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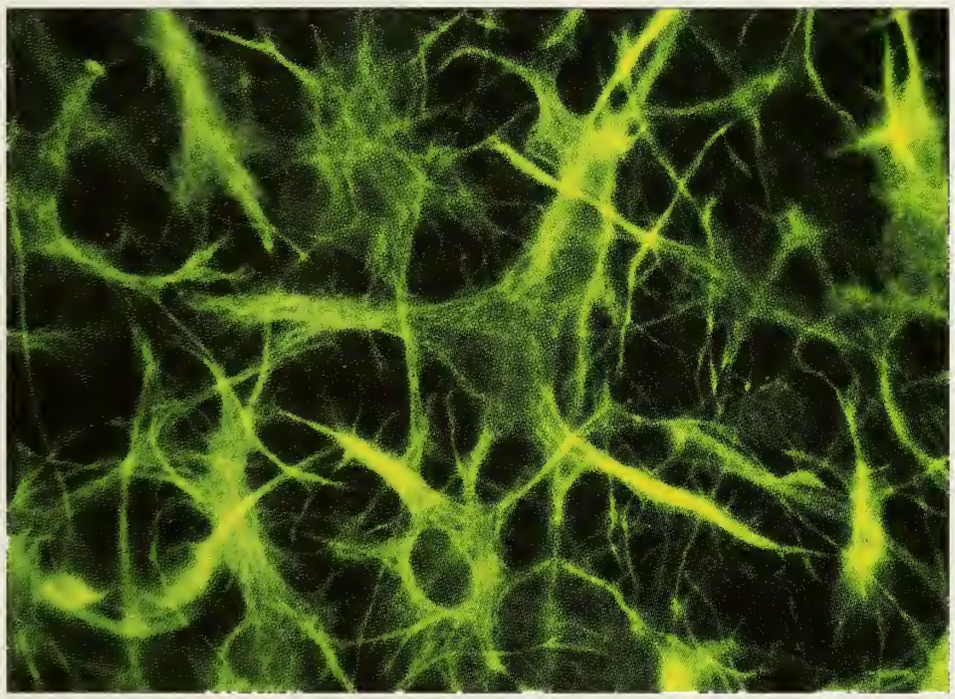

Alcohol and Glial Cells

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ALCOHOL AND GLIAL CELLS

Edited by
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This monograph is based on the workshop, "Alcohol and Glial Cells," sponsored by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) and held in Washington, D.C., November 6, 1993.

About the Editor: Francine E. Lancaster, Ph.D., developed this monograph and workshop while she was neuroscience program director in the Neurosciences and Behavioral Research Branch, NIAAA. She currently is Professor, Department of Biology, Texas Woman's University, Denton, Texas.

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FOREWORD

Glial cells make up about one-half of the volume of the brain, but until recently these cells gained little attention in the study of brain responses to alcohol. Although reports of gliosis following alcohol abuse are common, very little information is available on the influence of alcohol on glial cells. Damage to the white matter of the brain is a dominant feature of the alcohol-induced brain damage observed in chronic alcoholics and has been observed in children exposed to alcohol in utero.

Historically, more information has been available on the effects of alcohol on oligodendroglia and myelin than on the other types of glial cells. Interest in studying the responses of astrocytes to alcohol has been increased by new research techniques showing that astrocytes have ion channels, have receptors for neurotransmitters and hormones, produce or respond to cellular messengers, and have extensive processes which communicate with neurons and other glial cells. New attention has also been directed to a third glial cell type, the microglia. Microglia carry out immune responses in the brain, produce neurotrophic factors, and produce reactive oxygen species, including nitric oxide. This monograph presents evidence that microglia, as well as astrocytes, proliferate in response to alcohol exposure.

In an effort to bring researchers together to share their latest findings and ideas on alcohol and glial cells, the National Institute on Alcohol Abuse and Alcoholism (NIAAA) sponsored a symposium entitled "Alcohol and Glial Cells," held in Washington, D.C., November 6, 1993, in conjunction with the 1993 annual meeting of the Society for Neuroscience. *Alcohol and Glial Cells*, NIAAA Research Monograph No. 27, is based on papers presented at that symposium and papers invited from additional authors.

Scientists with a special interest in research on glial cells were invited to give overviews of the current status of glial cell research, to introduce some of the new techniques that are making research on glial cells more feasible, and to present their latest findings on the effects of alcohol on glial cells. The researchers who participated in the symposium and contributed chapters to this monograph spent many hours preparing and presenting their work at the symposium, and even more hours writing and revising the chapters for the monograph. NIAAA appreciates the effort, commitment, and contribution of each of these researchers. The development of this monograph was guided by Francine Lancaster. Her dedication, organization, and sound editorial judgment has made this publication possible.

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PREFACE

Acute and chronic alcohol exposure have a documented impact on nervous system function of adults and developing individuals. In the past, research on the effects of alcohol on the brain has concentrated on the study of neurons. The purpose of this monograph is to present updated information from the latest research findings on the effects of alcohol on glial cells and to introduce new research techniques for the study of the effects of alcohol on glial cells. To accomplish these goals, experts in glial cell research were asked to provide an overview of current developments in research on astrocytes, oligodendroglia, and microglia. Researchers with new data on the effects of alcohol on glial cells were invited to present their findings and to provide ideas for future research on alcohol and glial cells.

The first three chapters of the monograph provide an overview of research findings and techniques used to study astrocytes, oligodendroglia, and microglia. The reader is introduced to the study of alcohol and glial cells in the central nervous system.

Chapters 4 through 7 provide current findings on the influence of alcohol on glial cells during development. Information is presented from in vivo studies, from cell culture models, and from cells cultured from animals exposed to alcohol, or cells exposed to alcohol in culture.

Chapter 8 presents information on the role of astrocytes in alcohol-induced damage of the neuroimmune system. Evidence is provided to suggest that alcohol exposure may cause deficits in the ability of astrocytes to respond to infections in the brain.

Chapters 9 and 10 discuss the role of glial cells in alcohol-induced neuropathology. Two consequences of alcohol exposure on glial cells, up-regulation of neurotrophin production and excessive production of reactive oxygen species such as nitric oxide, are discussed. Data showing the up-regulation of neurotrophin expression by astrocytes in response to alcohol are used to support the development of astrocytic transplantation as a potential treatment for alcohol-induced cognitive losses. Evidence for induction of nitric oxide synthesis in glial cells is linked to alcohol-induced neuronal injury.

Chapter 11 provides compelling evidence for the involvement of astrocytes in hepatic encephalopathy. The role of astrocytes in removing ammonia from the nervous system and damage to this process by alcohol are discussed.

Chapter 12 presents a new imaging technique capable of separating glial and neuronal images in alcohol-induced brain atrophy. The implications of these findings are discussed in

chapter 13, which also presents information on alcohol-induced disturbances in neurosteroid production by glial cells and some ideas for future research.

I would like to express my gratitude to the participants in the symposium and contributors to this monograph. The efforts of these researchers to produce excellent and comprehensive overviews of research on alcohol and glial cells are greatly appreciated. An immense amount of time and effort was spent to prepare the presentations and chapters in the midst of busy schedules. Without their efforts, this work would have been impossible. I would like especially to acknowledge Dr. Harold Kimelberg for the extra time he spent in offering suggestions for the symposium and the monograph. The dedicated work and excellent attention to detail by Eve Nelson Shapiro and coworkers of CSR, Inc., were critical in producing the final product. I would like to acknowledge my colleagues at NIAAA, Diane Miller and Diana O'Donovan, for their excellent work on the monograph; Nancy Colladay for an outstanding job on the symposium; and Walter A. Hunt and William E.M. Lands for supporting this project from idea to completion.

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Chapter 1

Astrocytes and Their Functions, Past and Present

Harold K. Kimelberg, Ph.D., and Michael Aschner, Ph.D.

This chapter starts with a brief history of studies on glia, concentrating on the astroglia or astrocytes. This is followed by a brief review of recent research on the role of astrocytes in homeostatic mechanisms within the central nervous system (CNS), such as the maintenance of normal extracellular ion concentrations, the uptake of K^+, and the control of extracellular pH. Astrocytes in primary culture have been found to contain the whole pantheon of receptors, and also many of the uptake systems, for CNS transmitters; properties that were formerly thought to be exclusively neuronal. The uptake of transmitters, usually by Na^+-dependent mechanisms, and their subsequent inactivation by metabolism and possible functions for the receptors are then briefly surveyed. Also discussed are important roles of astrocytes during early brain development in neuronal migration and the production of neurotrophic factors important for neuronal division and differentiation, and the possible role of the astrocytes in the development and maintenance of the blood-brain barrier (BBB). The potential role of astrocytes in inflammatory or immunological responses in the brain is also surveyed, but there is some controversy regarding the relative importance of astrocytes and microglia in this regard. The role of astrocytes in brain pathology emphasizing the gliotic and swelling responses is then described. The small amount of information currently available on how ethanol may interfere with the role of astrocytes in brain development and how it may affect other astrocytic functions is reviewed.

BACKGROUND

Progress in science is supported on the twin pillars of methods and concepts (Medawar 1984). Advances in methodology have been critical to our current rapid advances in the understanding

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of the nervous system and these advances, and the biological concepts that we use to interpret the observations made come as much from other fields of biology as from studies within the field of neuroscience.

One concept unique to the nervous system is that its functions are overwhelmingly due to the properties of its electrically excitable cells, the neurons. There is, however, an even more numerous class of nonexcitable cells in the nervous system, collectively referred to as the neuroglia. This class comprises the astroglia, or astrocytes; the oligodendroglia, or oligodendrocytes; and the microglia.

Elucidation of the true nature of the neuroglia depended on advances in histological staining by Golgi and Ramon y Cajal in the period between 1870 and 1890. The term "neuroglia" came from an essentially erroneous concept of Virchow, which he proposed in 1850; namely, that neurons were embedded in a connective tissue to which he gave the name "neuroglia," or nerve glue. Although erroneous, this term—or its shortened form, "glia"—has persisted as the preferred generic term for these cells. Other terms used by Virchow were "Nervenkitt" and "Bindesubstanz" (Somjen 1988; Tower 1992).

According to Tower (1992), the term "astrocyte" was coined by Mihaly Lenhossek in Budapest in the 1890's, who also introduced the term "astroblast" for the developmental precursors of mature astrocytes. Two forms of astrocytes, namely the fibrous astrocyte and the protoplasmic astrocyte, were also recognized at around the same time by von Kolliker in Wurzberg and Andriezen in London (Tower 1992).

Examples of the morphological heterogeneity of astroglia within the CNS are shown in figure 1 for the cerebellum. These camera lucida drawings are from tissue sections stained by Ramon y Cajal's method. The fibrous astrocytes (f in figure 1) have many thin processes filled with bundles of the approximately 100-Å-wide filaments, which consist predominantly of the astrocyte-specific glial fibrillary acidic protein (GFAP). The protoplasmic astrocytes (s and v in figure 1) also have numerous but shorter processes, which in situ tend to insinuate in complex patterns between the neurons and their processes right down to the synaptic boutons where they contact the neuronal soma or dendritic processes. This was shown in a study by Derouiche and colleagues (1993) where the astrocytic processes were labeled with antibody to glutamine synthetase (GS) and could be detected close to and enclosing synapses (figure 2). The Bergmann glia (b in figure 1) are also astrocytic since they often stain positively for GFAP. They function like radial glia early in development by serving as guides for the migration of granule cell neurons (see the section on development and neurotrophic factors and the BBB, later in this chapter; see also the legend to figure 4). Unlike radial glia, however, they persist in the adult brain.

Bruckner and colleagues (1993) found that perineuronal nets that could be detected by staining polyanionic sites with colloidal iron or hyaluronan (hyaluronate) staining with plant lectins were also positive for GS or S-100 proteins. In this way the astrocytic processes appeared to form a perineuronal network over the soma and dendrites of many neurons. Panel a of figure 3 shows, at the light microscopic level, the complex net formed over neurons. The enclosing of synaptic boutons on the neuronal soma is shown, at the electron microscopic level, in panel b of figure 3. Thus, astrocytes and their processes are intimately associated with the neuronal interconnections that are critical to the functioning of the neu-

Figure 1. Different morphological forms of cerebellar astroglia from drawings based on staining using the Golgi and Ramon y Cajal methods. The top dotted line, labeled A, indicates the cerebellar surface. M = molecular layer; P = Purkinje cell layer; G = granule cell layer; W = white matter; b = Bergmann glia; s = smooth protoplasmic astrocytes; v = velate astrocytes; f = fibrous astrocytes in the white matter. Reprinted with permission from Wilkin, G.P., and Levi, G. Cerebellar astrocytes. In Fedoroff, S., and Vernadakis, A., eds. *Astrocytes: Development, Morphology, and Regional Specialization of Astrocytes.* Vol. 1. Orlando, FL: Academic Press, 1986. p. 247.

rons, and so could profoundly alter or modulate these functions.

The protoplasmic astrocyte has a much lower density of intermediate filaments and often will not stain for GFAP in situ, but will stain for the nonspecific intermediate filament protein vimentin (Bignami et al. 1980), GS, or S-100 protein (Bruckner et al 1993). It has also been suggested that vimentin predominates in the astrocyte early in development and is replaced by GFAP at later times, and that vimentin also increases in gliosis (Bignami and Dahl 1976; Bignami et al. 1980; see also the section on reactive gliosis and scar formation later in this chapter). The fibrous astrocytes are localized in situ to the white matter, whereas protoplasmic astrocytes are found exclusively in the gray matter.

It was recognized early on that the ends of astrocytic processes surrounded the blood

capillaries of the brain, either in gray or white matter, and these endings were called end feet. Other astrocytic processes were found to end around synapses, and other processes extend to the axonal nodes of Ranvier. These morphological specializations led researchers, as early as the turn of the century, to speculate that astrocytes might form the basis of the BBB and/or take up transmitters or other products from the extracellular fluid in the vicinity of synapses (Lugaro 1907).

By the first two decades of the 20th century the neuroglia had already been classified into the macroglia, consisting of the astroglia and the oligodendroglia, and the microglia. The oligodendroglia, or oligodendrocytes as they are more commonly called, are responsible for the formation of CNS myelin, which functions predominantly to speed up nerve conduction for a given cross-sectional axonal area, thus greatly conserving space within the brain and presumably allowing for more complex interneuronal connections. The microglia, originally described by Ramon y Cajal as the third glial element, was fully identified and described by del Rio Hortega in the period from 1920 to 1930 (Peters et al. 1976). According to the present state of knowledge, the microglia

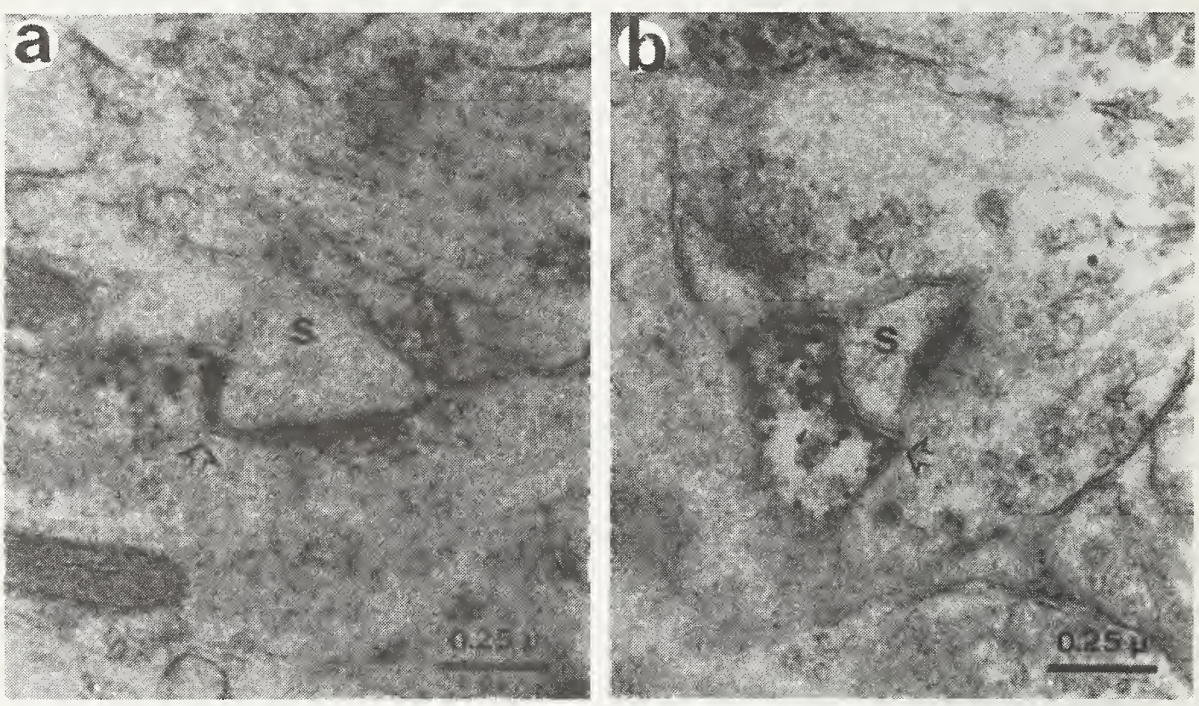

Figure 2. Astrocytic processes surrounding a presumed glutamatergic synapse in hippocampal slices cultured for 18 days from 2- to 4-day-old rat pups. Note the close apposition of two glutamine synthetase (GS)–positive astrocytic processes to the synaptic cleft and the pre- and postsynaptic elements. GS was detected by immunocytochemistry using a diaminobenzidine reaction. The diaminobenzidine precipitate was intensified with silver and the grains were replaced with gold particles for electron microscopy; the particles are seen as dark round dots. (a) GS–positive processes approach the synaptic cleft. (b) These processes partly enclose the pre- and postsynaptic elements. Open arrows indicate tips of astrocyte processes that protrude into the synaptic cleft. S = dendritic spines. Reprinted with permission from Derouiche, A.; Heimrich, B.; and Frotscher, M. Loss of layer-specific astrocytic glutamine synthetase immunoreactivity in slice cultures of hippocampus. *European Journal of Neuroscience* 5:122–127, 1993.

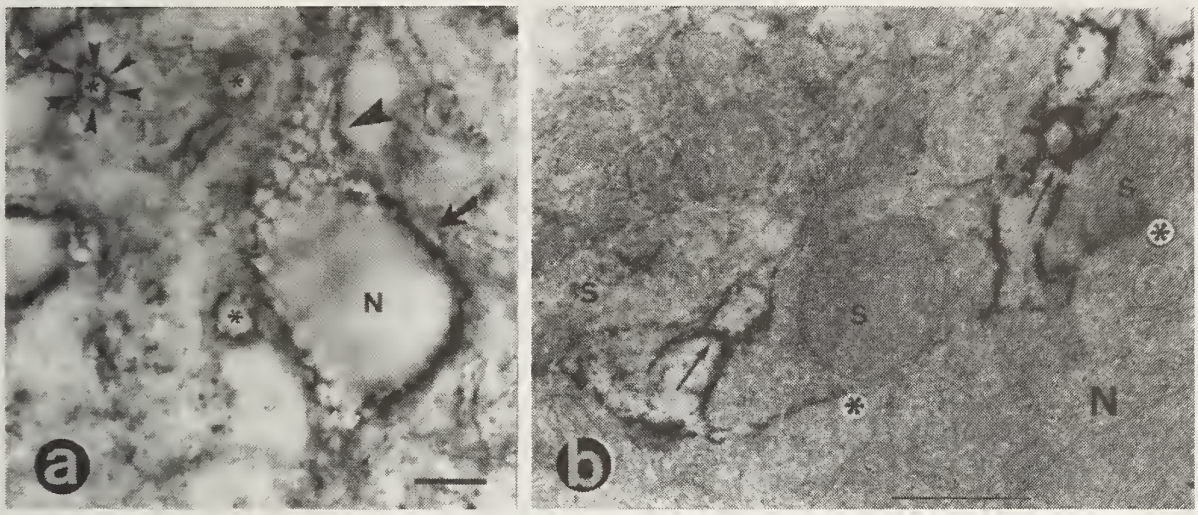

Figure 3. (a) Staining of polyanionic sites by colloidal iron, disclosing perineuronal nets over a large neuron (N) in the rat medial cerebellar nucleus. The top half of this neuron (large arrowhead) shows a surface view, and the lower half (arrow) shows a cross section through the same neuron. The asterisks indicate cross sections of large dendrites, which show a punctuate staining (small arrowheads). Normarski optics. Bar = 10 µm. (b) Staining at the electron microscopic level of lectin (*Vicia villosa*) binding in the visual cortex of the rat. The diaminobenzidine precipitate indicates the staining of polyanionic proteoglycans thought to be the same as the staining of polyanionic sites responsible for the perineuronal net shown in panel a. This cross-sectional area shows the presynaptic boutons (S) ensheathed by astrocyte profiles, which bind lectin. Membrane-associated (arrows) or extracellularly located precipitates predominate. Note that the synaptic cleft zones, indicated by asterisks, are free of label. Bar = 0.5 µm. Reprinted with permission from Bruckner, G.; Brauer, K.; Hartig, W.; Wolff, J.R.; Rickmann, M.J.; Derouiche, A.; Delpech, B.; Girard, N.; Oertel, W.H.; and Reichenbach, A. Perineuronal nets provide a polyanionic glia-associated form of microenvironment around certain neurons in many parts of the rat brain. *Glia* 8:183–200, 1993.

seem to have little to do directly with normal neuronal function, and are mainly involved in the response of the brain to injury. They also exhibit important immunological properties (Graeber et al. 1993). These three types of "glia" and their relations to the neuron and other elements of the CNS are shown diagrammatically in figure 4. The thickness of the cerebral cortex from pia to ependymal layer is too great for any one astrocyte to make all the contacts shown and also to function effectively to buffer K^+ (Reichenbach 1989). During early development the astrocytic radial glia do stretch vertically from the ventricles to the pial surface, but as the cortex develops it is thought that these glia lose their ability to span the cortex and change to stellate astrocytes (Hollander et al. 1991; Reichenbach 1989). Some glia retain their radial orientation, such as the Müller cells of the retina (Hollander et al. 1991), and to a more limited extent the Bergmann glia of the cerebellum (see figure 1).

