendritic arborization, the long apical dendritic process, and the postulated connections based on electrophysiological recordings.
have important consequences for the prevention of secondary pathology which normally follows the primary ischemic lesion.

The plastic nature of the CNS normally allows for the continuous remodeling of neural networks. It is not known how essential the "hard wired" circuitries of the adult CNS are for the spatial and temporal aspects of information processing but the data from the present study suggest that transplanted neurons have the potential to provide a bridge between target regions for the transfer of information.

Although the present study has laid the foundation for our understanding of the potential functional capabilities of transplanted tissue in the repair of ischemic brain lesions there are many aspects that require detailed investigation. It is also critical that we gain sufficient understanding of the natural responses to injury so that we may learn how to combine the appropriate interventions to manipulate these responses in the most beneficial way. A number of avenues are now open for further analysis.

1) Acute dissociation of the transplanted neurons, after their development in the adult host, will allow further electrophysiological investigation of their biophysical properties.

2) Systematic anatomical investigation will allow assessment of the degree and distribution of specific afferent and efferent fibre projection systems to and from the transplanted region.

3) A combination of electrophysiological and electron microscopic analysis will clarify the development of functional inhibitory synaptic contacts and the normality of their distribution on the transplanted neurons.

4) Preliminary investigations have suggested that the hippocampal regions, not initially affected by the ischemic insult, tend to become hyperexcitable. This increase in excitability may be important in mediating further degeneration. Electrophysiological analysis will determine whether the transplanted neurons have a functional role in influencing these events in a beneficial manner.
Finally, it may be possible to confirm that functional integration has been obtained by the transplanted neurons if amelioration of a behavioral deficit can be demonstrated. The role of the hippocampal formation in learning and memory mechanisms has been well documented. It is attractive to speculate that the reconstruction of synaptic circuitries by the transplanted neurons could mediate some degree of information processing.
9. REFERENCES


PUBLICATIONS

Papers


Abstracts


..........continued
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PUBLICATIONS