Thus, by the 1920's, the major forms of glia had been recognized and identified structurally by their histological appearance. Their basic structures and relationships with other critical parts of the nervous system were well recognized, and this led to the types of speculative suggestions on the function of glia already mentioned. Without critical experimental tests of such speculations or hypotheses, these suggestions were often wrong; however, in the

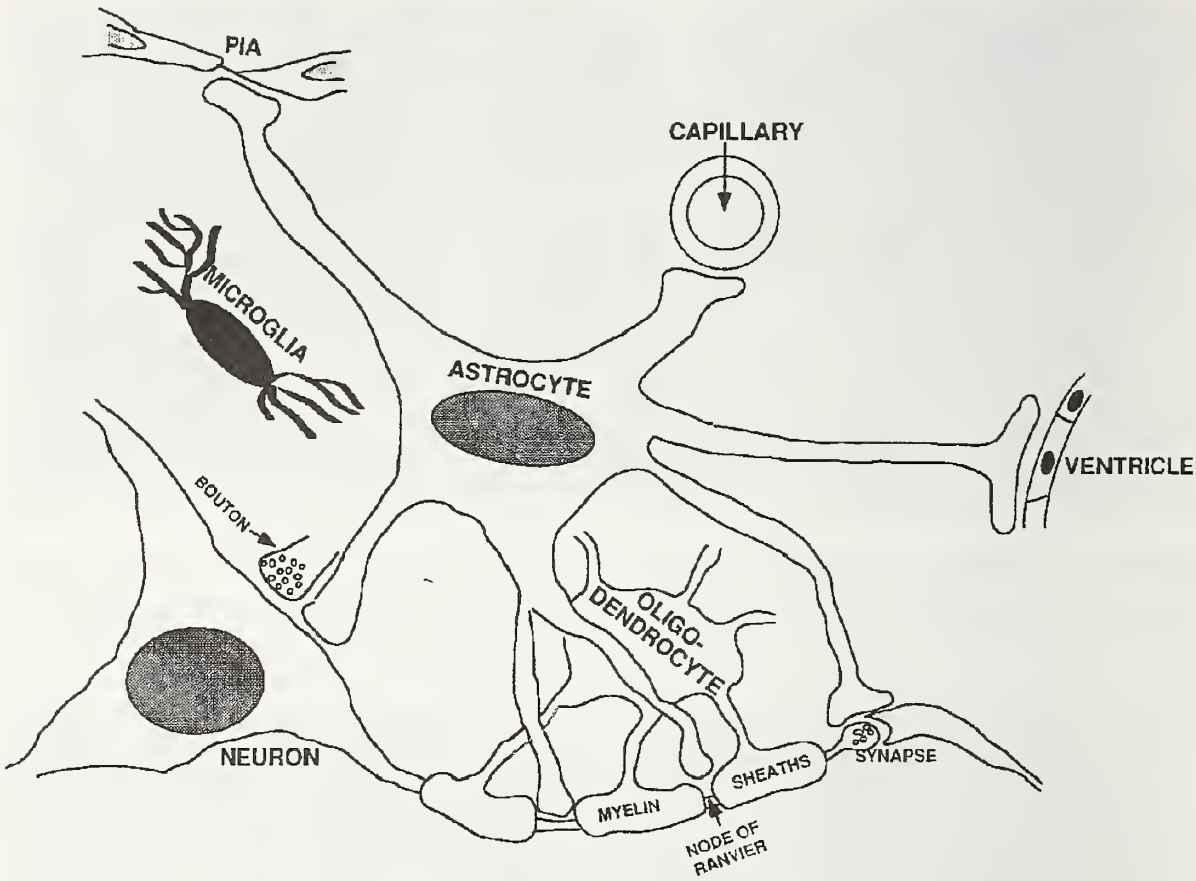

Figure 4. The three main types of glial cells (astrocyte, oligodendrocyte, and microglia) and their relationships to other components of the nervous system. The astrocyte in this figure is idealized and is not intended to imply that an astrocyte in situ will show all the relationships indicated for this cell, although more than one of these relationships is likely to be exhibited by an individual astrocyte. Thus, in the white matter there will be fibrous astrocytes, while the processes forming the glial limitans under the pia and those surrounding synapses will likely be from protoplasmic astrocytes. Both types of astrocytes have processes that surround blood vessels.

hands of a master of intuitive biology such as Ramon y Cajal, many of the suggestions were amazingly correct and perceptive.

This period of neuroglia research essentially closed in the 1930's. Advances in the period 1940–1960 in neurophysiology, such as elucidation of the ionic bases of the action potential and the actions of neurotransmitters at synapses, dominated the field of neuroscience and led most neuroscientists to use the experimental tools then becoming widely available, to study the rapid electrical changes characteristic of neuronal function. This was a perfectly acceptable strategy at that time, but, perhaps from a need to justify this "neuronocentric" approach, there was a tendency to incorrectly assert that the neuroglia, and particularly the enigmatic astrocyte, had little of importance to contribute to the overall function of the nervous system other than a general support role. Work of the past two decades has shown this view, as might have been expected, to be totally incorrect.

The modern *experimental* approach to glial cells, and specifically astrocytes, is generally considered to have started with the pioneering electrophysiological studies of Kuffler and his colleagues in the 1960's on astrocyte-like glial cells in primitive animals such as the leech and

lower vertebrates (Kuffler et al. 1966). This modern approach disposed of what was perhaps the last remnant of the old speculative approach, which had led electron microscopists and others to propose that astrocytes were high Na^+ cells which formed the extracellular space of the brain, since they were seen by electron microscopy as "enlarged watery structures" without any apparent extracellular spaces. The work of Kuffler and his colleagues clearly showed that this view, at least for the glia in the nervous system of leech and the optic nerve of amphibians, was wrong. The amphibian glial cells were found to have normally high intracellular K^+ concentrations and to be characterized by a membrane potential which was essentially the same as the Nernst potential for K^+, or -80 to -90 mV intracellular negative. Later work on "electrically silent" cells in the mammalian CNS identified putative glial cells which had the same characteristics; namely, nonexcitable cells with large negative membrane potentials which were apparently only sensitive to changes in $[K^+]_o$ (Somjen 1975). This finding led to one of the earliest functions proposed for glial cells: the control of extracellular K^+ by taking up K^+ released from active neurons (Kuffler et al. 1966, 1984).

The finding of an essentially pure K^+-dependent membrane potential implied that the cell membranes were impermeable to sodium, and possibly to chloride. In this hypothesis, it was postulated that localized release of K^+ from neurons during excitation would depolarize the astrocyte at this point with a "Nernstian" 60 mV depolarization for each tenfold increase in $[K^+]_o$. This process would set up a current loop with other, nondepolarized parts of the cell, and since the membrane was permeable only to K^+, there would be an inward current at the depolarized point carried by extracellular K^+ crossing the membrane. Since K^+ is the major electrolyte inside the cell, it would also be the major current carrier inside the cell, and the current loop would be connected by efflux of K^+ at some distant point since only K^+ could cross the membrane. The return part of the loop would be carried by major extracellular ions, such as Na^+ (see figure 5). This hypothesis was termed "K^+ spatial buffering," the essential aspect of which is that K^+ is transferred from a region of localized release to some distant point on the astrocyte, or even to other cells through the astrocytic syncytium (figure 5). This hypothesis also led to studies on other K^+ transport systems in glial cells with the emphasis that a major function of glial cells was to maintain $[K^+]_o$ homeostasis in the brain.

The next major advance in what we might term the modern experimental period of glial studies was that monolayer cultures prepared from neonatal or late-stage fetal rodent brains were found to consist predominantly, and with appropriate manipulations almost exclusively, of astrocytes (Booher and Sensenbrenner 1972). Confirmation of the astrocytic nature of these cultures came when it was discovered in the early 1970's (Eng et al. 1971) that the intermediate filaments of astrocytes were comprised of a unique protein, GFAP (Dahl et al. 1986; Eng 1988), and that almost all the cells in these primary cultures stained positively for GFAP. It is still unclear as to why these astrocyte cultures, which consist primarily of flat cells that many people think are analogous to protoplasmic astrocytes, stain so readily for GFAP whereas protoplasmic astrocytes in situ only stain sparingly for GFAP (Bignami and Dahl 1976; Eng 1987). However, the development of methods for routinely preparing ≥ 95 percent GFAP-positive cell cultures by methods such as that of McCarthy and De Vellis (1980) enabled the whole pantheon of experimental techniques blossoming in neuroscience and cell biology at that time, such

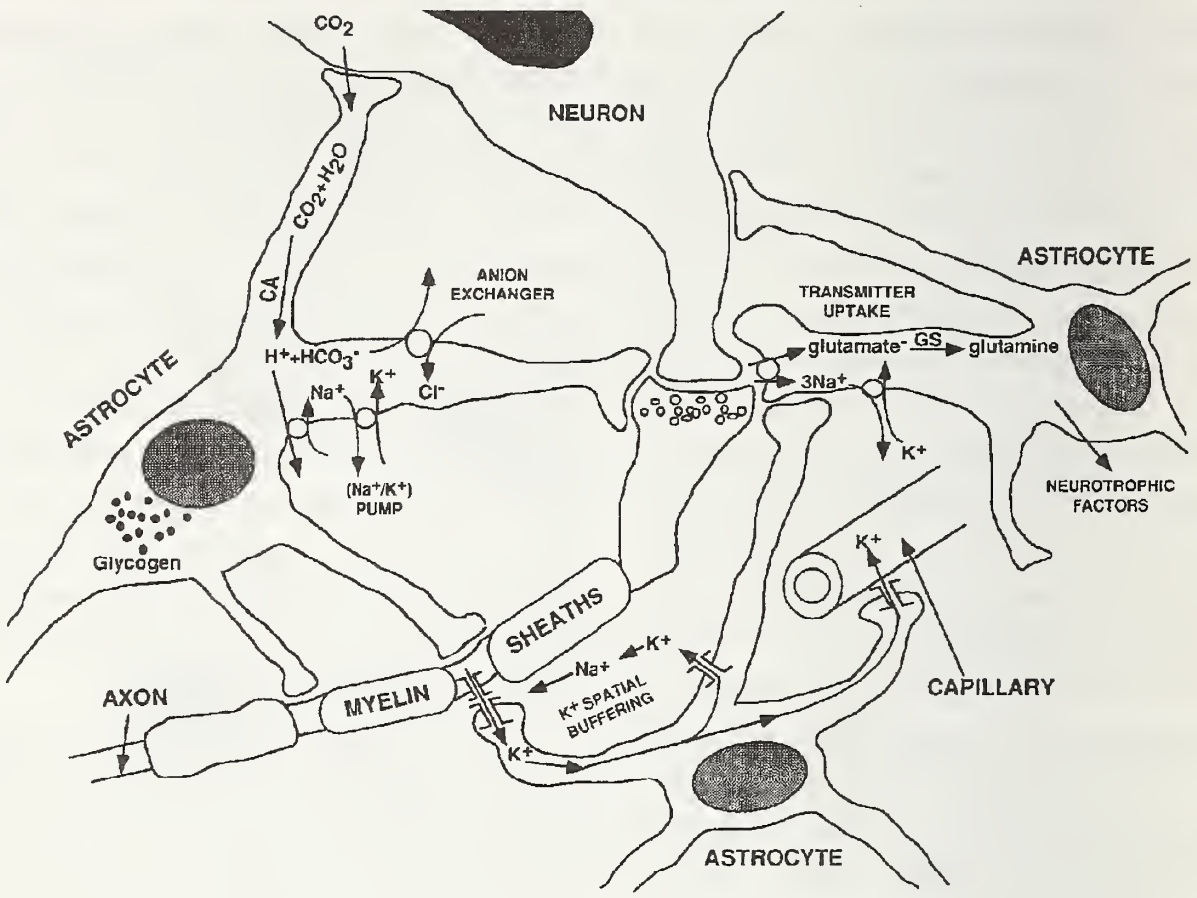

Figure 5. The main interrelationships of astrocytes and some of the major physiological functions of these cells. The K^+ channels and the transport of K^+ shown going from a node of Ranvier to the capillary indicates the K^+ spatial buffering hypothesis (see text). Uptake of transmitters is exemplified by glutamate. The release of neurotrophic factors is also shown. Conversion of CO_2 to HCO_3^- and H^+ accelerated by carbonic anhydrase (CA), which then activate the Na^+/H^+ and $Cl^-HCO_3^-$ exchangers leading to net uptake of NaCl, is shown in the astrocyte on the left. This can result in cell swelling. The pumping out of Na^+ by the Na^+/K^+ pump is also shown, and when the pump is compromised in ischemia or hypoxia NaCl can accumulate. Glycogen is specifically present in astrocytes in the brain, as shown.

as electrophysiology (including patch-clamp), molecular biology, transport studies using radiolabeled compounds, and immunocytochemistry, to be brought to bear on the study of these cultures. Also, these cultures were used as starting points for establishing pure cultures of the other glial cells, oligodendrocytes and microglia.

The cell culture approach led to lineage studies, which were initiated by reports of two types of astrocytes—type 1 and type 2—present in cultures from optic nerve. The two types of astrocyte were identified by morphology and immunological markers, and developmental lineages associated with each type were elucidated (Raff 1989). For example, a precursor cell (termed "O-2A") can lead to oligodendrocytes (O) or type 2 astrocytes (2A) depending on whether serum or certain factors are present. Thus, the stage was set for a burgeoning of in vitro work on the "neuroglia."

A major question in this growing field is how well the in vitro systems recapitulate the in vivo systems, for example, in examining when the astrocytes acquire the GFAP protein.

However, such cultures have two important and critical functions (Kimelberg 1983). First, these cultures have clearly shown to neuroscientists that astrocytes and other glial cells are likely to have a complexity of properties and functions equivalent to that of neurons. Second, cultures provide a convenient and easily used experimental tool to study such functions in detail. We are now entering the phase in which many of the ideas and concepts derived from the in vitro work are being tested in vivo. Thus, hybridization studies and immunocytochemistry in situ are corroborating the view that the astrocytes do indeed have many of the receptors found in the in vitro preparations and produce some of the specific products, such as cytokines, growth factors, and transmitters, that they produce in vitro. Electrophysiological studies in brain slices are also beginning to show which of the ion channels and channel-linked receptors seen in vitro are shared by astrocytes in vivo. Critical concepts regarding astrocyte specialization, functional heterogeneity, and the fascinating complexity of astrocyte-neuron and other glial-neuronal interactions are beginning to emerge from these studies. Many of these roles are summarized in table 1. We are undoubtedly at the dawn of a new "heroic" age of glia when these cells will come to be clearly recognized as equal partners with neurons in providing the cellular and molecular substrates underlying the complexities of nervous system function (Hertz and Richardson 1984).

PHYSIOLOGICAL FUNCTIONS

Regulation of Extracellular Ion Concentrations

As noted in the preceding section, the control of extracellular K^+ was one of the earliest physiological functions attributed to astrocytes. Consequently, K^+ transport in astrocytes has been extensively studied, and this has led to considerable information on a variety of K^+ transporting systems in astrocytes, including different K^+ channels, carrier systems for K^+, and the Na^+/K^+ pump.

K^+ Channels

K^+ channels are the most diverse ionic channel type, and a wide variety of K^+ channels have been found in astrocytes (Barres et al. 1990 [see their table 1]; Duffy and MacVicar 1993; Kimelberg et al. 1993 [see their table 1]). These channels include inward rectifying K^+ channels (K_{in}), Ca^{2+}-dependent K^+ channels (K_{Ca}), delayed rectifying K^+ channels (K_d), and transient K^+ channels (K_A). K^+ channels sensitive to adenosine triphosphate (ATP) have also been found in astrocytes. Some of these channels may be related to the K^+ spatial buffering phenomenon discussed in the preceding section. If there is a significant chloride permeability, net KCl uptake leading to swelling will occur when $[K^+]_o$ rises. Some of these channels should be responsible for the large negative K^+ diffusion potentials, characteristic of astrocytes (Barres et al. 1990). The work of Newman (1986) indicated K_{in} channels at very high densities in areas of the astrocyte that seem to be adapted to K^+ spatial buffering, namely at the capillary-facing astrocytic end feet. If the membrane potential is very close to the K^+ equilibrium potential, then the net outward leak of K^+ will always be very low. However, the net outward leak may be increased when there is depolarization of the astrocyte due to reasons other than an increased $[K^+]_o$, such as that due to receptor activation (see table 3). In this case there will be an outward flux of potassium, which will be replenished by uptake on the Na^+/K^+ pump (see the section on Na^+/K^+ pump later in this chapter).

Table 1. Properties and Roles of Astrocytes

Development

- Neuronal and axonal guidance and migration in development, especially associated with radial glia, which then develop into stellate astrocytes
- Influence or are necessary for synaptogenesis and neuronal development and survival and other CNS cells by cell-to-cell contact or production of a variety of growth factors
- Induction or maintenance of tight junctions between endothelial cells (blood-brain barrier)

Ion and pH homeostasis involving both voltage and ligand-gated ion channels for K^+, Na^+, Cl^-, HCO_3^-, and OH^-, and Na^+-H^+ and HCO_3^--Cl^- exchange carrier systems

Transmitter uptake and homeostasis

Receptors for transmitters

Release of transmitters and synthesis of neuropeptides

Immune responses—release of immune system signals, response to interleukins and possible antigen presentation

Phagocytosis associated with a number of lysosomal hydrolases

Compartmentation or parcellation of neurons, nonmyelinated axons, dendrites, and synapses (glomeruli)

Glycogen storage and metabolic interactions, including supply of substrates, to neurons

Pathology

- Swelling of astrocytes associated with trauma, ischemia, and hepatic encephalopathy
- Structural cellular injury leads to astrogliosis or glial scars formed by reactive astrocytes
- Environmentally induced neuropathies, e.g., parkinsonism via 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-toxin or developmental abnormalities due to alcohol via effects on radial glia?
- Role in growth or transplantation of neural or nonneural tissue to the brain and regeneration of damaged neurons
- Epilepsy and psychiatric disorders because of involvement with transmitter functions and neuronal excitability
- Protective or promotive roles in the aging brain and degenerative diseases such as Huntington's disease and multiple sclerosis

Na^+ Channels

Voltage-dependent Na^+ channels analogous to those found in neurons, where they are responsible for electrical excitability, surprisingly were found in astrocytes in primary culture (Bevan et al. 1985; Bowman et al. 1984). These channels were sometimes sensitive to tetrodotoxin (TTX), a specific, high-affinity inhibitor of neuronal channels, but it was found that there were also relatively TTX-insensitive Na^+ channels (Sontheimer 1991). The depolarizations needed to open these channels were always thought to be greater than would ever normally be seen in an astrocyte. However, Sontheimer and colleagues (1992) showed that astrocytes from certain regions of the brain, such as the spinal cord, have a very high density of Na^+ channels which would have a probability to be open at the cell resting membrane potential of these cells. It was hypothesized that the Na^+ channels may function in regulating entry of Na^+ in order to activate the Na^+/K^+ pump when active uptake of K^+ is required, such as when $[K^+]_o$ rises from its normal 3 mM level to 5–10 mM during periods of sustained neuronal activity. This would thus constitute a

self-regulating mechanism for active K^+ process clearance by astrocytes that does not require any special properties of the Na^+/K^+ pump (discussed later in this chapter) (Sontheimer 1991; Sontheimer et al. 1992).

Ca^{2+} Channels

Voltage-gated L-type Ca^{2+} channels were first identified in astrocytes in primary cultures by MacVicar (1984). This discovery was also surprising because it was thought that such channels in the CNS were specific to neurons and were responsible for such properties as the depolarization-induced Ca^{2+} influx at nerve terminals required for exocytotic release of transmitters, and also for Ca^{2+} action potentials. The function of the Ca^{2+} channels in astrocytes has not yet been satisfactorily explained, but the occurrence of large changes in $[Ca^{2+}]_i$ levels when glutamate receptors are stimulated or with mechanical stimulation or cell swelling raises the possibility that part of the Ca^{2+} under these conditions may be entering via channels (Cornell-Bell and Finkbeiner 1991; Duffy and MacVicar 1993; Finkbeiner 1992). Also, a number of cellular processes in astrocytes, as in all other cells, require the release of Ca^{2+} from intracellular stores, which then acts as an intracellular messenger. Ca^{2+} channels would be necessary for the entry of Ca^{2+} to replenish the intracellular stores after their depletion.

Anion Channels

As in other cells, anion channels in astrocytes have been less studied than cation channels, but it is now becoming clear that anion channels do subserve important functions in cells. A number of anion channels have now been identified in astrocytes, including small conductance chloride channels (Cl_s) and a high conductance chloride channel (C_H) (Kimelberg et al. 1993*b*; Jalonen 1993). These channels transport Cl^- and HCO_3^-, but the C_H may also transport organic anions such as amino acids. The roles of these channels may include the uptake of K^+ with HCO_3^- or chloride or the release of potassium chloride and/or amino acids during volume regulation when these cells lose intracellular solutes in order to regain their normal volume after swelling.

Ion Carriers

Carriers are distinct from channels in that a concerted movement of several ions usually occurs, rather than the independent diffusional movement of an ion down its electrochemical gradient characteristic of channels (Hille 1992). One important ion carrier is the $Na^+ + K^+ + 2Cl^-$ uptake system usually utilized by cells for active uptake of Cl^-. This ion carrier occurs in astrocytes in primary culture (Kimelberg et al. 1993*b*) and has been found by immunocytochemistry in Bergmann glia in situ (Zalc et al. 1984). Intracellular Cl^- in astrocyte cultures has been found to be severalfold greater than expected from electrochemical equilibrium (Kimelberg 1981). Kettenmann and colleagues (Berger et al. 1992*b*; Kettenmann et al. 1988) have proposed that this high Cl^- may serve as a source to maintain extracellular Cl^- levels, based on the finding of $GABA_A$ receptors on astrocytes both in vitro and in situ. Activation of astrocytic $GABA_A$ receptors would lead to release of Cl^-, maintaining extracellular concentrations at the same time as GABA causes influx of Cl^- into neurons via activation of their $GABA_A$ receptors. Since neurons generally have low $[Cl^-]_i$, they show hyperpolarizing, inhibitory GABA-induced responses. The high Cl^- in astrocytes may also be required for the efflux of KCl for volume regulation, as proposed by Kimelberg and Frangakis (1985).

Na^+/K^+ Pump

As in all mammalian cells, astrocytes contain an active Na^+/K^+ pump responsible for accumulating K^+ and pumping out Na^+ from cells (see figure 5). The pump consists of isoforms

of α and β subunits. Sweadner (1991) showed that the α_1, α_2, and α_3 forms are distributed in a complex manner among different cells of the CNS. It appears that neurons can exhibit all three isoforms, either individually or in various combinations, and astrocytes and other glia cells express α_1 or α_2 or both, but not α_3. The β_1 messenger RNA (mRNA) has a broad distribution in brain, whereas the β_2 mRNA is much more localized. While β_2 is the same as an adhesion molecule on glia (AMOG) (Schachner 1991), the β_2 isoform is not specific to glia (Sweadner 1991). This finding raises interesting questions regarding the relationship between ion transport and cell adhesion.

In terms of the kinetics of the different isoforms, there is evidence both for and against a specialized role of glial Na^+/K^+ ATPase in the uptake of K^+ by astrocytes (Sweadner 1991). As with other Na^+ pumps, this system seems to be driven mainly by intracellular Na^+. It has a high affinity for K^+ on the outside ($K_{1/2}$ 1 mM), and a midactivation level for Na^+ of about 10 mM on the inside. Thus, with a $[K^+]_o$ of around 3 mM there will be only a small amount of activation between 3 and 10 mM K^+, 10 mM being the saturation level for activation of the Na^+/K^+ pump by K^+. It has been found that some astrocytes have a high density of TTX-sensitive Na^+ channels, and it has been suggested that these channels are responsible for maintaining the intracellular Na^+ levels required for the functioning of the Na^+/K^+ pump (Sontheimer 1991; Sontheimer et al. 1992). Since these Na^+ channels are voltage activated, increased $[K^+]_o$, which would depolarize the astrocyte membrane potential, could regulate the influx of Na^+ and thereby indirectly regulate the Na^+/K^+ pump via increases in $[Na^+]_i$. This is an interesting suggestion since this process would control active K^+ uptake by astrocytes without requiring any specialization of the astrocytic Na^+/K^+ pump, which so far has not unequivocally been shown (Sweadner 1991).

pH

Other carrier systems for Cl^- or Na^+ involve co- or exchange transport with the pH equivalents, H^+, HCO_3^-, or OH^-. These systems are the Na^+/H^+ and Cl^-/HCO_3^- or OH^- exchangers and a variety of electrogenic or nonelectrogenic co-transport systems of Na^+ plus $nHCO_3^-$, where n can be from 1 to 3 (Boyarsky et al. 1993; Newman 1991). It has been suggested, based on the existence of these transport systems and the fact that astrocytes in situ can undergo very large pH changes in ischemia, often in the opposite direction to the extracellular pH, that these cells are critically important in maintaining pH homeostasis in the brain (Chesler and Chen 1991). In ischemia the astrocytes become very acidic, whereas in spreading depression they undergo large intracellular alkaline shifts (Chesler 1990). The operation of such pH transporting systems would also lead to volume changes. For example, the simultaneous operation of the Na^+/H^+ and Cl^-/HCO_3^- exchangers, driven by intracellular hydration of CO_2 to H^+ and HCO_3^-, would lead to a net uptake of Na^+ and Cl^-. Extracellularly HCO_3^- combines with H^+ to form CO_2 again, thus leading to a cycle conserving CO_2, HCO_3^-, and H^+ and leading to a net uptake of Na^+ and Cl^-. In trauma or ischemia, when it is likely that the Na^+/K^+ pump would be compromised due to falling energy levels, the Na^+/K^+ pump would have a decreased ability to pump out Na^+ (Kimelberg 1992). This would lead to cell swelling, and astrocytic swelling is commonly seen in such pathological states.

Transmitter Uptake Systems

Uptake of transmitters by astrocytes has been recently reviewed (Kimelberg et al. 1993*b*). There are uptake systems for a number of

amino acid transmitters, such as glutamate, glycine, taurine, and GABA. These systems are Na^+ dependent and can also be electrogenic. There is considerable evidence for extremely active electrogenic uptake of glutamate in isolated Müller cells (Nicholls and Attwell 1990), and uptake into astrocytes in situ measured by autoradiography and immunocytochemistry (Ottersen 1989). Recent molecular biology studies have identified at least three members of a glutamate transporter family, of which two are found exclusively in astrocytes (Kanner 1993).

Uptake of a number of monoamine transmitters has been reported in cultured astrocytes (see Kimelberg et al. 1993*b* and table 2). These systems resemble those found in nerve terminals, being both Na^+ dependent and inhibitable by a variety of clinically relevant antidepressants, such as fluoxetine (Prozac) for serotonin. Uptake systems for adenosine (Matz and Hertz 1990), taurine (Holopainen 1988; Lee et al. 1992; Shain and Martin 1990), and histamine (Huszti et al. 1990) have also been described. It is clear that the glutamate transport system is very likely to have an important role in controlling glutamate action in the brain in situ. The relevance of the other uptake systems has not yet been established, and the relative rates of uptake of any of the astrocytic systems, including glutamate, as compared to neuronal uptake cannot be directly assessed at this time (table 2).

Receptors for Transmitters

An almost complete set of CNS neurotransmitter receptors has been found on astrocytes

Table 2. Transmitter Uptake in Astrocytes

Transmitter	Uptake System Characteristics, and Where Studied	Study
Glutamate	Na^+-dependent isozymes found both in situ and in vitro	Kimelberg et al. 1989; Schousboe et al. 1977; Hertz et al. 1978*a*
Taurine	Na^+-dependent system in vitro and in vivo	Holopainen 1988; Lee et al. 1992; Shain and Martin 1990
GABA	Na^+-dependent system in vitro	Hertz et al. 1978*b*; Larsson et al. 1980; Levi et al. 1983
Adenosine	No Na^+-dependence in vitro	Matz and Hertz 1990
Serotonin	Na^+-dependent system in vitro	Katz and Kimelberg 1985; Kimelberg 1988*b*; Kimelberg and Katz 1985
Norepinephrine and dopamine	Na^+-dependent system in vitro	Kimelberg and Pelton 1983; Pelton et al. 1981
Histamine	Na^+-dependent system in vitro	Huszti et al. 1990

in vitro (Kimelberg 1988*a*; Murphy and Pearce 1987), and a number of these, specifically the β-adrenergic receptors and the ionotropic kainic acid/α-amino-3-hydroxy-5-methyl-4-isoxazole proprionate (KA/AMPA) receptors, have also been located in astrocytes in vivo (see tables 3 and 4). Thus, these astrocytes are poised to respond to transmitters that were thought to be an exclusive property of post- or presynaptic neuronal membranes.

The perisynaptic location of many astrocyte processes, which form glial nets around neurons (Bruckner et al. 1993; see figure 3), puts these receptors and the transmitter uptake systems in direct apposition to their sites of release, namely presynaptic boutons on the neuronal soma or dendrites. Indeed, electron microscopic immunocytochemistry studies (Aoki 1992) showed that β_2 receptors are located on astrocyte processes close to the synaptic cleft (figure

Table 3. Adrenergic and Other Aminergic Receptors in Cultured Astrocytes and Astrocytes In Situ, and Some Functional Effects of Their Activation

Receptor Type	Second Messenger	Functional Effects	Study
β_1,β_2 (1° astrocytes cultures prepared from rat brain and studied by ligand binding) β_2 cat visual cortex in situ by immunohistochemistry	↑cAMP	Morphological changes (change from flat to process-bearing cells); increased enzyme activities; GFAP phosphorylation; inhibition of glutamate uptake; glycogenolysis (long-term exposure to agonist → glycogenesis)	Aoki 1992; Browning and Ruina 1984; Magistretti et al. 1993; McCarthy 1983; Narumi et al. 1978
α_1 (type 1 and 2 cultured astrocytes)	↑IP_3 ↑Ca^{2+}	Membrane potential depolarization; glycogenolysis, (long-term → glycogenesis)	Bowman and Kimelberg 1987; Hosli et al. 1982; Magistretti et al. 1993;
α_2	↑Ca^{2+}		Ritchie et al. 1987; Salm and McCarthy 1992
Muscarinic (M_1; M_2) (rat cerebral cortex in situ by immunohistochemistry)	↑IP_3, ↑Ca^{2+}	Inhibits cAMP; increases depolarization; activates phospholipase D	Murphy and Pearce 1987; Pearce et al. 1985; Van der Zee et al. 1993
5-HT_{1a}, 5-HT_2	↑IP_3 ↑Ca^{2+}	Stimulate growth of 5-HT neurons; ↑release S-100 protein; membrane hyperpolarization; ↑K^+ channels	Deecher et al. 1993; Ogura and Amano 1984; Walz 1988; Whitaker-Azmitia et al. 1993
H_1H_2	↑Ca^{2+}	Glycogenolysis; depolarization; hyperpolarization	Arbones et al. 1990; Hosli and Hosli 1988; Inagaki 1991

Table 4. Amino Acid and Peptide Receptors in Cultured Astrocytes and In Situ, and Some Functional Consequences of Their Activation

Receptor Type	Second Messenger	Functional Effects
$GABA_A$ (ionotropic)	Anion channel	Membrane depolarization: receptor activation leads to efflux of Cl^- and HCO_3^-. Regulation of Cl^- concentrations and pH in vicinity of active neurons.
$GABA_B$ (metabotropic)	↑IP_3 ↑Ca^{2+}	Membrane hyperolarization, but ionic basis of response unclear. Possible increased K^+ conductance. Inhibition of agonist-evoked Ca^{2+} fluxes, PI metabolism, and eicosanoid release.
Glutamate (KA/AMPA ionotropic)	Cation channel	Membrane depolarization in response to GLU, KA, AMPA (not NMDA); receptor activation leads to influx of Na^+ and Ca^{2+}, and also K^+ efflux. May function to regulate ion concentrations at nodal regions of axons, or reversal of GABA transport system.
Glutamate (metabotropic)	↑IP ↑Ca^{2+}	Functions to regulate release of astrocyte-derived releasing factor, inhibits proliferation, induces filopodia formation.
Natriuretic	↑cGMP	Receptor activation leads to increased cGMP and guanylate cyclase, but functions are as yet unclear.
Angiotensin II, endothelins, bradykinins, substance P	↑IP ↑IP ↑Ca^{2+}	Affect release of plasminogen activator inhibitor; function in the release of prostaglandins and potentiates norepinephrine-induced increases in cAMP.
VIP	cAMP	Functions to stimulate glycogenolysis and mitogenesis; increases neuronal survival (via secretion of neurotrophic factors).
Opioid	Delta subtype (metenkephalin)	Functions in glycogen metabolism and suppresses astrocytic DNA synthesis.

Note: VIP = vasoactive intestinal polypeptide; IP = inositol phosphates; GLU = glutamate.

6), and Derouiche and colleagues (1993) described glutamine-positive astrocyte processes directly next to, or even penetrating to a limited extent into the synaptic clefts of glutamatergic synapses (see figure 2).

It is of great interest to ask what effects the activation of these receptors have on astrocyte properties. As expected, these receptors first involve the activation of second messenger systems, which can then lead to a variety of

functional changes. The elucidation of such functional effects, however, goes hand in hand with further advances in our understanding of astrocyte properties in general. What is known so far is summarized for a variety of receptors in tables 3 and 4. The activation of the KA/AMPA glutamate receptor has been shown to lead to membrane potential depolarization and Na^+ and K^+ inward currents (Bowman and Kimelberg 1984; Sontheimer et al. 1988), and recent work has shown that this may be a glial-specific type of AMPA receptor which also transports Ca^{2+} (Muller et al. 1992). Addition of glutamate to astrocytes has also been shown to lead to self-propagating Ca^{2+} waves through the astrocyte syncytium (Cornell-Bell and Finkbeiner 1991; Finkbeiner 1992), possibly through the same receptor type. Ca^{2+} waves allow astrocytes to signal changes over a wide region of the brain, much as a neuronal network might function. Consequently, there have been speculations that astrocytic Ca^{2+} waves are involved in information processing (Van den Pol et al. 1992).

The effects of activation of adrenergic receptor activation on the shape of astrocytes in vitro (Narumi et al 1978; Shain et al. 1987) suggest that astrocytes may also have the property of changing shape in vivo. Such changes could potentially be involved in learning by altering the distribution or effectiveness of synapses, and there have been a number of studies that have shown an increase in astrocyte number in the brains of rats taught tasks, as compared to nonlearning rats (Diamond et al. 1966). GFAP-positive processes have also been shown to increase in animals that have gone through odor preference training (Matsutani and Leon 1993). Parenthetically, it

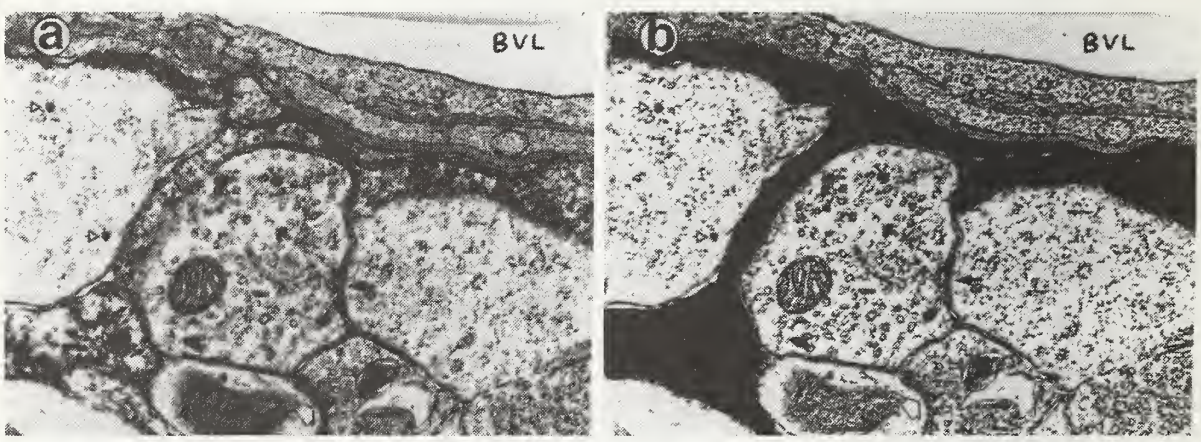

Figure 6. (a) The localization of β_2-adrenergic receptors on astrocytic processes surrounding a catecholamine terminal (middle profile to immediate left of solid arrow). The β_2 receptor is visualized by antibodies directed against a synthetic peptide corresponding to amino acids 404–418 of hamster lung β_2-adrenergic receptor. The catecholamine terminal forms a symmetric synaptic junction with a dendrite to its right (solid arrow). Astrocytic processes above and below the terminal contain peroxidase reaction product reflecting positive immunoreactivity for β_2 receptors. (b) The astrocytic processes positive for β_2 receptors reactivity are filled in in black. The small arrowheads in each panel indicate nonrecurring silver grains that reflect background labeling. The large arrowhead in each panel indicates a noncatecholamine synapse. BVL = blood vessel lumen. Reprinted with permission from Aoki, C. Beta-adrenergic receptors: astrocytic localization in the adult visual cortex and their relation to catecholamine terminals as revealed by electron microscopic immunocytochemistry. *Journal of Neurosci* 12(3):781–792, 1992.

should be noted that it does seem unlikely that the increased number of glia reported for Einstein's preserved brain (Diamond et al. 1985) has any relationship to the well-known intellectual superiority of this famous scientist. More prosaically, the activation of any astrocyte property, such as the activity of K^+ or Ca^{2+} channels and increased glycogenolysis, could also result from receptor-mediated increases in intracellular Ca^{2+}, inositol 1,4,5 trisphosphate (IP_3), or cyclic adenosine monophosphate (cAMP) levels (see tables 3 and 4). Increased enzyme activities imply a precise coordination of astrocyte function in the CNS, which supports a very critical role for these cells in such diverse actions as control of neuronal excitability and brain development.

Effects of Ethanol on Astrocyte Function

The effects of ethanol on receptor mechanisms in astrocytes do not appear to have been studied so far. However, a number of studies have shown that ethanol in vitro acutely stimulates GABA-mediated chloride fluxes and inhibits NMDA and to a lesser extent KA-activated ion fluxes (Deitrich et al. 1989). These effects are generally more sensitive than blockade of voltage-dependent Ca^{2+} channels, which is also seen (Gonzales and Hoffman 1991). These inhibitory effects on excitatory amino acid receptors may explain recent reports of ethanol protecting against excitotoxicity in cerebral cortical cultures (Lustig et al. 1992), and inhibition of NMDA- and KA-evoked noradrenaline release (Fink et al. 1992). Ethanol is also known to increase Ca^{2+} levels in brain cells (Dildy-Mayfield et al. 1991), and has been shown to stimulate [^{3}H] 5-hydroxytryptamine (5-HT) uptake by synaptosomes, which was thought to be due to an increase in intracellular Ca^{2+} and/or a rise in K^+ channel conductances (Alexi and Azmitia 1991). These examples are selected from numerous observations of how ethanol influences different physiological functions of the nervous system (Deitrich et al. 1989). It would be of great interest to see whether ethanol alters the effects of glutamate and GABA on the ion fluxes and channels in astrocytes described in this chapter's section on regulation of extracellular ion concentrations.

There have been very few studies on the effect of ethanol on the physiological functions of astrocytes. Shain and colleagues (1987*b*) reported that increasing concentrations of ethanol lead to stimulation of taurine release from primary astrocyte cultures. A recent study in our laboratory showed that this effect may be due to swelling of astrocytes caused by ethanol, which is most marked when ethanol is added isosmotically (Kimelberg et al. 1993*a*). Figure 7 shows the swelling of a primary astrocyte monolayer, as measured by an increased voltage difference across a small chamber above the cells using an electrical impedance method (O'Connor et al. 1992), caused by exposure to a hypotonic or an isosmotic ethanol-containing solution with a constant perfusion system. The smaller degree of swelling for ethanol may be due to a smaller osmotic effect or a less confluent monolayer. However, the efflux of [^{3}H] D-aspartate (panel B of figure 7), with which the cells were preloaded, when measured simultaneously is slightly greater for the isosmotic ethanol as compared with the hypotonic medium. This suggests that the smaller swelling seen for ethanol is due to a less confluent monolayer, since the efflux is measured as a percent of the total present at each time point and this would correct for less cells being present.

In in vitro studies, usually ethanol is simply added to solutions, which results in a hyperosmotic solution. There is generally no correction made for this change, since it is assumed that

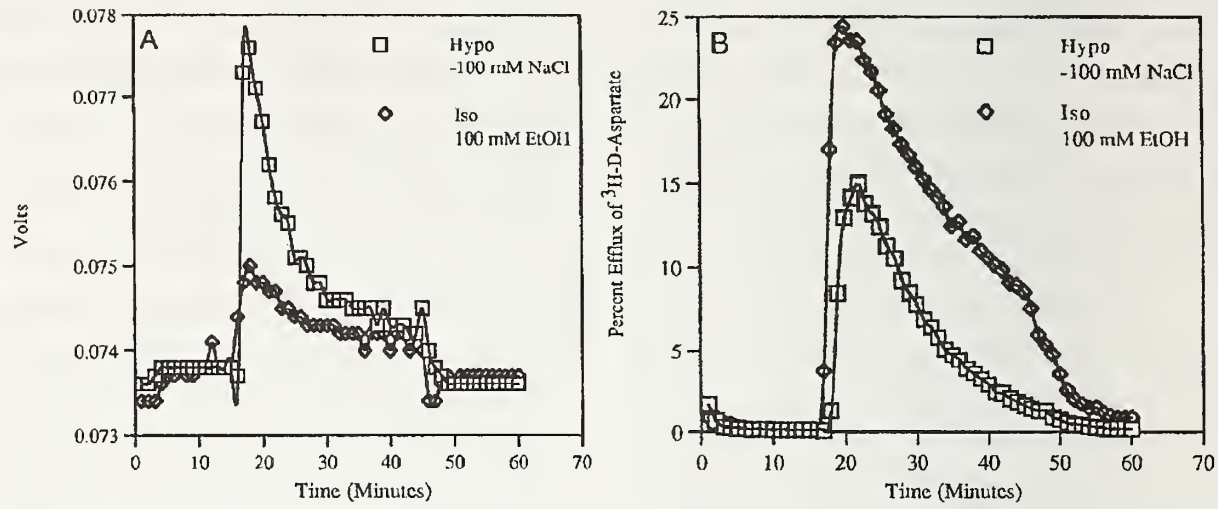

Figure 7. (A) Volume changes in primary monolayer astrocyte cultures as measured by the extracellular impedance method (O'Connor et al. 1992) for cultures exposed to hypotonic (100 mM NaCl removed) or isosmotic ethanol (EtOH) (100 mM ethanol replaces 50 mM NaCl) solutions. (B) The simultaneous efflux of [^{3}H] D-aspartate, with which the monolayer cultures were preloaded, is shown as a percent of the amount present at the beginning of each time point (1 minute). Differences in the degree of swelling for the ethanol-treated cultures could be due to a smaller osmotic effect, since the ethanol concentration was only 100 mM or 100 mOsm while in the hypotonic solution 100 mM NaCl (200 mOsm) were removed. However, the percent efflux of [^{3}H] D-aspartate does not show marked differences, so it is likely that the smaller response in the ethanol-treated cultures reflects a less confluent monolayer, since the volume increase is not measured in relative terms as it is for the percent efflux (Vitarella, Aschner, and Kimelberg unpublished observations).

ethanol rapidly equilibrates to the same concentrations across the cell membrane. However, at high concentrations of ethanol (> 100 mM) there is a few-millimolar reduction in electrolyte concentrations and therefore a small isosmotic component. Thus, some of the effects of ethanol on very volume-sensitive properties of cells, such as the release of taurine from astrocytes (Pasantes-Morales and Schousboe 1989), could be due to cell swelling. This result is different from the effects of ethanol on K^+-induced release of glutamate and aspartate from hippocampal slices, which was inhibited by increasing hyperosmotic ethanol (Martin and Swartzwelder 1992). It is possible that the hyperosmotic ethanol in the latter study induced a shrinkage of the cells, although ethanol, being a membrane-permeant molecule, should rapidly enter cells and result in equalization of concentrations inside and outside the cell, thus producing no net osmotic load. This equalization is the likely reason why the cells can tolerate large concentrations (100–200 mM) of hyperosmotic ethanol. However, the inside of the cell is now 100–200 mM hyperosmotic compared to normal conditions, and thus will have a slightly decreased dielectric constant. Direct inhibitory effects of ethanol via specific ethanol receptor sites on any components of the transmitter release mechanism in synaptic terminals or a generalized effect of changes in the dielectric on these same components, such as Ca^{2+} channels, could also explain the inhibitory effects in slices.

Alexi and Azmitia (1991) showed that ethanol treatment of synaptosomes increases uptake of [^{3}H]-5-HT. In our laboratory we found a similar effect of both isosmotic and hyperosmotic ethanol for fluoxetine-sensitive uptake of

[^{3}H]-5-HT in cortical astrocytes. This increase in uptake was markedly different from the effects of isosmotic ethanol on aspartate uptake, where there was marked inhibition, which we attribute to swelling of the cells (Kimelberg and Goderie unpublished observations) since hyperosmotic (i.e., normal added ethanol) up to 100 mM had no effect on glutamate or aspartate uptake. Alexi and Azmitia (1991) attributed their effects of increased [^{3}H]-5-HT to increased intracellular Ca^{2+} levels, and it is possible that a similar mechanism is occurring in astrocytes. Thus, it would be interesting to examine the effects of isosmotic and hyperosmotic ethanol on $[Ca^{2+}]_i$ levels and Ca^{2+} currents in astrocytes. Chronic treatment of brain stem astrocyte cultures with 65 mM ethanol was found to inhibit fluoxetine-sensitive [^{3}H]-5-HT uptake, although the effect disappeared when expressed per milligrams of cell protein (Lockhorst and Druse 1993).

DEVELOPMENT AND IMMUNOLOGY

Development and Neurotrophic Factors and the BBB

Development

Interactions between astrocytes and neurons are of the utmost importance in the developing CNS. In the immature CNS, neuronal cell body migration and axonal outgrowth occur on radial glia, which later on lose their longitudinal orientation and are thought to form mature astrocytes (Rakic 1990; Rakic and Siedman 1973; Reichenbach 1989). Neuronal migration is the basis of CNS pattern formation and layering and in the cortex is characterized by astrocyte-guided translocation of nerve cells from the site of cell division (subventricular zone) to their final destination. This process also is exemplified by the "outside-in" migration of cerebellar granule cells along radial fibers of the Bergmann glial cells (Hatten et al. 1990; Rakic 1990).

Astrocyte-neuron interactions are now recognized as being due to a number of adhesion and recognition molecules that are expressed by both cell types. Astrocyte-neuron contact mediated by adhesion molecules is exemplified by the migration of cerebellar granule cells along Bergmann fibers. Translocation of neuronal cell bodies requires the coordinated temporal and spatial expression of different adhesion molecules, e.g., nerve cell adhesion molecule (N-CAM), astrotactin, and L1 (Chuong 1990; Rakic 1990; Stitt and Hatten 1990). Similarly, neurite outgrowth along astrocytic cell surfaces and the extracellular matrix (ECM) is characterized by a specific spatiotemporal elaboration of several adhesive molecules including L1, N-CAM, N-cadherin, and integrin-class ECM receptors (Smith et al. 1990). Adhesion molecule binding mediates a number of additional processes that are also critical for development and regeneration, such as nerve fiber fasciculation (e.g., fasciclins and cadherins), pathway cues for guidance and target connectivity (e.g., L2/HNK-1 carbohydrate epitope), demarcation of topographic boundaries between laminar neuronal assemblies (e.g., J1 antiadhesion molecules), astroglial differentiation (e.g., L1), regulation of intra- and extracellular ion composition (e.g., AMOG; see the section in this chapter on the Na^+/K^+ pump), and nerve-target adhesion (e.g., N-CAM) (Gloor et al. 1990; Kruse et al. 1985; Schachner 1991).

Adhesion molecule interactions promote growth cone motility along astrocytic surfaces, and also provide the neurite with directional cues and other pertinent information regarding the surrounding microenvironment.

Processing of this information clearly requires complex signal transduction and intracellular integration. It is not known how this occurs, although it has been shown that the cytoplasmic domains of certain adhesion molecules (e.g., integrins, N-CAM 180, L1) are linked to various cytoskeletal elements (Chamak et al. 1987; Pollerberg et al. 1987). In addition, specific binding-induced changes in these membrane-cytoskeleton linkage complexes might be responsible for alterations in cytoskeletal components that are the basis of cell-cell stabilization and growth cone extension (Chuong 1990). In vitro evidence suggests that adhesion molecule binding influences second messenger turnover (i.e., inositol phosphates, Ca^{++}), which might also mediate appropriate and necessary changes in neurite metabolism and membrane ion channel function (Acheson and Rutishauser 1988; Acheson and Thoenen 1983; Schuch et al. 1989).

It is now clear that central neurons modulate astrocyte differentiation and proliferation (Gasser and Hatten 1990; Hatten 1985), as well as ion channel and neurotransmitter receptor phenotypes (Sontheimer 1991; Thio et al. 1993). Astrocyte-neuron interactions are also viewed as essential in determining the characteristic morphology of the latter. Morphological features of mesencephalic neurons (e.g., branching and varicosities) appear to be dependent upon whether cocultured astroglia are prepared from mesencephalic tissue (homotopic origin) or from a different brain region (heterotopic origin) (Denis-Donini et al. 1984). Soluble factors from astrocyte cultures have also been shown to promote neurite extension by neurons (Miller et al. 1990). On the other hand, adult astroglial scar tissue inhibits neurite extension (Rudge and Silver 1990).

Astrocytes are also implicated in the lineage development of oligodendrocytes. Comparing the differentiation of enriched oligodendroglia in subculture with their counterparts remaining on an astrocyte underlayer, the oligodendrocyte lineage exhibits signs of impaired cytoskeletal progression and plasticity in the absence of the astrocyte bed layer, exemplified by the prolonged retention of vimentin (indicating immaturity) (Ingraham and McCarthy 1989). With respect to myelinogenic products, newly separated cells within the oligodendrocyte lineage fail to initiate or sustain production of the oligodendrocyte-specific differentiation marker, galactocerebroside (Ingraham and McCarthy 1989; Saneto and De Vellis 1985). Coculture experiments suggest that galactocerebroside expression and the loss of vimentin, two events that normally coincide with terminal oligodendrocyte differentiation in vivo (Raff et al. 1984), require direct cell-to-cell contact with astrocytes (Aloisi et al. 1988).

Other in vitro studies indicate that astrocytes can exert a major mitogenic influence on oligodendrocyte development. When O-2A progenitor cells are dissociated from optic nerve and cultured, they stop dividing and some cells prematurely differentiate into oligodendrocytes, unless provided with a sufficient number of astrocytes. It is postulated that astrocytes provide the oligodendrocyte lineage with the necessary mitogens for proliferation. Evidence suggests that this mitogen is platelet-derived growth factor (PDGF) (Raff 1989).

Effects of Ethanol on Glial Cell Development

With the growing awareness of the important role of radial glia and other types of astrocytes on neuronal development, attention has focused on the possible effects of ethanol on astrocytes because of interest in fetal alcohol syndrome and the marked effects ethanol has on brain development. The information so far has come

from studies in which either ethanol was added to glial cell cultures or animals were exposed pre- or postnatally to ethanol, and then the development and structure of the glial cells were examined by electron microscopy (see also chapters 4 and 6). Studies in situ have demonstrated marked alterations of the Bergmann glia in rat cerebellum after pregnant rats were fed with an ethanol-containing diet as compared with an isocaloric diet throughout gestation (Shetty and Phillips 1992; see chapter 4). As indicated previously in this chapter, these alterations of Bergmann glia should significantly affect migration of granule cells during development. Abnormalities in other, non-Bergmann glia astrocytes have also been observed (Shetty and Phillips 1992).

Smith and Davies (1990) showed that perinatal exposure of rats to ethanol altered the dendritic arbors of the CA1 pyramidal cells of the hippocampus and also reported a marked "watery" astrocytic swelling, which may be of relevance to our findings that such astrocytic swelling causes release of excitatory amino acids in vitro (see the section on effects of ethanol on astrocyte function, earlier in this chapter).

Ledig and colleagues (1991; see also chapter 7) found that primary astrocyte cultures, used as an in vitro model for glial changes, treated and grown for 1 week with 30 mM ethanol exhibited only small morphological changes, but did show markedly decreased levels of nonneuronal enolase and the astrocyte-specific enzyme GS. Isenberg and colleagues (1992) reported that 65 mM ethanol added to the growth media for up to 7 days from plating resulted in marked inhibition of the growth of C_6 glioma cells. This effect was not seen with the same concentrations of other membrane-permeable components, such as urea and dimethyl sulfoxide.

Guerri and colleagues (1990) reported decreased proliferation, protein synthesis, and DNA synthesis in primary astrocyte cultures prepared from animals prenatally exposed to ethanol and in cultures in which the cells were exposed to ethanol in vitro. The in vivo effects led the authors to suggest that ethanol consumption produces significant damage in the cortical astrocyte progenitor cells of the fetus. Phillips and Krueger (1992) found, in an in situ study, that pre- and postnatal exposure to ethanol led to delayed development of oligodendroglia and a modest increase in astroglial cell number in rat optic nerve. This delay in development was considered significant. Saez and colleagues (1991) reported a marked decrease in GFAP and a disorganization of the cytoskeleton in primary astrocyte cultures prepared from 21-day-old fetuses from female Wistar rats that had been treated chronically with ethanol for 4–5 weeks. There were also decreases in vimentin, a major component of the intermediate filaments in these primary cultures, as well as in tubulin. Thus, both in vitro and in vivo studies suggest that ethanol may have deleterious effects on the development of astrocytes, which in turn should have a marked disruptive effect on the organization and cytoarchitecture of the CNS.

Role of Astrocytes in the BBB

As noted in preceding sections, the perivascular end feet of astrocyte processes surround brain capillaries, which unlike other capillaries do not allow the free passage of small solutes between the individual endothelial cells. This lack of free passage is termed the "blood-brain barrier" and has been known from the work of Ehrlich and Goldman at the turn of the century (Goldstein and Betz 1986). It was thought at one time that the astrocytic end feet actually formed the BBB, because this was the most obvious distinguishing feature between brain

capillaries and all other capillaries in the body. However, electron microscope studies in the 1950's using electron-dense markers showed that the barrier to the diffusion of these markers resided in tight occluding junctions (zonula occludens) between the endothelial cells, and that there was free passage of such markers between the astrocytic end feet (Goldstein and Betz 1986).

Another interesting astrocytic feature of the BBB is the very high density of intramembranous particle assemblies in the astrocytic membranes facing the blood capillaries (Landis and Reese 1982). The nature of these systems is unknown, but it has been speculated that they might be K^+ channels since a high K^+ conductance has been found on these astrocyte membranes (Newman 1986; see also figure 5).

Astrocytic end feet processes may play an important role in the induction of the BBB, which occurs in late gestation or early postnatal life in most mammals, as in humans and rats, respectively. Transplantation experiments showed that the formation of the BBB depended largely on the CNS environment, since it formed in capillaries growing into CNS tissue transplanted into systemic tissue (Stewart and Wiley 1981). Janzer and Raff (1987) showed that injection of primary astrocyte cultures into the rat anterior eye chamber or the chick chorioallantoic membrane induced a permeability barrier in the endothelial cells of the capillaries of these tissues, which would otherwise lack such a barrier. More recently, however, these experiments have been criticized and the view has been expressed that the available evidence only supports a more limited role of astrocytes in the maintenance of the BBB rather than its induction (Holash et al. 1993).

Recent work has used both endothelial cell and astrocyte cultures to form cocultures to study whether, in these more defined systems, astrocytes are truly responsible for induction of tight junctions between the endothelial cells and lead to an effective BBB. Formation of an effective barrier would be characterized by a high electrical resistance of around 2,000 $\Omega \cdot cm^2$, indicative of a low conductance to even small ions (Risau and Wolburg 1990). Shivers and colleagues (1988) reported that culturing of bovine aorta or pulmonary artery endothelial cells in media conditioned with primary astrocyte cultures showed ultrastructural features indicative of synthesis and insertion into the plasma membrane of tight junction components. However, no actual assemblies of tight junctions were seen. Growth of cultures of bovine brain capillary endothelial cells on one side of a filter, with primary astrocyte cultures on the other, was reported to lead to a transfilter resistance of $661 \pm 48\ \Omega \cdot cm^2$ (Dehouck et al. 1990). However, these authors also reported that the endothelial cells grown alone had a resistance of $416 \pm 58\ \Omega \cdot cm^2$. Since the astrocyte cultures formed multilayers of overlapping cellular sheets, it may well be that the small increase in resistance, especially taking into account the variability of the measurements, was due to the additive effect of the resistance of the astrocytes to the endothelial cell monolayer, thus adding two resistances in series. The resistance of an astrocyte multilayer of a similar thickness without the endothelial cells was not reported. In the coculture study, induction of the BBB-specific enzyme gamma-glutamyl transpeptidase was found in the brain endothelial cells when astrocytes and brain capillary endothelia, but not aortic artery endothelial cells, were cocultured (Dehouck et al. 1990).

Thus, although it is clear that induction of the BBB does occur at certain stages of development, the role that astrocytes may play in it is still unclear (Abbott 1991). Brightman (1991) concluded that "the precise role of perivascular

astrocytes in the induction and maintenance of brain endothelium as a structural and functional barrier has yet to be fully elaborated." This conclusion was based on some of the questions raised in this section, and on the finding that brain endothelial cells cultured alone can have resistances as high as 700–800 $\Omega \cdot cm^2$ without any astrocytes present (Karnovsky et al. 1987), and that some brain capillaries and other organs surrounded by astrocytes do not form a BBB. On the other hand, it does appear that morphological differentiation and induction of specific BBB proteins can be produced by primary astrocyte cultures in endothelial cells in vitro (Dehouck et al. 1990; Lobrinus et al. 1992; Tagami et al. 1992). As pointed out by Abbott (1991), the evolutionarily earlier barriers are formed between the glial cells as seen in the cephalopod mollusks and not between the endothelial cells. Abbott speculated that during evolution the barrier shifted from glial cells to endothelial cells in the vertebrates, perhaps to allow greater complexity and control of the CNS interstitial environment by the glial cells, superimposed upon a barrier that prevented interference by large changes in the blood.

Immune and Inflammatory Responses

Cells of the CNS constitutively express very low levels of antigens encoded for by major histocompatibility complex (MHC) genes whose products play a fundamental role in the induction and regulation of immune responses in the body. However, the long-standing view that the brain is insulated from the effects of the immune system is now being challenged (Fierz et al. 1985; Fontana et al. 1986; Schnyder et al. 1986).

A prominent feature of inflammatory and degenerative diseases of the CNS is the accumulation of macrophages, recruited from circulating blood monocytes and/or from the resident CNS macrophages (microglia). The signals leading to this accumulation are poorly understood. Nevertheless, astrocytes have been implicated as active participants in this process in view of their ability to secrete an interleukin-3 (IL-3)–like factor, which induces growth of cultured mouse peritoneal exudate cells and brain tissue macrophages (Frei et al. 1985). Astrocytes also secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), as evidenced by induction of colony formation in bone marrow cells and growth of FDC-P1 cells (Malpiero et al. 1990). A cytokine necessary for growth and differentiation of macrophages, GM-CSF has been found to lead to an accumulation of macrophages at the site of inflammatory lesions, and it enhances the phagocytic, cytotoxic, microbicidal, and other functional activities of mature macrophages. Therefore, GM-CSF produced locally by astrocytes may provide an essential element for the recruitment and activation of macrophages. Using Northern blots, Malpiero and colleagues (1990) demonstrated the presence of the mRNA for GM-CSF in cultured astrocytes. It would appear, therefore, that after the initial penetration of T cells into the CNS, astrocytes could further support the intracerebral T-cell activation process. When primary astrocyte cultures derived from newborn mice are treated with lipopolysaccharide (LPS) derived from *E. coli*, the astrocytes secrete interleukin-1 (IL-1) (Fontana et al. 1982).

Astrocytes have also been considered as the brain's antigen-presenting cells (APC's)—cells with the ability to present antigens to lymphocytes (Erb et al. 1986; Fontana et al. 1987). Study of the capacity of astrocytes to function as APC's has been facilitated by the development of myelin basic protein (MBP)–specific T-lymphocyte lines from mice or rats immunized with

MBP in complete Freund's adjuvant. Astrocytes from Lewis rats cocultured with a syngenic, MBP-specific, Ia-restricted T-cell line of Lewis rat origin stimulate T-cell proliferation; this process is antigen specific and restricted to the MHC (Fontana et al. 1984). During such cocultivation of T cells and astrocytes, the latter are induced by the preactivated T cells to express MHC class II molecules (also called Ia antigens) (Fierz et al. 1985; Fontana et al. 1984). Furthermore, interferon-γ (IFN-γ)–containing supernatants of lectin-stimulated spleen cells can induce murine astrocytes in culture to express Ia antigens (Hirsch et al. 1983), underscoring the dependence of astrocytes as APC's on the presence of Ia-inducing signals, such as IFN-γ. However, the validity of the studies depends on the absolute astrocyte purity of the cultures and the absence of microglia (Giulian and Baker 1986).

Although in vitro studies are supportive of astrocyte participation in CNS immune responses and in the etiology of autoimmune disorders such as multiple sclerosis (Massa 1989; Massa and ter Meulen 1987), there is controversy about their *actual* role in these processes in situ. Controversy still exists about which cells are the "real" APC's in the CNS. In both rats (Vass and Lassmann 1990) and humans (Lampson and Hickey 1986), microglia can express MHC class II antigens in situ. Microglia constitute approximately 5 percent of the resting total glial cell population and are considered to be the macrophages of the brain. Their major function is as a scavenger cell, ingesting cellular debris, a process which may be important for tissue modeling in the developing CNS. Microglia may also be involved with inflammation and repair in the adult CNS due to their phagocytic ability, release of neutral proteinases, and production of oxidative radicals. Microglia have been shown to express MHC antigens upon activation. They may act as APC's, and they are known to secrete a number of immunoregulatory cytokines and to respond to cytokine stimulation.

Astrocytes may be significant in modulation of the immune response, because in the adult CNS they far outnumber microglial cells and are, therefore, more immediately accessible for antigen-presenting functions. In vitro studies demonstrate that the amount of Ia expressed on IFN-γ–treated astrocytes correlates with the in vivo susceptibility of the CNS to immune-mediated encephalitis. Others have argued that the microglia are the more likely source of IL-1 during acute-phase brain injury, because microglia are the first brain cells to appear in increased numbers at sites of trauma or infection. A better definition of the respective roles played by microglia and astrocytes in CNS immune responses will have to await further studies. (See chapter 3 for further discussion and references on the microglia.)

PATHOLOGY

Reactive Gliosis and Scar Formation

Gliosis, also known as reactive gliosis or reactive astrocytosis, represents a major response of astrocytes to brain injury (Eng 1988; Norton et al. 1992; Reier 1986). Its hallmark is the accumulation of the intermediate filament protein, GFAP in reactive astrocytes (Eng 1988; Norton et al. 1992). Gliosis occurs in response to a number of etiologies including, but not limited to, physical trauma, chemical damage, and vascular damage (O'Callaghan 1993). Gliosis occurs in astrocytes throughout the CNS, although in general it is more extensive in white matter than gray matter (reviewed by Kimelberg and Norenberg in press).

Reactive gliosis after brain injury is normally detected within the first 24 hours. In rats, a peak response is reached within 3–4 days (Amaducci et al. 1981), subsiding over the succeeding 14–21 days. Increased GFAP is noted as early as 30 minutes after an injury (Amaducci et al. 1981). Expression of GFAP mRNA is elevated within 6 hours of injury, peaking at 1–3 days (Condorelli et al. 1990; Rataboul et al. 1988). Increments in mRNA are not confined solely to the lesion site, and can be encountered some distance from the site of injury (Cavicchioli et al. 1988). The degree and the reversibility of the response are closely correlated with the intensity of the injury. Thus, in mild injuries scar tissue is localized predominantly in the vicinity of the injury and resolves within weeks (Streit and Kreutzberg 1988), whereas in severe injuries gliosis is permanent and widespread (Petito et al. 1990).

It has been generally assumed that reactive gliosis is a property unique to mature and fully differentiated astrocytes, and astrocytes in the developing animal have been generally believed to be nonresponsive (Barrett et al. 1984; Bignami and Dahl 1976). It is now evident, however, that damage to the CNS can result in enhanced expression of GFAP in a variety of experimental conditions, followed by GFAP abatement to normal levels with time (O'Callaghan 1993).

Vimentin is normally found in cells of mesenchymal origin and, although widely expressed in developing astrocytes, it disappears upon cell maturation (Pixley and De Vellis 1984). However, upon injury astrocytes regain the capacity to synthesize vimentin (Petito et al. 1990), providing a sensitive marker for reactive gliosis.

The enhanced expression of GFAP in reactive gliosis is primarily caused by hypertrophy and not hyperplasia. Most recent studies with [^{3}H]-thymidine autoradiography combined with GFAP immunocytochemistry indicate that a very small number of astrocytes, perhaps as few as 1 percent, divide in the process of reactive gliosis (O'Callaghan 1993). Hyperplastic responses (Aldskogius 1982; Cavanagh 1970) may be more prevalent where the integrity of the BBB is compromised, allowing for the entry of blood-borne factors—including mitogens and other agents (e.g., PDGF, thrombin, fibronectin)—into the CNS. In keeping with enhanced proliferative activity under these conditions are the findings of the presence of the early response genes, such as *c-fos* (Dragunow et al. 1990) and *ras* p21 (Charman et al. 1988), in reactive gliosis with breaching of the BBB.

Other characteristics of reactive gliosis include increases in nuclear diameter, elevated DNA levels, elevated oxidoreductive enzyme activity, and increased glycogen. It was proposed over 100 years ago by Golgi (see Kuffler et al. 1984) that glial cells might act as an energy source for neurons. Although unequivocal data from vertebrate CNS are lacking, it seems possible that metabolic support for increased neuronal activity is derived from breakdown of astrocytic glycogen stores with subsequent intercellular transfer of glucose (Magistretti et al. 1993). Increased nuclear diameter and increased numbers of mitochondria, endoplasmic reticula, lysosomes, microtubules, dense bodies, and lipofuscin pigments are also common findings in reactive gliosis (Nathaniel and Nathaniel 1981). An increase in gap junctions has also been documented (Lafarga et al. 1991; Nathaniel and Nathaniel 1981), perhaps facilitating the spread of signals or substrates through an astrocytic network.

The molecular mechanisms involved in reactive gliosis remain elusive. Triggers may originate from damaged cellular components of the CNS (e.g., astrocytes, neurons, oligodendrocytes,

endothelial cells), invasion of the CNS by blood-borne factors following the disruption of the BBB, microglial activation, inflammation, or the production of cytokines (Goldmuntz et al. 1986; Murabe et al. 1981).

Microglia, along with many other CNS cell types, produce IL-1 (Arai et al. 1990), which has a wide range of target cells and is considered an important mediator of inflammatory responses. IL-1 is known to increase intracellular adhesion molecule 1 (ICAM-1) expression on human astrocytes (Frohman et al. 1989). In addition, IL-1 is a strong inducer of cytokine production by astrocytes. Astrocytes themselves have the ability to produce IL-1 in response to LPS stimulation (Benveniste 1993), thus representing a potential autocrine mechanism for astrocytic proliferation and growth. Astrocytes also produce tumor necrosis factor α (TNFα) in response to IFN-γ, LPS, and viruses and are known to be responsive to TNFα derived from microglia (Benveniste 1993). The architecture of the BBB, with astrocytic processes abutting cerebrovascular endothelium, suggests that astrocyte-derived TNFα may influence neighboring endothelial cells, alter BBB permeability, and promote inflammatory infiltration into the CNS. Astrocytes are also known to produce IL-6 in response to TNFα, IFN-γ, LPS, virus, and Ca^{2+} ionophores (Benveniste 1993). IL-6 has a mitogenic effect on astrocytes and may thus also contribute to reactive gliosis. The list of factors implicated as a trigger for gliosis continues to grow (Kimelberg and Norenberg in press).

The significance of reactive gliosis remains elusive. Unlike connective tissue scars, astrocytic scars do not provide great tensile support (Kimelberg and Norenberg in press). Nevertheless, reactive gliosis may delineate the damaged tissue, walling off the injured area from intact tissue and providing a barrier against entry of nonneuronal components into the CNS (Cavanagh 1970).

A limited number of studies have probed the role of gliosis in CNS regeneration. Extracts of gliotic tissue have been shown to promote the functional recovery of cortical tissue upon ablation (Kesslak et al. 1986). Membranes derived from anisomorphic gliosis (i.e., following a stab lesion) promoted neurite outgrowth, while those membranes derived from isomorphic gliosis (i.e., Wallerian degeneration) failed to promote neurite outgrowth (Boloventa et al. 1992). The presence of a sulfated proteoglycan in isomorphic gliosis (and its absence in anisomorphic gliosis) has been implicated in the inhibition of neurite outgrowth (Boloventa et al. 1992). Thus, it may be inferred that factors suppressing such proteoglycans would be beneficial in promoting neurite outgrowth; this possibility awaits confirmatory studies in situ.

Astrocytic Swelling in Ischemia and Trauma

Astrocytic swelling occurs uniformly in response to a wide variety of pathological states, such as trauma and ischemia, and may reverse slowly with time (Kimelberg and Ransom 1986). Although there have been many detailed descriptions of the morphology of this response, the functional consequences are only now being studied (Kimelberg 1992; Kimelberg and Ransom 1986). With the use of primary astrocyte cultures, the consequences and the mechanisms of astrocytic swelling are beginning to yield to experimental analysis. It appears, at least in its exaggerated form, that such swelling is deleterious and can be viewed as a pathological extension of more limited and controlled volume changes that are otherwise part of the normal homeostatic function of astrocytes. Thus, inhibition of astrocytic swelling

by an anion transport inhibitor, L-644,711, is associated with improved outcome in animal head injury and focal ischemia models (Kimelberg 1992; Kohut et al. 1992). In astrocyte cultures, L-644,711 prevents increased release of aspartate and glutamate due to hypotonic media, high K^+ media, or ethanol-induced cell swelling (see the next paragraph), and thus may reduce excitotoxic injury in vivo (Kimelberg 1992; Kimelberg et al. 1990*a*, 1990*b*, 1993*a*).

We recently reported that exposure of astrocyte cultures to isosmotic ethanol will lead to the same type of release of aspartate, glutamate, or taurine as high K^+ or hypotonic-media–induced release (Kimelberg et al. 1993; see also the section on effects of ethanol on astrocyte function, earlier in this chapter). This appears to be due to a swelling response since it is not seen with hyperosmotic ethanol, where ethanol is simply added to the solution with no reduction in electrolytes to maintain osmolarity. However, the K^+-induced amino acid release, which seems to be due to swelling, is increased by hyperosmotic ethanol (Kimelberg et al. unpublished observations), so some synergistic effect may be present. If such processes occur in situ, it would contribute to the harmful effects of ethanol on the nervous system; thus, it is important to consider the question of whether or not ethanol is isosmotic in the brain and the effect of ethanol-induced swelling.

SYSTEMS FOR STUDYING ASTROCYTES

In Situ Studies

As noted at the beginning of this chapter, methodology is critical to advances in our understanding in the empirical sciences. Thus, the question, "How does one study astrocytes?" is an important one. As noted in the introduction, a large impetus was given to studies of glial cells by Kuffler and colleagues (1966), who demonstrated that glia in invertebrates and lower vertebrates could be studied by the electrophysiological techniques current in the mid-1960's. These techniques have advanced considerably, and investigators are now studying the electrophysiological properties of mammalian astrocytes in situ by whole-cell patch-clamp techniques and also by imaging with intracellular pH or ion-sensitive fluorescent dyes, using confocal microscopy sometimes simultaneously with electrophysiological recordings (Berger et al. 1992*a*, 1992*b*; Clark and Mobbs 1992). The traditional methods of light microscopy, using specific labeling with astrocyte markers, and electron microscopy still allow one to look at astrocyte morphology and their interactions with neurons in situ. When coupled with immunocytochemistry or in situ hybridization, these techniques also allow one to localize specific proteins or their mRNA's to astrocytes and to look for evidence of increased synthesis by measuring changes in mRNA levels.

In Vitro Studies

The identification in the mid-1970's of primary cultures prepared from neonatal rodents as being predominantly astrocytic provided preparations of cells in sufficient numbers to allow for a variety of biochemical, electrophysical, molecular, and cell biological studies. The bulk of the information on astrocytes so far has come from these preparations. The methodologies and characteristics, as well as the advantages and disadvantages, of astrocyte cultures are summarized in table 5. In addition, primary cultures can be prepared from different brain regions; such cultures have provided the first indications of marked

Table 5. Summary of In Vitro Preparations for Studying Astrocytes

Methods for Preparing Astrocytes

- Microdissection
- Bulk isolation (density gradient centrifugation, selective adhesion, affinity chromatography, automated cytoflurography, and magnetic microspheres)
- Tissue prints
- Primary cultures
- Cultured cell lines

Protein Characteristics of Astrocytes

- GFAP
- S-100 (Ca^{2+}-binding protein and growth factor)
- GS (glutamine synthetase)
- A2B5 (galactocerebroside specific to oligodendrocytes or glial progenitor cells)
- GD3 (cell surface ganglioside)
- GC3
- GQiB (cell surface galactocerebroside for glial progenitor cells)
- Glutathione-*S*-transferase
- Pyruvate carboxylase
- Vimentin—most prevalent in dividing astrocytes

Advantages of Purified Astrocyte Preparations

- Defined system
- Cellular homogeneity
- Effects can be studied at the cellular level during proliferation or at confluence
- Temporal assessment—long-term versus acute exposure to substances
- Biochemical assessment—correlation between toxicity and parent compound or metabolite; studies of endogenous metabolism, and effects of changes in extracellular media composition

What Can Be Studied in Primary Astrocyte Cultures

- Cell morphology
- Protein synthesis and release into the extracellular medium of growth factors
- Effects of medium composition on growth/differentiation
- Energy metabolism
- Interaction of neurotransmitters with receptors
- Neurotransmitter uptake and release
- Electrophysiology of single cells and cell-to-cell communication
- Second messenger systems
- Ion transport by fluxes of radionuclides
- Effects of growth factors
- Regional specificity—cultures from different brain regions

Disadvantages of Purified Astrocyte Preparations

- Selection for surviving cells
- Immaturity and hypersensitivity of cultured cells due to lack of contact with other cells
- Disappearance or appearance of properties due to culture conditions, such as effects of serum factors

physiological and metabolic heterogeneity in astrocytes, to complement the known heterogeneity of their morphology in situ. Preparations from different brain regions also lend themselves to looking at the effects of coculturing with neurons, endothelial cells, or other cells and the ef-

fects of addition of different growth and mitogenic factors.

Studies on primary astrocyte cultures have always had the implicit caveat that one is uncertain as to how their properties are altered by growing them for several weeks in vitro. This has led several workers to study acutely isolated astrocytes obtained from brain by dissociating tissue from animals of varying ages and then allowing the cells to adhere to suitably coated surfaces (Barres et al. 1989; Newman 1986; Tse et al. 1992). The use of antibodies to specific markers, such as GFAP, GS, and S-100 protein allows one to unequivocally identify which cells are astrocytes (table 5). However, the small yield of these acutely isolated preparations only allows one to do experiments involving measurements from one or small groups of cells such as immunocytochemical studies, electrophysiology studies, and experiments involving autoradiography or fluorescent imaging. As advances in these techniques allow more precise information to be obtained, these acutely isolated cell preparations can be used to complement studies on primary astrocytes, comparing, for example, which types of channels are present both in vitro and in vivo.

CONCLUSIONS

This brief review cannot do justice to the great increases in experimental data driving changes in concepts now being made within the "glial" field, and this is just as applicable to astrocytes alone. However, it seems that the conceptual advances have to some extent stabilized, because the main themes of astrocyte functions listed in table 1—which we believe represent current thinking—are similar to those emphasized in a general review of astrocytes by Kimelberg and Norenberg published in 1989. The field is entering a more mature phase characterized by greater and greater details, leading to further refinements of our thinking.

In contrast to the previously emphasized support roles of astrocytes for neurons, new information now provides evidence that astrocytic contact with neurons significantly affects the morphological and functional differentiation of the latter. This reciprocity between neurons and astrocytes suggests that the morphological and physiological attributes of neurons are a product of this cell-cell interaction and vice versa. In addition, such reciprocity appears to exist with other CNS cell types such as oligodendrocytes, microglia, and endothelial cells. The wide diversity of astrocytic functions in maintaining homeostasis and the number of functions attributed to these cells are very large. The potential of astrocytes in modulating damage and repair is also reflected in this review.

Thus, it seems likely that ethanol could have a number of effects on astrocyte function, comparable to the effects reported for neuronal systems (Deitrich et al. 1989). The small amount of information currently available on such effects makes the expansion of such studies timely and worthwhile at this juncture, in view of the large number of properties that we now attribute to astrocytes and that have been experimentally verified. The large number of second messenger systems that are influenced by ethanol (Hoek and Rubin 1990) and the effects on membrane channels and transmitter effects (Deitrich et al. 1989; Gonzales and Hoffman 1991) can provide a rich field for investigations of the action of alcohol on astroglial function.

The problem of how ethanol affects biological systems is certainly part of the complexity of living cells. An understanding of the mechanisms of such effects can lead to effective and rational

treatments of alcoholism, without which such treatments can only be of a rough-and-ready kind. Furthermore, the application of these studies to "glial cells" and astrocytes in particular will lead to a greater understanding of the human brain and perhaps in time help us understand those brain-generated tragic forces that lead so many members of our society to seek solace from its pressures and conflicts by ultimately poisoning their minds with that simple—and, in terms of its biological effects, still so little understood—molecule, ethanol.

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Chapter 2

Oligodendrocytes and Myelin in the Neonatal and Adult Central Nervous System: A Framework for Examining the Effects of Toxins

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Along with a battery of detrimental effects, alcohol abuse is associated with myelin-related deficits. During central nervous system (CNS) development, myelin formation is delayed as a result of exposure to high alcohol levels. In adulthood, myelin loss, or demyelination, is correlated with chronic alcohol abuse. Effects of alcohol on the development of myelin-forming cells are discussed in chapter 4. To encourage and guide further research on the mechanisms underlying either situation, this chapter provides a broad outline of CNS myelin formation, maintenance, damage, and repair.

MYELIN FORMATION

During CNS development, myelination is the culminating event of a cascade of exquisite cell-cell interactions. Oligodendrocytes, the cells that form myelin in the CNS, are generated through a precise balance of proliferation and progression along the oligodendrocyte lineage. Mature oligodendrocytes must then coordinate the synthesis of myelin-specific proteins and lipids during a specific window of early postnatal development. Finally, the specialized oligodendrocyte processes repeatedly encircle appropriate axons and then the membranes compact to form myelin sheaths. Perturbation of this complex developmental process causes abnormal or insufficient myelin formation or, in the case of alcohol exposure, delayed myelin acquisition.

Oligodendrocyte Development

Glial precursor cells arise from germinal zones (e.g., the subventricular zone in the mammalian forebrain) and migrate into formative white matter regions. In vivo and in vitro studies of developing rodent CNS have characterized the

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oligodendrocyte lineage, which is comprised of cells at various stages in the progression from germinal zone cell to myelinating oligodendrocyte (for a detailed discussion and citations, see Dubois-Dalcq and Armstrong 1992). Several stages within the lineage can be distinguished by stage-specific antibodies. Oligodendrocyte progenitor cells in the subventricular zone are recognized by antibodies against the ganglioside GD_3. As the cells migrate away from this site, GD_3 is progressively lost. Cells at the leading edge of myelinogenesis are labeled by the monoclonal antibody O4 (Warrington and Pfeiffer 1992). These cells go on to express the myelin-specific glycolipid, galactocerebroside (GC), which is a marker of mature oligodendrocytes. Fully differentiated oligodendrocytes synthesize several myelin-specific proteins to form myelin sheaths. Myelin-associated glycoprotein (MAG) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) are the earliest myelin proteins to appear, followed by myelin basic protein (MBP) and proteolipid protein (PLP).

In vitro studies have identified factors that can regulate oligodendrocyte lineage cell behavior and mimic in vivo developmental processes (for further discussion and citations, see Dubois-Dalcq and Armstrong 1992; Goldman 1992; Richardson et al. 1990). Very early glial precursors proliferate rapidly in neonatal rodent brain glial cultures to form clusters that are immunolabeled by antibodies against the polysialylated form of neural cell adhesion molecule (PSA-NCAM) (Trotter et al. 1989) and express vimentin (Grinspan et al. 1990). These cells develop into bipolar, highly migratory cells which have been designated "O-2A progenitor cells" for their potential to develop into oligodendrocytes or type-2 astrocytes, depending upon the culture conditions. O-2A progenitors can be recognized in vitro by anti-GD_3 antibodies (R24 and LB1), and other frequently used antibodies such as A2B5 and NG2. Migration of O-2A progenitor cells is directed toward increasing concentrations of platelet-derived growth factor (PDGF). O-2A progenitor cells proliferate rapidly in the presence of PDGF and/or basic fibroblast growth factor (bFGF).

In vivo, proliferation of O-2A progenitors can be regulated by neuronal activity, which may modulate local PDGF levels (Barres and Raff 1993). Transforming growth factor β_1 (TGF-β_1) can attenuate PDGF-induced proliferation. A more mature progenitor stage, which is positive for O4 immunoreactivity (O4+), proliferates in response to exogenous insulin-like growth factor I (IGF-I). These three developmental stages (PSA-NCAM+, A2B5+, and O4+) each exhibit cell-surface immunoreactivity with the HNK-1 monoclonal antibody, which may be useful as a general marker of oligodendrocyte lineage precursor cells (see figure 1).

Mitogens for fully differentiated GC+ oligodendrocytes include dorsal root ganglion neurons, axolemma-enriched fractions, and bFGF (Vick and DeVries 1992; Wood and Bunge 1986*a*, 1986*b*; Wood and Mora 1993). The neuron-derived factors that regulate proliferation of GC+ oligodendrocytes have not yet been identified. Interestingly, media conditioned by different neuronal cell types contain different growth factors which can act as mitogens for different stages within the O-2A lineage (Hardy and Reynolds 1993). However, astrocyte-derived factors may also regulate proliferation of O-2A lineage cells since astrocytes synthesize PDGF-A (Richardson et al. 1988), bFGF (Ferrera et al. 1988), IGF-I (Chernausek 1993), and TGF-β_1, -β_2, -β_3 (Constam et al. 1992).

Progression along the oligodendrocyte pathway of differentiation may be regulated by neuron-glial interactions and growth factor signals. Oligodendrocytic differentiation and synthesis of myelin-specific proteins are promoted by treatment with IGF-I (McMorris and Dubois-Dalcq 1988) or with oligodendrocyte

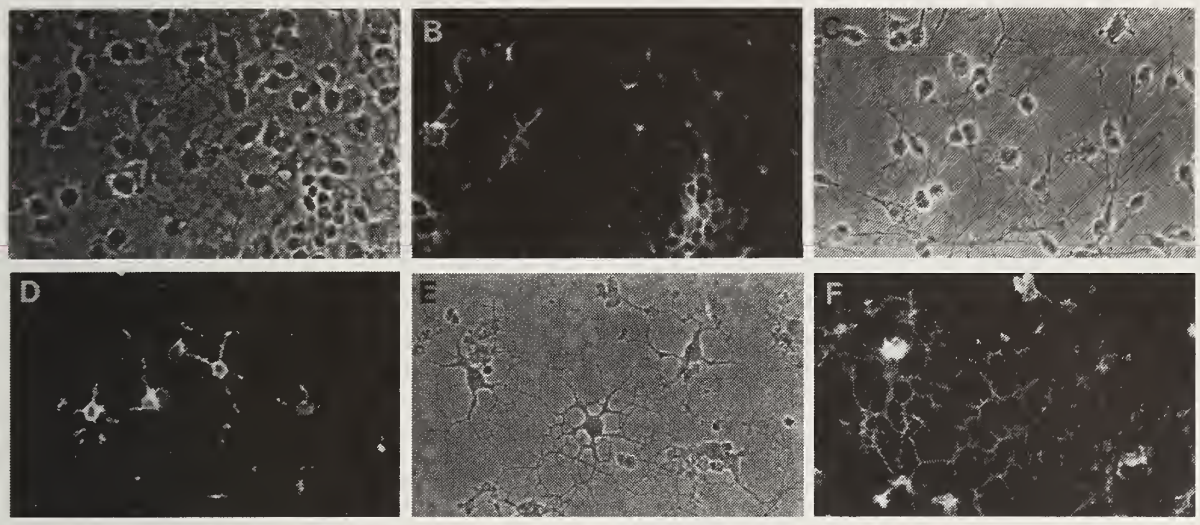

Figure 1. Phase contrast and immunocytochemistry of several stages of differentiation within the oligodendrocyte lineage. Panels A and B show the same field for a primary culture of glial cells derived from postnatal day 2 rat brain. The cells were fixed with 4 percent paraformaldehyde after growing for 11 days in Dulbecco modified Eagle medium (DMEM) supplemented with 10 percent fetal bovine serum. In panel A, process-bearing oligodendrocyte lineage cells are visible on top of a monolayer of flat astrocytic cells. In panel B, the majority of oligodendrocyte precursors are immunolabeled with the monoclonal antibody HNK-1, which is visualized with 7-amino-4-methyl-coumarin-3-acetic acid (AMCA). Early oligodendrocyte progenitors (equivalent to the PSA-NCAM+ to A2B5+ transition stage) are present in an HNK-1+ cluster toward the bottom right of the picture. The HNK-1+ multipolar cells in the upper left region are more mature oligodendrocyte precursors (equivalent to the A2B5+, O4+ stage). The HNK-1+ cells are not immunolabeled by antisera against glial fibrillary acidic protein (GFAP), which is a marker for astrocytic cell types (not shown). Panels C and D both show the same field of a secondary culture of oligodendrocyte progenitor cells. This secondary culture was prepared by shaking a primary culture, similar to that shown in panels A and B, and then reseeding the dislodged process-bearing cells. The cells were fixed in 4 percent paraformaldehyde after growing for 3 days in defined medium (Armstrong et al. 1990) supplemented with platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). These growth conditions maintain the oligodendrocyte lineage cells in a nondifferentiated state. In panel C, the cells possess a bipolar/tripolar morphology, which is characteristic of oligodendrocyte progenitors. In panel D, many of these cells are immunostained with the O4 monoclonal antibody, which is visualized with rhodamine. The majority of cells in these cultures are also A2B5+, GC-, and GFAP- (not shown). Panels E and F both show the same field of a secondary culture of oligodendrocytes. This secondary culture was prepared as described for panels C and D. The cells were fixed with 4 percent paraformaldehyde after growing for 3 days in defined medium that was not supplemented with PDGF and bFGF. Oligodendrocyte precursors differentiate into oligodendrocytes in these growth conditions. In panel E, the cells exhibit a network of branched processes, which is characteristic of oligodendrocytes. In panel F, these cells are immunolabeled by an anti-GC monoclonal antibody (RmAb), which is visualized with fluorescein.

growth factor (Giulian et al. 1991). Conversely, differentiation of oligodendrocyte precursors can be inhibited by treatment with PDGF and bFGF (Bögler et al. 1990) or with retinoic acid (Noll and Miller 1994), or by interaction with surface galactolipids (Bansal and Pfeiffer 1989). Maturation and expression of myelin-specific components are inhibited by bFGF (McKinnon et al. 1990) and by transforming growth factor α (TGF-α) or epidermal growth factor (EGF),

which both act through the EGF receptor (Sheng et al. 1989). Progression, or regression, within the oligodendrocyte lineage correlates with a range of physiological changes. As already described, each stage within the lineage exhibits a characteristic pattern of immunoreactivity and migratory or proliferative responses. In addition, each stage within the lineage expresses a distinct set of ion channels (reviewed in Ransom and Sontheimer 1992), which presumably facilitates the differential expression of phenotypes within the lineage.

As with many cell types, a large proportion of newly formed oligodendrocytes die off during normal development. Trophic factors are required for long-term survival of oligodendrocyte lineage cells (Barres et al. 1993). PDGF and IGF's are sufficient to maintain the survival of oligodendrocyte precursors in vitro. Oligodendrocytes may require a more complex set of survival signals, including IGF-I and neurotrophin-3, along with either ciliary neurotrophic factor, leukemia inhibitory factor, or interleukin-6. The full complement of survival factors that influence the oligodendrocyte lineage is likely to be even broader than this set, and the specific role of each factor in preventing apoptosis must still be determined.

Synthesis of Myelin Components

Myelin has a distinct composition of proteins and lipids, compared with other cell membranes (reviewed in Morell 1984). The major myelin proteins are PLP, MBP, CNPase, and MAG. Minor, or less abundant, myelin proteins include PMP22/gas3 (reviewed in Lemke 1993), myelin/oligodendrocyte glycoprotein (MOG) (Gardinier et al. 1992), and oligodendrocyte-myelin glycoprotein (OMgp) (Mikol et al. 1988; Viskochil et al. 1991). Myelin is highly enriched in lipids, which are mainly ethanolamine plasmalogen, phosphatidylcholine, and sphingomyelin phospholipids (reviewed in DeWille and Horrocks 1992).

Synthesis of each of these myelin components must be coordinately regulated during early postnatal development in order to elaborate normal myelin membranes. Synthesis of the major myelin proteins appears to be regulated mainly at the level of transcription (reviewed in Hudson 1990). Transcription can be controlled by DNA-binding proteins, which bind to each myelin-specific gene. Transcription is activated when an appropriate complement of *trans*-acting proteins binds to *cis*-regulatory enhancer or promoter elements of each gene (reviewed in Mitchell and Tjian 1989). Similarly, transcription is suppressed when specific proteins bind to silencer elements in a gene. Transcriptional regulation of the genes for MBP and PLP has been most thoroughly studied. It is not yet clear how transcription of each of these genes is regulated, or how expression of these and other myelin-specific genes can be coordinately controlled. A large number of sites, spanning regions up to 5,400 base pairs of 5'-flanking sequence in the case of the PLP gene, have been identified as putative enhancers/promoters or silencers (Berndt et al. 1992; Cook et al. 1992; Devine-Beach et al. 1990; Fors et al. 1993; Nave and Lemke 1991; Tamura et al. 1991).

Recent studies have identified a number of DNA-binding proteins that are found in brain, or in some cases in oligodendrocyte lineage cells, and that bind to putative *cis*-regulatory sites in myelin-specific genes (table 1). These studies, which have examined individual myelin-regulatory sites, may have broader significance for understanding the effects of alcohol. Many DNA-binding proteins are present in more than one cell type but can still regulate tissue- or cell-type-specific gene expression because they bind differentially in one cell type

versus another, due to, for example, posttranslational modifications or the presence of other proteins. Conversely, a consensus sequence for a given *cis*-regulatory element may be found in many genes but may differentially regulate transcription depending upon the presence and position of other regulatory elements and the complement of active DNA-binding proteins. Therefore, it is possible that the effects of alcohol may be exerted on a wide variety of genes, including those involved in myelination, by mechanisms involving shared DNA-binding proteins or *cis*-regulatory elements.

Myelin gene transcription may also be regulated through molecules, which have diverse biological roles. Retinoic acid and cyclic adenosine monophosphate (cAMP) influence expression of myelin-specific genes by mechanisms that are likely to be indirect because the response is relatively slow, requiring several days (Sato-Bigbee and Yu 1993; Zhang and Miskimins 1993; Zhu et al. 1992). However, triiodothyronine (T_3) appears to directly regulate the expression of myelin protein genes by transcriptional control (Farsetti et al. 1991) or by increasing messenger RNA (mRNA) stability and/or translational efficiency (Tosic et al. 1992). These molecules, and their activation pathways, may again suggest areas of overlap for mechanisms underlying the effects of alcohol. In fact, inhibition of retinoic acid synthesis has been hypothesized as a mechanism underlying multiple developmental abnormalities in fetal alcohol syndrome (Duester 1991; Pullarkat 1991).

Unexpectedly, recent studies have shown that expression of myelin genes is not strictly limited to postnatal myelinating cells. Some myelin genes are transiently expressed at relatively low levels for short periods of development prior to their up-regulation associated with postnatal myelination. An alternatively spliced isoform of the PLP gene, called DM-20,

Table 1. Summary of Recent Studies Identifying Proteins That Are Present in Brain or Oligodendrocyte Lineage Cells and Bind to 5'-Flanking Sequences of Myelin-Specific Genes.

	Potential Regulators of Myelin Gene Transcription	
Gene	**DNA-Binding Protein**	**Promoter Binding Site**
MBP, PLP MAG, OMgp[1] CNPase[3]	SP1-like	5'-NN(A/G)GGGAGG(A/C)(A/G) (GA box[2])(DR1/2 box[1])
PLP	MYT1	5'-AAGGATCAGTTGGAAGTTTCCAGGACATCTTC[4]
MBP	MB1-binding proteins	5'-AGGGAGGACAACACCTTCAAAGACAGGCCCTCTGAG[5] (MB1)
MBP	T3 receptor	5'-GGACCTCGGCTGAGGACACGGCGG[6] (MBP-TRE)
MBP	NFI-B[7]	5'-TGGCAAGGCGCCCA[8]
???	SCIP/Tst-1/Oct-6[9,10,11]	5'-GA(A/T)T(T/A)ANA[12] octomer motif

[1]Janz and Stoffel 1993; [2]Henson et al. 1992; [3]Monoh et al. 1993; [4]Kim and Hudson 1992; [5]Haas et al. 1993; [6]Farsetti et al. 1992; [7]Inoue et al. 1990; [8]Zhang and Miskimins 1993; [9]Monuki et al. 1989; [10]Collarini et al. 1992; [11]Suzuki et al. 1990; [12]He et al. 1991.

is normally expressed in brain during early postnatal development. Surprisingly, DM-20 has also been detected in embryonic mouse brain (Ikenaka et al. 1992; Timsit et al. 1992) and in heart tissue from several-week-old mice (Campagnoni et al. 1992). Similarly, spliced isoforms of the MBP gene have been detected in embryonic mouse brain (Nakajima et al. 1993), as well as in bone marrow and spleen (Campagnoni et al. 1993; Zelenika et al. 1993). Expression of MBP and PLP isoforms that is not correlated with myelinogenesis indicates that these genes may have additional functions during development.

Myelin Sheath Formation

Myelination is initiated as an oligodendrocyte process contacts and encircles an axon that has reached a critical diameter of approximately 1 μm (reviewed in Raine 1984*a*). The distal extension of the oligodendrocyte process flattens, spreads, and continues to extend so that the axon becomes repeatedly encircled (Bunge et al. 1989). The initial encircling of an axon by an oligodendrocyte process is likely to be directed through axon-glial interactions that involve MAG (Owens et al. 1990). The spiraled membranes of each oligodendrocyte process fuse to form a tightly compacted, multilamellar sheath referred to as a "myelin internode." Various myelin proteins are differentially distributed within myelin, which presumably reflects their distinct functions (reviewed in Hudson 1990 and Lemke 1993). Myelination progresses at slightly different rates in different areas of the CNS but is generally coordinated along particular nerve fiber tracts (reviewed in Raine 1984*a*).

MYELIN MAINTENANCE

Myelin is a relatively stable membrane but it is a metabolically dynamic structure. The protein and lipid myelin components described earlier in this chapter turn over throughout life. Therefore, oligodendrocytes must continue to support the synthesis and transport of these proteins and lipids, and their insertion into the myelin sheaths. Surprisingly, each individual component may have a turnover rate that is independent of the other components, even in the normal mature CNS (reviewed in Benjamins and Smith 1984). During this turnover, cytochrome P-450 IIE1 in oligodendrocytes and myelin may contribute to the reutilization of ketone bodies that are produced slowly during myelin breakdown (Cammer et al. 1991).

Interestingly, an array of enzymatic activities is associated with myelin membranes. Enzymes active in myelin membrane fractions include CNPase (for which the substrate has not been identified) and a number of enzymes with a wide range of functions, including roles in phospholipid synthesis, posttranslational modification of proteins, and second messenger pathways (reviewed in Ledeen 1992). Myelin also may possess mechanisms for signal transduction, because recent studies have demonstrated the presence of muscarinic cholinergic receptors and GTP-binding proteins (reviewed in Ledeen 1992).

MYELIN DAMAGE

Damage to myelin that cannot be compensated for by normal myelin maintenance will result in a loss of myelin sheaths, or demyelination. Demyelination can result from mechanisms that directly impair oligodendrocytes or myelin sheaths (reviewed in Raine 1984*b*). Examples of this type of primary demyelination include autoimmune responses directed against myelin components (Alvord et al. 1992; Shin and Koski 1992), viral infection of oligodendrocytes (Fazakerly and Buchmeier 1992), and

hereditary or toxic metabolic diseases (reviewed in Norton and Cammer 1984 and Raine 1984*b*).

Demyelination can also result from an indirect insult of oligodendrocytes or myelin. When axons are damaged and undergo Wallerian degeneration, the associated myelin degenerates and is phagocytosed (reviewed in Smith and Benjamins 1984). Another mechanism of demyelination has been referred to as "bystander demyelination" (reviewed in Norton and Cammer 1984). This form of demyelination occurs during cell-mediated immune reactions as invading macrophages, which may be directed against a non-oligodendrocyte/myelin antigen, release lytic enzymes and agents that damage the neighboring oligodendrocytes and myelin. Similarly, during inflammatory responses or blood-brain barrier impairment, activated microglia may release nitric oxide and/or tumor necrosis factor α and cause oligodendrocyte lysis (Compston 1993; Merrill et al. 1993).

Complications of alcoholism are correlated with diseases of myelin, presenting as vitamin B_{12} deficiency, central pontine myelinolysis, or Marchiafava-Bignami disease (reviewed in Norton and Cammer 1984 and Raine 1984*b*). In fact, magnetic resonance imaging studies demonstrate CNS white matter lesions, which indicate early involvement of the brain in asymptomatic alcoholics (Gallucci et al. 1989).

MYELIN REPAIR

The ability of the adult CNS to repair myelin, or remyelinate, is often complicated by ongoing demyelination and by factors in the lesion environment that may inhibit remyelination. However, evidence of remyelination has been documented following both acute and chronic episodes of demyelination; for example, in multiple sclerosis patients (Prineas et al. 1993; Raine 1990; Raine and Wu 1993). Since mature oligodendrocytes in normal tissue have processes apparently anchored to many different axons, it has been difficult to conceptualize and identify the source, or sources, of remyelinating cells following demyelination and depletion of oligodendrocytes within the lesions.

In normal adult rodent brain, oligodendrocytes turn over very slowly (McCarthy and Leblond 1988). Immature oligodendrocytes or oligodendrocyte precursors persist in rodent and human adult CNS, but probably comprise less than 2 percent of the cells in normal adult CNS tissues (Armstrong et al. 1992; Miller et al. 1985; Wood and Bunge 1991). Remyelination appears to involve more than just recruitment of the population that is slowly turning over and differentiation of the available precursors. Efficient remyelination, which is achieved in experimental animals, requires that the proliferation of oligodendrocyte lineage cells be greatly increased in response to demyelination (reviewed in Wood and Bunge 1991).

Several mechanisms for generating oligodendrocytes may contribute to this robust regenerative response. In vitro studies indicate that the proliferation of both immature and mature oligodendrocytes can be up-regulated by the appropriate signals. Immature oligodendrocytes from adult rodent CNS, called O-2A^{adult} progenitors, are immunolabeled by the antibodies A2B5 and O4. O-2A^{adult} progenitors differ from neonatal O-2A progenitors in that they differentiate more slowly, exhibit a longer cell cycle, and move more slowly in response to PDGF (reviewed in Noble et al. 1992). In the presence of both PDGF and bFGF, a subset of the O-2A^{adult} progenitor population reverts to the neonatal phenotype (i.e., proliferates rapidly and becomes highly motile) (Wolswijk and Noble 1992). Unfortunately, similar treatments of PDGF and bFGF did not elicit proliferation

of oligodendrocyte precursors from adult human CNS (Armstrong et al. 1992). Mature GC+ oligodendrocytes exhibit responses that differ from those of the O-2A^{adult} progenitors. Proliferation of mature GC+ oligodendrocytes is triggered optimally by coculture with neurons (Wood and Bunge 1986*a*, 1986*b*) or growth in medium supplemented with an axolemmal-enriched fraction or bFGF (Vick and DeVries 1992; Wood and Mora 1993). Interestingly, bFGF can also induce mature oligodendrocytes to dedifferentiate to a more immature state, as monitored by loss of GC immunoreactivity (Armstrong et al. 1992; Wolswijk and Noble 1992; Wood and Mora 1993). Future studies must determine whether reversion of oligodendrocyte lineage cells to more immature stages takes place during remyelination, and the extent to which these phenotypic changes correlate to specific functions in the remyelination process.

Following demyelination and the generation of remyelinating cells, the formation of new myelin sheaths seems to recapitulate the developmental process. As the oligodendrocytes differentiate, myelin-specific proteins appear in the same temporal patterns as in development (Ludwin and Sternberger 1984). In fact, isoforms of the alternatively spliced myelin genes that are expressed during early stages of myelination are also expressed at early stages of remyelination (Jordan et al. 1990). During remyelination the myelin sheath is compacted normally but is disproportionately thin relative to the axon diameter (reviewed in Raine 1984*a*).

CONCLUDING COMMENT

In this overview of the cellular and molecular mechanisms of myelination and remyelination, I have attempted to demonstrate the complexity of the system. As a result of this complexity, one can interpolate many potential points at which toxins can perturb this system. Although some toxins appear to act through singular mechanisms, alcohol has widespread physiological effects in the developing and adult CNS. Clearly, future researchers will be challenged to advance and integrate knowledge of these two very complicated areas in order to reveal the mechanism(s) underlying the toxic alcohol effects that result in delayed myelination during development and demyelination in adulthood.

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Chapter 3

Microglia in the Pathological Brain

Wolfgang J. Streit, Ph.D.

The first detailed investigation of microglial cells, termed "the third element" by Cajal in 1913 to distinguish a third cellular component besides neurons and astroglial cells, was conducted by del Rio-Hortega in the 1920's and 1930's. In the following decades, the relatively few scientists interested in microglia were concerned primarily with their ontogenesis, which was believed to be mesodermal as opposed to neuroectodermal (del Rio-Hortega 1932).

Since the 1980's the neurobiology of microglia has been undergoing a true renaissance, and microglia are currently the subject of an ever-increasing number of research efforts examining many diverse aspects of their cell biology. This renewed interest in the third major glial cell type stems largely from the realization that microglia serve a function within the central nervous system (CNS) that in many regards is very similar to the function served by cells of the immune system in other organs. Accordingly, the potential significance of microglia for the study of neuropathological and neurotoxicological conditions cannot be underestimated.

Using examples of some well-studied CNS disease models, this chapter provides the noninitiated reader with an overview of microglial cell functions. In addition, this chapter attempts to formulate a working hypothesis as to how microglia could be involved in fetal alcohol syndrome.

METHODS FOR VISUALIZING MICROGLIA IN TISSUE SECTIONS

A crucial event in the rediscovery of microglia was the advent of histochemical methods allowing the reliable and reproducible visualization of microglia in situ. Prior to the development of such methods—which are primarily enzyme histochemical, lectin histochemical, and immunohistochemical in nature (see Streit in press for more detailed descriptions)—it was difficult for nonspecialists to identify microglia. Although some characteristic morphological criteria of microglia made identification of these cells possible in plastic-embedded sections at both light and electron-microscopic levels (Blakemore 1975;

Ling et al. 1973), this type of study was time consuming, confined to small sample areas, and inherently limited by the fact that morphology was the singular criterion in the identification process.

The availability of histochemical procedures—which not only allow the monitoring of microglial changes in morphology, but also provide us with information about phenotypic changes occurring within surface molecules embedded in the microglial plasma membrane—has enabled researchers to follow patterns of microglial activation during neuropathological situations in much greater and meaningful detail. The degree of sophistication with which we perform histochemistry of microglia soon will gain yet another dimension through the histochemical detection of microglial secretory molecules, such as cytokines and growth factors. These secretory products are known to mediate intercellular communication among cells of the immune system, and their presence within the CNS is currently undergoing intense study.

SENSITIVITY OF MICROGLIAL CELLS TO NEURONAL INJURY: THE FACIAL NERVE MODEL

A key event in the effort to characterize microglial responses to neural injury or disease is *microglial activation,* which can be broadly defined as the summary of morphological, phenotypic, and secretory changes detectable in microglial cells following stimulation. Unfortunately, little is known about the molecular nature of the stimuli that activate microglia. To learn more about what causes microglial activation, two basic experimental approaches have been taken.

One approach involves the maintenance of microglial cells in vitro; by exposing purified cultures to various chemical stimuli, any effects on their morphology, phenotype, and secretory activity can be measured. An in-depth discussion of this strategy is beyond the scope of this chapter, and the reader is referred to chapter 10 and to Giulian (1993) and Banati et al. (1993).

The other approach that has enabled researchers to study the spatiotemporal sequence of events comprising microglial activation in situ can be found in an in vivo model of reproducible neuronal injury, namely transection of the axons of facial motor neurons. Following axotomy of the facial nerve outside of the CNS, a massive microglial reaction occurs within the confines of the facial nucleus in the ventral brain stem. Characteristic features of the microglial response within the facial nucleus include altered microglial morphology and enhanced immunoreactivity for the CR3 complement receptor within 24 hours after nerve section (Graeber et al. 1988*a*), followed by a proliferative burst of microglia on days 3 and 4 after axotomy (Kreutzberg 1966). Unlike microglia, astrocytes do not undergo mitosis in this paradigm (Graeber et al. 1988*b*).

Beginning also during the first week after motor neuron injury is the de novo expression of major histocompatibility complex (MHC) antigens on the microglial cell surface (Streit et al. 1989). It is important to note that MHC class I antigens are expressed differently from MHC class II antigens, in terms of both their temporal appearance after injury and cell type. Expression of MHC class I antigens occurs within days after injury in a burstlike fashion and involves many, if not all, of the proliferating microglia. In contrast, expression of MHC class II antigens occurs much more slowly over several weeks and is more selective than MHC class I expression, involving only a fraction of the total microglial and perivascular

cell population (for definitions of cell types, see Streit in press).

Because the presence of class II antigens is used as one critical, identifying criterion for antigen-presenting cells, the limited population of microglia that becomes positive for MHC class II antigens can be considered to represent an immunologically competent set of microglia capable of carrying out the function of antigen presentation. At the same time, one needs to realize that the mere expression of MHC II antigens in situ does not permit the conclusion that MHC II–positive microglia actually perform antigen presentation functionally. Such functional antigen presentation can only be ascertained through lymphocyte proliferation assays. Based on the observations of a differential expression of MHC class I and class II by microglia in vivo, it is apparent that microglia represent a cell population that is heterogeneous in terms of immunological competence. Presumably, the selective up-regulation of MHC class II antigens on only some microglial cells is due to a special susceptibility of a certain microglial cell pool to some unknown stimulating factor(s).

What Causes Microglial Activation?

In the facial nucleus paradigm, it is usually assumed that "emergency signals" must originate from injured facial motor neurons to activate microglia in their immediate microenvironment. This seems like a logical assumption since axotomy of the facial nerve is a lesion that does not involve the brain directly, but is confined to the peripheral axons of facial motor neurons. Somehow a signal is transmitted from the axotomy site, possibly via axonal transport, to the cell bodies of facial motor neurons, which are stimulated to undergo increased protein synthesis. Such increased protein synthesis could result in the generation of chemical messenger molecules diffusing out from the injured neurons into the immediate microenvironment. At present, the nature of any hypothetical neuronal emergency messenger molecules remains elusive.

One possible candidate molecule which has been examined specifically in the context of MHC antigen expression is gamma interferon, because it is known from in vitro studies that gamma interferon is one of the most potent inducing substances of MHC antigens on a number of different cell types, including microglial cells. When gamma interferon is infused systemically at high doses, it causes widespread up-regulation of MHC class II molecules on microglia in the CNS (Steiniger and van der Meide 1988). However, studies demonstrating an increase in gamma interferon–like immunoreactive material in motor neurons after axotomy detected a molecule different from bioactive gamma interferon, which made it unlikely that gamma interferon can originate from motor neurons to serve as an inducing signal for MHC expression on microglia after nerve section (Kiefer et al. 1991).

As an additional possibility to neuronally derived signals which activate local glial cells, the possibility that exogenous (i.e. blood-derived) factors may gain selective entry to the facial nucleus system should be considered. Although it has been ascertained that the integrity of the blood-brain barrier in the region of the facial nucleus remains intact when assessed by conventional tracing methods with intravenously applied horseradish peroxidase and/or Evans blue, recent observations using intraneural injections of Fluorogold have raised the possibility of an alternate pathway by which substances from the periphery may gain access to the CNS after a lesion (Streit and Graeber 1993). The injection of Fluorogold into the

facial nerve followed by axotomy (either crush or cut) results in the selective labeling of perivascular cells found in close association with blood vessels in the region of the facial nerve root. The fact that no such labeling is seen in the contralateral uninjured facial nerve root or anywhere else excludes the possibility of a systemic effect (for example, that Fluorogold leaking from the injection site into the bloodstream is taken up nonspecifically by perivascular macrophages of the cerebral vasculature). Instead, this finding shows that Fluorogold can reach the perivascular space of the CNS following application to an axotomized peripheral nerve. The implication of this observation is that other substances produced at the wound site, such as growth factors, cytokines, and other pro-inflammatory agents, could also reach the CNS perivascular space via the same route and promote the development of the microglial reaction in the facial nucleus system.

TRANSFORMATION OF MICROGLIA INTO BRAIN MACROPHAGES FOLLOWING ACUTE NEURONAL DEGENERATION

Although there is some evidence to suggest that astrocytes and oligodendrocytes can engage in phagocytic activity, microglial cells have traditionally been thought of as the main source of indigenous brain macrophages. We were able to demonstrate experimentally the macrophage function of microglia in situ using the facial nerve paradigm (Streit and Kreutzberg 1988). Acute and complete degeneration of facial motor neurons can be brought about by the intraneural injection of toxic ricin, the lectin from *Ricinus communis* (RCA_{60}), which is transported retrogradely and causes death of facial motor neurons through inhibition of protein synthesis (Streit and Kreutzberg 1988; Wiley et al. 1982). Thus, the administration of toxic ricin affords a model of irreversible neuronal injury, in contrast to an axotomy which is a reversible lesion that allows motor neurons to regenerate. Under these conditions of acute neuronal death, the reactive microglial cells in the facial nucleus undergo transformation into brain macrophages. The obvious function of these microglia-derived brain macrophages is to remove the neuronal debris generated by ricin-induced degeneration.

The ricin experiments were followed by another set of experiments designed to show that the brain macrophages in the facial nucleus are indeed derived from indigenous microglia and not from blood-borne monocytes. Using the cytostatic agent adriamycin which, like RCA_{60}, is transported retrogradely in neuronal processes (Bigotte and Olsson 1982), it was shown that simultaneous intraneural injections of adriamycin and RCA_{60} cause neuronal degeneration accompanied by a much attenuated macrophage response—that is, only a few macrophages are detectable immunohistochemically (Graeber et al. 1989). This finding, together with a significant reduction in the incorporation of ^{3}H-thymidine by proliferating microglia, showed that inhibiting microglial proliferation with adriamycin causes decreased formation of brain macrophages, and therefore justified the conclusion that brain macrophages are predominantly derived from microglia.

THE MICROGLIAL RESPONSE TO ISCHEMIA: A CONTRIBUTING FACTOR IN THE DEVELOPMENT OF DELAYED NEURONAL DEATH?

Transient global forebrain ischemia induced by four-vessel occlusion in the rat has been used as

a model to study the phenomenon of delayed neuronal death (Kirino et al. 1984). Unlike the acute ricin-induced neurodegeneration in the facial nucleus which is followed by a microglial response, ischemia-induced neuronal death in the rat hippocampus is preceded by a microglial response. Activated microglia can be detected in the hippocampus as early as 20 minutes after reperfusion, and within 24 hours microglial activation is prominent in areas of imminent neuronal death, notably in the pyramidal cell layer of the CA1 sector (Gehrmann et al. 1992; Morioka et al. 1991). Because histologically detectable degeneration of pyramidal neurons does not occur until 2–4 days after ischemia, the question arises of whether the presence of activated microglial cells in the region could be a contributing factor in the demise of pyramidal cells. The demonstration that microglia can secrete glutamate when stimulated in vitro (Piani et al. 1991) may lend credence to this idea, since high glutamate levels are believed to represent one of the earliest events in the excitotoxic cascade that eventually leads to delayed neuronal death in the pyramidal cell layer (Choi 1988).

In addition to glutamate, microglia in vitro have been shown to secrete a number of other potentially damaging molecules, including reactive oxygen species (Colton and Gilbert 1987) and certain, yet unidentified, neurotoxic substances whose biological activity can be blocked by NMDA receptor antagonists, such as MK-801 (Giulian et al. 1993). The fact that systemic administration of MK-801 to animals prior to the induction of ischemia can prevent not only the delayed neuronal death in the hippocampus, but also the accompanying microglial reaction, could therefore be considered as evidence favoring a neurotoxic role of microglia in situ (Streit et al. 1992).

However, other in vivo and in vitro observations argue against a neurotoxic effect being exerted by activated microglia. For example, microglia stimulated in tissue culture have been shown to produce neurotrophic molecules, including nerve growth factor and basic fibroblast growth factor (bFGF) (Araujo and Cotman 1992; Mallat et al. 1989). The latter factor is of particular interest within the current context regarding hippocampal neurons, since a recent study by Ray and colleagues (1993) has shown bFGF to induce the survival and proliferation of embryonic hippocampal progenitor neurons. The seemingly conflicting in vitro findings on microglial secretory activity demonstrating production of both neurotoxic and neurotrophic molecules raise the question as to which ones of the microglial secretory products are released in situ following forebrain ischemia. While the answer to this question awaits investigation, it is interesting to note that the early presence of activated microglia in the hippocampus after ischemia is not limited to regions that eventually show neuronal loss (i.e., the CA1 pyramidal layer); rather, activated microglia are also found in the infragranular layer of the dentate gyrus where neurons survive the ischemic insult (Morioka et al. 1991).

Similarly, following focal cerebral ischemia induced by occlusion of the middle cerebral artery (MCA), activated microglia are widespread throughout various brain regions, including areas outside of the MCA territory (Morioka et al. 1993). Thus, the in vivo observations show that the presence of activated microglia does not always indicate imminent neuronal degeneration, but may in a broader sense signal neuronal injury which could be either reversible or irreversible. It remains to be determined if there are subtle characteristics of activated microglia that could allow one to predict the degree of neuronal injury in its early phases. At this point, the conclusion that can be drawn is that the detection of activated

microglia in situ is a most sensitive indicator of disturbances in neuronal homeostasis.

DO MICROGLIA SERVE PARADOXICAL FUNCTIONS IN TUMORS AND IN NEURAL TRANSPLANTS?

The introduction of foreign tissue into the brain is inevitably accompanied by physical lesioning of the CNS tissue which in itself causes microglial activation, as well as astrocytic gliosis or glial scar formation. This response of glial cells to physical trauma is transient and, immunologically speaking, nonspecific; that is, the cellular reaction does not occur as a response to the introduction of specific cells or antigens, nor does it, as far as we know, elicit an antigen-specific, systemic immune response.

In contrast, specific interactions of microglial cells with foreign tissue components likely occur after neural grafting and during the early stages (sensitization phase) of the immunological rejection process, which eventually causes destruction of neural allo- or xenografts. Detailed immunohistochemical and electron microscopic studies by Lawrence and colleagues (1990) strongly suggest that the systemic immune response targeting a neural allograft is initiated and propagated by CNS microglial cells. Such a catalytic role of microglia in facilitating immune rejection is closely linked to their proposed function as CNS antigen-presenting cells (see the section on sensitivity of microglial cells to neuronal injury). Upon the introduction of a neural allograft, microglia are the first line of parenchymal immunocompetent cells which encounter foreign antigens, and they subsequently ingest, process, and present alloantigens. This type of microglial activity occurring during neural tissue transplantation accounts in large part for development of the subsequent systemic immune response which ultimately rids the CNS parenchyma of foreign histoincompatible cells and tissues.

Similar to implantation of neural allografts, the inoculation of glioma cells to produce an experimental brain tumor represents an introduction of foreign cells into the CNS. However, the glioma cells, unlike transplanted donor CNS tissue, are not subject to immunological rejection, and despite widespread microglial activation and infiltration of tumor tissue by activated microglia, malignant brain tumors induced by tumor cell inoculation grow without restraint and eventually kill the animal (Morioka et al. 1992*b*). This is puzzling because tumor cytotoxicity has been shown to be one of the functions of microglial cells, at least in vitro where microglia can effectively kill tumor cells by at least two different mechanisms (Frei et al. 1987; Sutter et al. 1991).

We have begun to address the question of whether microglia are tumor-cytotoxic in vivo by studying the composition and extent of the inflammatory cell infiltrates accompanying experimental rat gliomas induced by intracerebral inoculation of RG-2 cells. RG-2 gliomas are well-vascularized and rapidly growing, malignant tumors which show a remarkable degree of similarity between individual animals in their size and growth characteristics, as well as in the extent of their inflammatory cell infiltrates. Therefore, they provide a reproducible glioma model that is well-suited for the study of tumor-immune interactions. Our histological observations show that RG-2 gliomas are infiltrated and surrounded by large numbers of activated microglia. In terms of morphology and phenotype, these tumor-associated microglial cells demonstrate the gamut of changes characteristic of microglial activation, including transformation into

large brain macrophages and strong expression of MHC class II antigens and other leukocyte antigens (Morioka et al. 1992*a*).

Expression of MHC class II antigens occurs preferentially on microglia-derived brain macrophages located within the tumor mass, and is absent from peritumoral, reactive microglia which have not undergone transformation to the macrophage stage. In addition, microglial cells within the tumor show upregulation of leukocyte common antigen, CD4 antigen, and the ED2 epitope. These activated microglia can be seen undergoing mitosis, and they surround cords of tumor cells invading peritumoral areas of normal CNS tissue. Thus, the phenotype and morphology of microglia in the glioma clearly indicate that tumor-associated microglial cells are activated to a point where they could be functioning as antigen-presenting cells and might also engage in other immune functions, such as tumor cytotoxicity. However, despite this widespread microglial activation in and around the gliomas, there is no evidence that would suggest the presence of an effective antitumor defense mechanism; that is, there is unrestrained growth of tumors which can reach enormous proportions.

From these observations it is apparent that the documentation of morphological and phenotypic changes alone is not sufficient to make conclusions about the actual functions being performed by activated microglia in situ. In order to incorporate findings from in vitro studies on microglial secretory activity into the in vivo concept, we have initiated an investigation aimed at determining the levels and cellular sources of cytokines/growth factors in the RG-2 glioma environment. Our studies to date, performed at the messenger RNA (mRNA) level, have focused on the detection of transforming growth factor β (TGF-β) in the rat glioma, because this cytokine is known to be prominently associated with human gliomas (Kiefer et al. 1994). It is thought that TGF-β might mediate some of the immunosuppressive activity commonly associated with human gliomas. Our findings obtained with Northern blot analysis and in situ hybridization have revealed that tissue samples from RG-2 gliomas indeed show a very high content of TGF-β mRNA in the main tumor mass, and to a lesser extent in peritumoral tissue. In situ hybridization histochemistry has been confirmatory in showing a most intense accumulation of silver grains in the glioma area with little activity in the surrounding normal brain tissue. Thus, it appears that the rat glioma, like human gliomas, produces large amounts of TGF-β. In accordance with the known immunosuppressive action of TGF-β (Fontana et al. 1984; Merrill and Zimmerman 1991), we hypothesize that glioma-derived TGF-β suppresses microglial tumor cytotoxicity in situ. This hypothesis would be one possible explanation for the apparent paradoxical behavior of microglia in neural allografts and in brain tumors.

DOES ETHANOL TOXICITY AFFECT MICROGLIA? SOME PRELIMINARY FINDINGS AND HYPOTHESES

Little is known about a potential involvement of microglia in ethanol-induced neurotoxicity. Chronic ethanol treatment of adult rats appears to exacerbate the effects of normal aging on microglia—that is, microglia develop abnormal morphologies with truncated cellular processes at an accelerated pace (Kalehua et al. 1992). Interestingly, a microglial reaction does not seem to accompany the reported loss of hippocampal neurons that occurs as a result of chronic ethanol treatment. Although this may seem surprising in view of the substantial loss

of neurons reported (Walker et al. 1980), it is not at all astonishing when considering the slow and chronic nature of the lesion. Other studies examining microglial responses to ischemic injury also document an attenuated microglial response in thalamic areas where neurodegeneration takes a prolonged time course (e.g., Morioka et al. 1993).

In contrast to the long-term and chronic administration of ethanol to adult animals, it may be more instructive to examine microglial cells during fetal alcohol syndrome, because the effects of ethanol toxicity may be more profound during development. In addition to anticipating a microglial response to acute ethanol-induced neuronal damage, it is possible that ethanol may have a direct effect on microglia themselves. Preliminary data have been obtained in my laboratory on rat brain sections from developmental stages E18, P0, and P2; these sections were stained with horseradish peroxidase–labeled *Griffonia simplicifolia* I-B_4 isolectin (GSA I-B_4-HRP), a selective marker for microglial cells (Streit 1990). Although GSA I-B_4-HRP is selective for only microglia and no other glial cell types, the lectin does have an affinity for the endothelial surface of cerebral blood vessels. Simultaneous staining of the vascular channels and microglial cells with GSA I-B_4-HRP in the developing CNS could be a reflection of the common derivation of these structures from primordial mesodermal tissue.

Microglial cells are believed to originate through a series of transformations from so-called ameboid microglia which are found aggregated in clusters in specific locales, such as the supraventricular corpus callosum, during early postnatal life of the rat (Hurley and Streit 1991). Although the origin of ameboid microglial cell clusters remains a matter of dispute, the successive developmental transformations of *ameboid* microglia to an *intermediate* stage, and to the fully differentiated *ramified* (adult) microglial cell is well-accepted (for a review of microglial ontogeny, see Theele and Streit 1993). In the course of our preliminary lectin histochemical comparison of ethanol-treated and control animals, we have noted that clusters of ameboid microglial cells in the supraventricular corpus callosum from animals after chronic prenatal ethanol treatment (CPET) contain larger numbers of cells than control animals. In addition, microglial cells in other locales are more differentiated in animals after CPET than in control animals; that is, these microglia have a morphological appearance resembling more closely the adult ramified phenotype (figure 1).

Due to simultaneous visualization of the vasculature and microglial cells with lectin histochemistry, we felt compelled to also look for any changes in vascular development that may occur as a consequence of CPET. Vasculogenesis during the embryonic stage involves the formation of primitive vascular cords which invade the developing neuroectoderm from the pial surface. The distal ends of these vascular cords typically grow numerous tentacles of sprouts, which eventually fuse with other growth tips to form connecting channels (Mato et al. 1989). In the brains of animals after CPET, the development of the vasculature appears to be at a more advanced stage than in age-matched control animals, as judged by the increased presence of more highly developed and larger tufts of vascular sprouts (figure 2).

These observations of enhanced development of the vasculature and of microglia after CPET may, at first sight, seem counterintuitive when considering the numerous studies indicating a general slowing of CNS development as a consequence of CPET. However, the fact that slowing of brain development concerns primarily

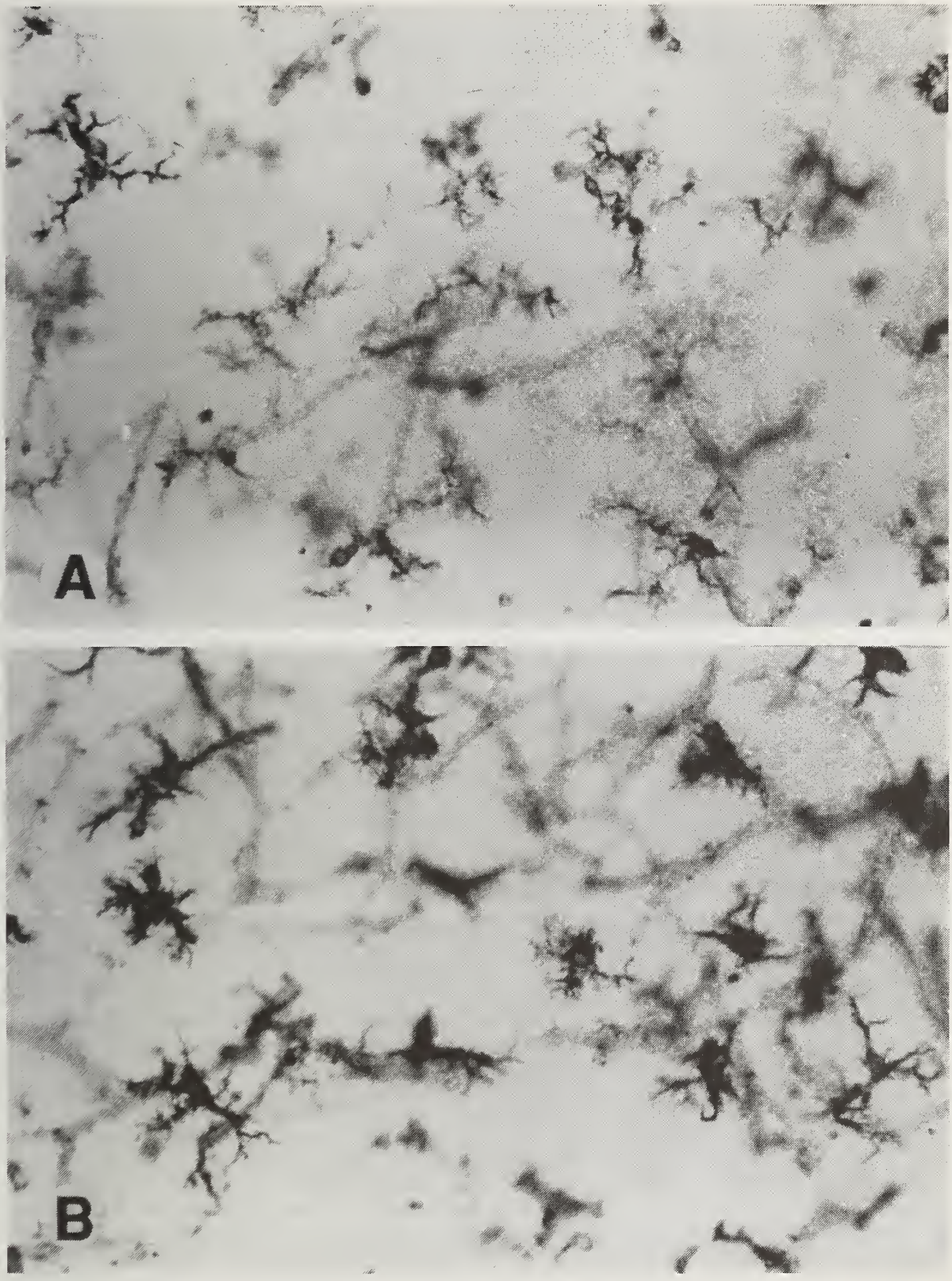

Figure 1. Lectin staining (GSA I-B_4-HRP) of microglial cells in the parietal cortex of newborn (P0) rats. Microglia appear to be better differentiated (more finely branched) in animals after chronic prenatal ethanol treatment (A) than in chow-fed control animals (B). x325.

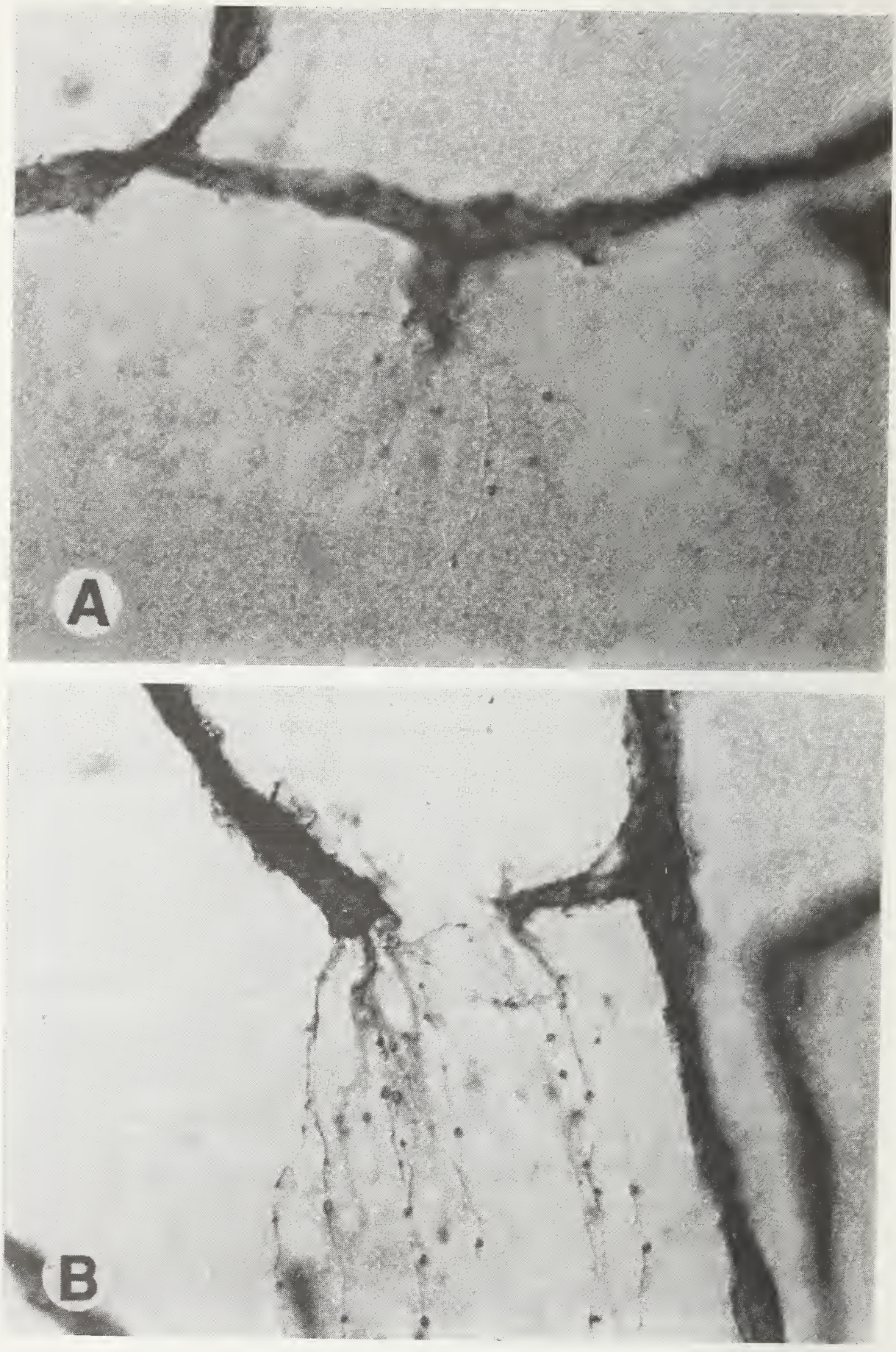

Figure 2. Lectin staining (GSA I-B_4-HRP) of vascular channels in the parietal cortex of newborn (P0) rats. Vascular tentacles are less developed in chow-fed control animals (A) than in animals after chronic prenatal ethanol treatment (B). x810.

neuroectodermal components and not mesodermally derived structures, such as the vasculature and microglial cells, provides a fundamental difference in the evaluation of developmental changes. In view of our preliminary observations, it may be justified to speculate that CPET actually accelerates the prenatal development of mesodermal CNS tissue components. The biological implications could be that enhanced vascular development may serve to increase nutrient and oxygen delivery to developing neurons suffering from the toxic effects of ethanol.

Regarding the enhanced differentiation of microglia, it is reasonable to speculate that this may be associated with a different pattern of secretory activity which could on one hand promote microglial differentiation through autocrine mechanisms, and on the other hand influence the production of astroglia-derived neurotrophic substances. Furthermore, neurotrophic influences coming directly from microglia might be attenuated during fetal alcohol syndrome, whereas neurotoxic effects could be increased. Thus, a decrease in microglial neurotrophic action occurring concomitantly with increased microglial neurotoxicity could aggravate direct ethanol neurotoxicity.

It is apparent that a thorough investigation of these (still hypothetical) effects of ethanol will require an analysis of microglial secretory substances. As in other models of neuropathology described earlier, a two-pronged approach using in vitro and in vivo studies seems warranted. Microglial cells derived from animals with fetal alcohol syndrome could be cultured and their secretory activity, in terms of neurotrophic and neurotoxic actions, compared with that of microglial cultures derived from untreated animals. In addition, these studies should be supported by an in vivo analysis of microglial cytokines and growth factors which may be detectable at the mRNA level using in situ hybridization.

CONCLUSIONS: THE MICROGLIAL IMMUNE NETWORK OF THE CNS

From the examples of neuropathology described in this chapter, as well as from other examples (see Graeber and Streit 1990), a proposed role of microglia as indigenous immunocompetent cells of the CNS seems justified. The assignment of such functions as phagocytosis, antigen presentation, and tumor cytotoxicity to microglia is likely to represent only the beginning of what promises to be an array of immunological activities carried out by these cells. A challenge for the immediate future will be to devise methods for the reliable and reproducible demonstration of microglial secretory molecules in situ—to add another degree of sophistication to histochemical investigations in order to relate in vitro data on the release of cytokines/growth factors with a given neuropathological circumstance.

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Chapter 4

Effects of Alcohol on Glial Cell Development In Vivo: Morphological Studies

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Fetal alcohol syndrome (FAS), due to maternal ethanol consumption during gestation, is characterized by growth deficiencies; a characteristic pattern of facial malformations; and behavioral, learning, sensory, and motor dysfunctions indicative of developmental brain damage (Driscoll et al. 1990; Jones et al. 1973; Streissguth et al. 1978). Among the most distressing features of FAS are the central nervous system (CNS) dysfunctions (Driscoll et al. 1990; Streissguth et al. 1978) and the fact that CNS pathology and mental retardation may be present without the more obvious external abnormalities (Clarren et al. 1978; Peiffer et al. 1979; Streissguth et al. 1991). Many of the detailed alterations of nervous system structure and function caused by developmental alcohol exposures have now been described in both humans and experimental animals (Driscoll et al. 1990; Riley et al. 1986; Streissguth 1986).

An understanding of the mechanisms by which alcohol alters the development of the CNS and the cellular basis of those alterations is necessary and important to understand the specific vulnerability of the nervous system and its individual parts to alcohol, to extrapolate results from animal studies to human implications (and the possible development of after-the-fact treatments), and to better appreciate the basic roles of each cell type in the response of the developing CNS to toxic compounds. It is important to understand the effects of alcohol not only on the development of nerve cells, but also on the development of the supporting glial cells and the elaborations of those cells, such as myelin and limiting membranes, which are critical for the normal development of CNS function. Glial cells in the developing CNS are crucial in such roles as providing a framework for the forming nervous system, assisting in guidance of neuronal migration, producing myelin, producing trophic factors crucial for development, and contributing to the maintenance of ion, pH, and neurotransmitter homeostasis within the CNS.

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EVIDENCE OF GLIAL CELL INVOLVEMENT IN DEVELOPMENTAL BRAIN DEFECTS DUE TO ALCOHOL EXPOSURE

Although numerous experimental studies have examined the effects of alcohol on neuronal development (reviewed in Miller 1992; Pentney and Miller 1992; West and Goodlett 1990), potential effects on glial development (reviewed in Phillips 1992) have not been as well studied. Studies of both humans and experimental animals provide evidence that alterations of glial development may contribute to the effects of alcohol on the developing brain.

Postmortem studies of CNS tissues from human FAS victims, although limited, have consistently provided evidence of abnormal glial placement, primarily associated with the meninges (meningeal neuroglial heterotopia) (Clarren 1986; Clarren et al. 1978; Peiffer et al. 1979; Wisniewski et al. 1983); in some cases, reactive gliosis has also been noted (Peiffer et al. 1979; Wisniewski et al. 1983). Similar glial heterotopias have been reported in a primate model of FAS (Clarren and Bowden 1984) but have not been reported in experimental studies of laboratory rodents exposed to alcohol during development. White matter areas are smaller in brains of FAS victims examined postmortem (Clarren et al. 1978; Clarren 1986; Peiffer et al. 1979; Wisniewski et al. 1983) and in brains of FAS patients examined with magnetic resonance imaging (Mattson et al. 1992). Such decreases in white matter areas indicate potential for alcohol-induced delays or changes in glial and myelin development, as well as in axon development.

Studies of experimental animals exposed to alcohol during development have provided evidence of glial involvement in alcohol-induced CNS alterations. Effects on myelin acquisition and maturation (Hofteig and Druse 1978; Lancaster et al. 1984; Phillips 1989; Phillips et al. 1991; Samorajski et al. 1986) could be caused by alcohol-induced effects on myelin-producing oligodendroglial cells. Alcohol-induced abnormalities in neuronal migration (Miller 1986, 1993) could relate to abnormal development of radial glia, which are important in normal neuronal migration (Rakic 1985). The most dramatic reductions in brain size occur in experimental animals when alcohol exposures are provided during the brain growth spurt (Bonthius and West 1990; West 1987), a period of development when rapid glial proliferation and maturation and the acquisition of myelin account for most of the increase in brain size (Dobbing and Sands 1979), thus implicating effects on those processes. The recruitment of glial cells is known to be extremely sensitive to undernutrition (Clos et al. 1991), and since many of the behavioral and structural effects of developmental alcohol exposures are similar to those of undernutrition (Wiggins 1986), it is logical to expect that alcohol could exert effects on glial recruitment.

Despite the evidence from the studies cited here, there have been relatively few in vivo studies designed to explicitly characterize the effects of alcohol on the morphological maturation of glial cells or other cells in their lineages. Rather, the majority of studies have used in vitro models to characterize the response of developing glial cells, particularly astrocytes, to alcohol.

IN VITRO STUDIES OF THE EFFECTS OF ALCOHOL ON DEVELOPING GLIA

Developing Astrocytes in Culture

The responses of developing astrocytes in culture are only briefly summarized here, as chap-

ters 1, 6, 7, and 8 discuss the specifics in more detail. Most of the in vitro studies have examined the responses either of cells directly exposed to alcohol in vitro or of cells cultured from newborn rats that were exposed to alcohol during gestation (via maternal exposure). Both types of alcohol exposures have generally caused similar effects, most often either delays in the production of specific proteins or delays in astrocytic proliferation and/or maturation.

More specifically, exposures to alcohol have been reported to cause the following effects in cultured, developing astrocytes: delays in the morphological maturation of the cells (Davies and Cox 1991; Davies and Ross 1991); decreases in either DNA synthesis or cell proliferation (Davies and Cox 1991; Davies and Vernadakis 1984; Guerri et al. 1990; Kennedy and Mukerji 1986*b*; Renau-Piqueras et al. 1988); generalized decreases in protein synthesis (Davies and Vernadakis 1984; Guerri et al. 1990; Kennedy and Mukerji 1986*a*, 1986*b*; Renau-Piqueras et al. 1988); decreases in the accumulation of glial fibrillary acidic protein (GFAP) (Renau-Piqueras et al. 1989), the intermediate filament protein specific for astrocytes; decreases in membrane-bound enzymes (ATPases and 5'-nucleotidase) (Guerri et al. 1989; Renau-Piqueras et al. 1988); and decreases in the astrocyte-specific marker enzyme, glutamine synthetase (GS) (Davies and Vernadakis 1984; Guerri et al. 1989; Kennedy and Mukerji 1986*a*; Renau-Piqueras et al. 1988).

Because cells grown in culture following gestational exposures to alcohol exhibit similar effects as cells exposed to alcohol in vitro, it is possible that the effects of alcohol on the prenatal astrocyte progenitor cells influence astrocyte maturation as much as direct insult to the maturing cells (Guerri et al. 1990). Not all studies of cultured astrocytes have produced the same results, however, and there are reports that alcohol does not affect DNA synthesis (Bass and Volpe 1988), GFAP concentration (Chiappelli et al. 1991; Lipsky et al. 1988), or GS activity (Chiappelli et al. 1991). It has also been reported that in vitro exposure of cultured immature astrocytes can cause an increase in mRNA for GFAP (Fletcher and Shain 1993).

Developing Oligodendroglia in Culture

There have been fewer studies of alcohol-exposed cultured oligodendroglia than of cultured astrocytes. Both types of in vitro experiments, culture of cells exposed to alcohol during gestation and direct exposure of developing cells to alcohol in vitro, indicate that developing oligodendroglia are more sensitive to ethanol insult than developing astrocytes (Chiappelli et al. 1991; Davies and Cox 1991).

Maturation of oligodendroglia was delayed in mixed glial cell cultures derived from newborn rats exposed during gestation, as evidenced by cell counts of galactocerebroside-stained cells and by a delay in the appearance of process-bearing cells (Chiappelli et al. 1991). Delays in the accumulation of transferrin and myelin basic protein also indicated alcohol-induced delays in the early production of myelin. There was also some evidence of cellular degeneration of oligodendroglia in the same study (Chiappelli et al. 1991). A recent preliminary report indicated that cell cultures from the hippocampus, cerebral cortex, and brain stem of similarly exposed rats contained fewer oligodendroglia, as revealed by galactocerebroside staining (Aquilino and Cianci 1992). Because oligodendroglia are generally not present until after birth, these results indicate that the effect of alcohol, in these instances, could be on the prenatally existing glial progenitor cells.

When mixed glial cell cultures from newborn animals were directly exposed to alcohol

for 56 days, the effects were similar to those caused by prenatal exposures (Davies and Ross 1991). There was a reduction in oligodendroglial acquisition, which the authors attributed to either a selective vulnerability of the maturing oligodendroglia or a paucity of mitogenic factors derived from abnormally late developing astrocytes (Davies and Cox 1991). These results imply that alcohol can also directly affect cells present in cultures of postnatal cells, whether the cells are still-present progenitor cells or differentiating and maturing oligodendroglia.

EXPOSURE MODELS FOR STUDYING GLIAL DEVELOPMENT IN VIVO

Glial cell proliferation (especially oligodendroglia), most of glial cell maturation, and the early production of myelin occur during the most rapid phase of the brain growth spurt. This dramatic increase in brain mass occurs during the third trimester in humans and during the first 7–10 postnatal days in the rat (Dobbing and Sands 1979; Wiggins 1986). Thus, any exposures of rats to alcohol that are intended to parallel the exposure of the developing CNS during all of human gestation or that are intended to influence the proliferation and maturation of glial cells should include the first 10 postnatal days, as well as gestation. Lactational exposures of postnatal animals to alcohol are confounded by the possibility of undernutrition because alcohol causes both an alteration in the feeding behaviors of the pup and decreases in the maternal production of milk (Swiatek et al. 1986). Most other methods of exposing the pups to alcohol (e.g., gavage, vapor inhalation, and injection) are also complicated by the probability that the exposed animals will not nurse properly.

The use of artificial rearing of postnatal animals minimizes the problems of controlling for malnutrition because all diet constituents and fluids are isocalorically pair fed to alcohol-exposed and control animals (Phillips 1992; West et al. 1984). Both groups are exposed in identical fashion to the additional variables of an artificial diet and an isolated rearing environment. West and his colleagues (Bonthius and West 1990; Goodlett et al. 1990; West et al. 1989) have further refined artificial rearing methods so that the pattern and timing of the postnatal alcohol exposures can be controlled to produce relatively high blood alcohol concentrations for relatively short times. Such "binge-like" alcohol exposures can be more harmful to CNS maturation than higher daily doses delivered in a more chronic fashion, and have been used to define the vulnerability of specific neuronal populations in the rat cerebellum (Bonthius and West 1990; Goodlett et al. 1990; West et al. 1989). Such methods, coupled with specific immunocytochemical labeling related to time-dependent developmental events, such as the proliferation or maturation of specific cells or cell elaborations, offer a useful opportunity to further characterize the mechanisms by which alcohol affects glial development.

EFFECTS OF ALCOHOL ON MYELIN DEVELOPMENT

It is logical to suspect that damage to myelin or to the oligodendroglia that produce myelin contributes to the functional brain damage associated with developmental alcohol exposures. Myelination is known to be a particularly vulnerable period of brain development (Wiggins 1986), and many of the neurological and behavioral abnormalities reported in FAS (Driscoll et al. 1990; Streissguth et al. 1978) are similar to those caused by retarded myelin development following malnutrition (Cravioto and DeLicardie 1975; Wiggins 1982).

Postmortem examinations of CNS tissues from FAS victims have shown that in some

cases CNS white matter structures, including optic nerve (Coulter et al. 1993), were abnormally small (Clarren 1986; Clarren et al. 1978; Peiffer et al. 1979; Wisniewski et al. 1983). These results provide indirect evidence of smaller or fewer myelinated nerve fibers, but detailed studies of myelin development in such human tissues have not been possible.

Biochemical studies to analyze the accumulation of myelin-specific molecules have most often used whole-brain myelin samples from laboratory rodents after alcohol exposures limited to prenatal development. Such exposures occur before myelination and even before the rapid wave of oligodendroglial proliferation that precedes myelination (Tennekoon et al. 1980). Thus it is difficult to interpret the varying results. Subtle, yet specific abnormalities and delays in the biochemical profile of myelin (in near-normal levels of total brain myelin) have been reported following prenatal exposures to alcohol (Druse 1981; Druse and Hofteig 1977; Gnaedinger and Druse 1984; Gnaedinger et al. 1984; Hofteig and Druse 1978). A more obvious 30 percent lag in myelin protein synthesis was demonstrated in postnatal day 16 (P16) rats prenatally exposed to alcohol, but by P30 the rate of synthesis had recovered (Lancaster et al. 1982). The effects were much less apparent, however, when nutritional controls were provided by cross-fostering, implying that the overall effects of the prenatal exposure were minimal (Lancaster et al. 1984). However, when myelin samples were taken from localized regions of the brain on P29 after prenatal exposures, myelin protein concentrations were decreased in the brain stem, but not in the cerebrum or cerebellum (Lancaster et al. 1989). It seems probable that the whole-brain myelin samples used in many biochemical studies could mask any regional vulnerability of myelin development to alcohol and yet produce the subtle but not statistically significant differences often reported. Such regional variations could be related to differences in the timing of the exposures relative to different times of myelin maturation in different CNS regions.

Postnatal alcohol exposures, provided by lactation during the time of most active myelination in much of the brain, caused severe decreases in whole-brain myelin concentrations at 15–52 days, compared with cross-fostered control animals. These postnatal effects were much more dramatic than those from prenatal exposures in the same experiment (Lancaster et al. 1984).

We used morphological methods to examine the development of myelin and glial cells in the rat optic nerve during and following a full three-trimester–equivalency exposure (Phillips et al. 1991). The exposure involved gestational exposure to alcohol in a pair-fed liquid diet followed by postnatal exposure to alcohol (of the artificially reared pups) in a gastrostomy-fed liquid diet provided on P1–10. In these studies alcohol exposure produced the following effects in rat optic nerve:

- Optic nerve cross-sectional areas were generally smaller from gestational day 20 (G20) through P90.
- There was a delay in myelin acquisition at P10 and P15 (as measured by the number of mature myelinated fibers per unit area) that was compensated for at P20 and P90 (figure 1).
- Myelin thickness relative to axon diameter was decreased up to 15 percent at P10–P20 and, permanently (by approximately 10 percent), at P90 (figure 1).

When the numbers of nerve fibers in various stages of myelination (initiation of contact, enwrapment, addition of spirals, partial compaction, complete compaction) were counted, the numbers of fibers in the intermediate

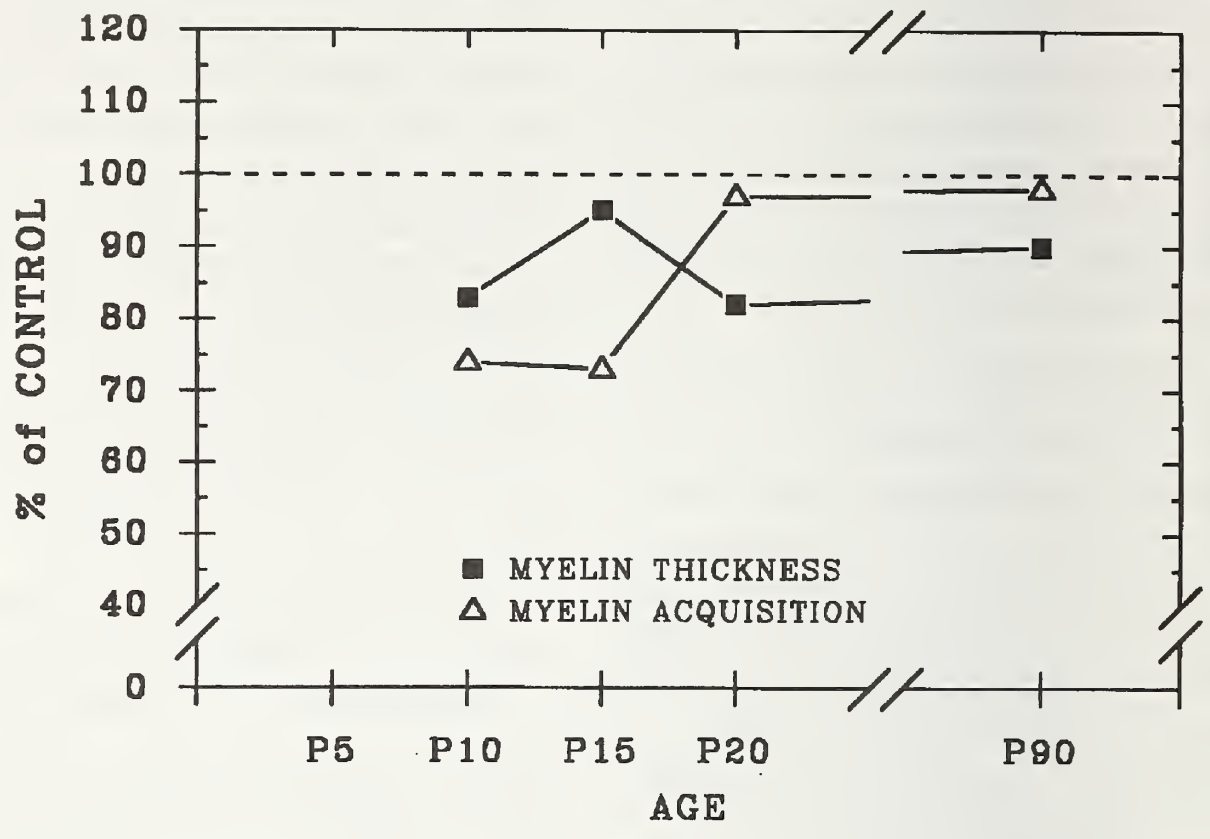

Figure 1. Effects of a three-trimester–equivalency alcohol exposure on the acquisition of myelin and on myelin thickness in rat optic nerve, expressed as percentage of control. The myelin acquisition data represent counts of the number of mature myelinated nerve fibers per unit area. The myelin thickness data are based on averages of regression lines of myelin thickness plotted as a function of axon diameter. There were alcohol-induced delays in myelin acquisition and decreases in myelin thickness.

stages of myelination were not affected by alcohol (figure 2).

These findings indicate that the delay in the acquisition of mature myelin was due to a delay in the initiation of myelination and not in the progression of myelination through enwrapment and compaction. Axon diameters were consistently less in alcohol-exposed animals during development, although statistically significant only at P5. The implication is that decreased axonal size could contribute to the delay in the initiation of myelinization, since the triggers for myelin acquisition are related to axon size (Matthews and Duncan 1971). The finding of permanent alterations in myelin thickness following developmental alcohol exposure is particularly notable because so many other structural effects of developmental alcohol exposures represent delays, not long-term alterations.

Studies of optic nerve tissues from artificially reared animals exposed for a limited third-trimester–equivalency exposure (on P5–9) produced similar findings in terms of myelin acquisition (Phillips 1989). Thus, the effects in the full three-trimester–equivalency exposure must be mostly due to the postnatal exposure. This implies that the greatest effects of alcohol, in terms of myelination, are either on the interactions between axons and oligodendroglia that signal the rapid acquisition, maturation, and metabolism of oligodendroglia required for myelination (Barres and Raff 1993; Goto et al. 1990; McPhilemy et al. 1990) or on the proliferation and maturation of the

oligodendroglia or their precursor cells that are present postnatally.

We found similar alcohol-induced delays in myelin acquisition and decreases in myelin thickness in the spinal cord corticospinal tract of full three-trimester–equivalency exposed rats (Phillips unpublished data). The same effects were not present in the adjacent dorsal column general sensory fibers. Myelin thickness relative to axon diameter was permanently decreased by approximately 10 percent at P90 for some axon diameters in the corticospinal tract, but not in the general sensory tract. These findings again point to the possibility of selective vulnerability of myelin. This selective vulnerability might be related to differences in the cells that give rise to the axons or to differences in the timing of development, since the sensory tract myelinates much earlier than the corticospinal tract (Matthews and Duncan 1971).

EFFECTS OF ALCOHOL ON OLIGODENDROGLIAL DEVELOPMENT

Oligodendrocytes form later in development than the majority of astrocytes, and develop from radial glia (Choi et al. 1983; Hirano and Goldman 1988), from glioblasts (Phillips 1973; Skoff 1990; Skoff et al. 1976*a*; Vaughn 1969), or from a common O-2A progenitor cell that

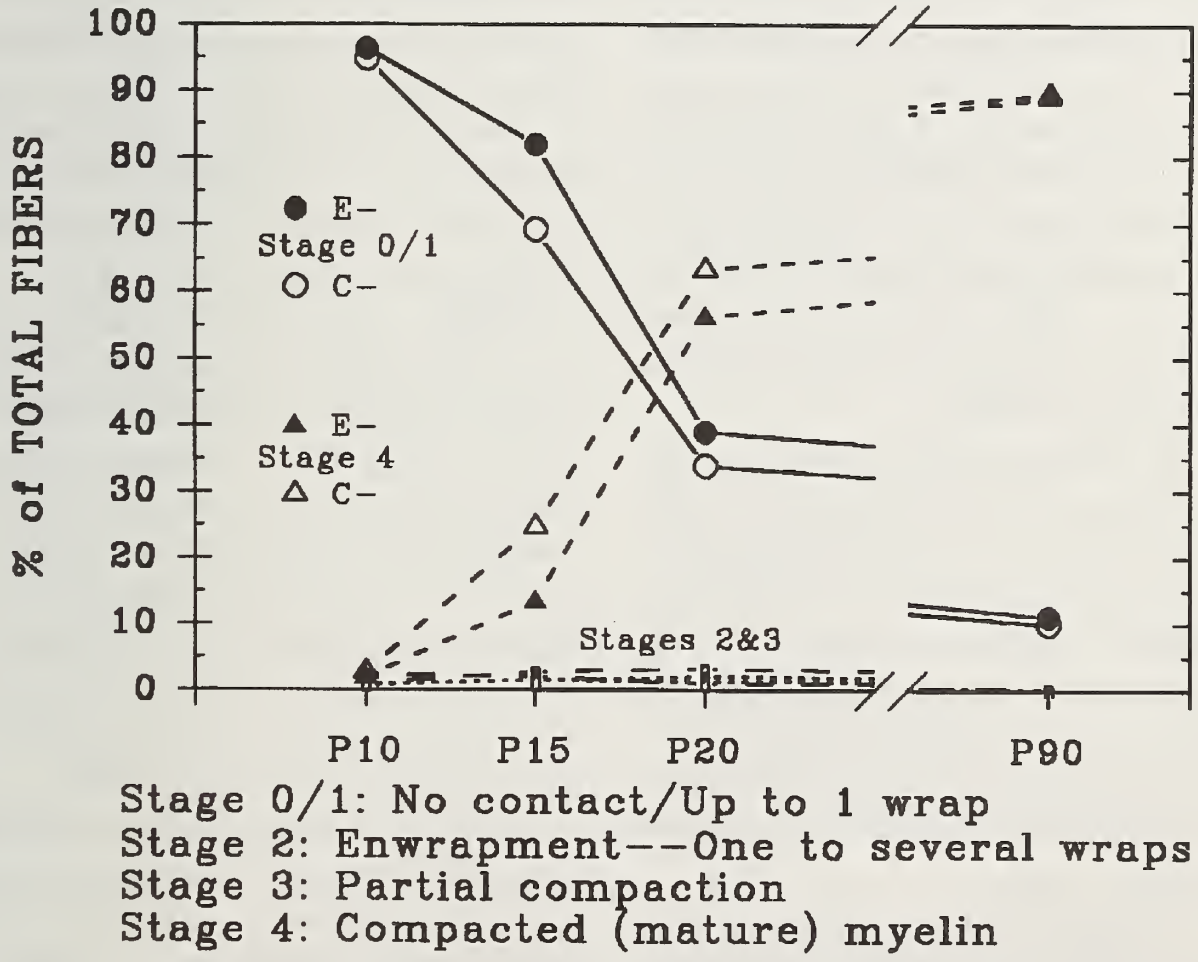

Figure 2. Effects of a three-trimester–equivalency alcohol exposure on progressive stages of myelin development in rat optic nerve for P10–P90. There were alcohol-induced delays in the loss of unmyelinated fibers and in the acquisition of mature myelin, while intermediate stages of enwrapment and compaction were not affected. E = ethanol-exposed. C = control.

has been reported to give rise to a second, late wave of astrocyte formation (Raff 1989). The primary wave of oligodendroglia formation is related to the development and maturation of axons that require myelin ensheathment (Phillips 1973; Skoff 1990; Skoff et al. 1976*a*; Tennekoon et al. 1980; Vaughn 1969).

In our electron microscopic studies (Phillips and Krueger 1990, 1992), we examined samples of tissue from the optic nerve and typed the glial cells as microglia, glioblasts, immature oligodendroglia, active oligodendroglia, mature oligodendroglia, immature astrocytes, or mature astrocytes, using the morphologies characterized in other studies of developing CNS white matter (Phillips 1973; Skoff 1990; Vaughn 1969). The percentage of each cell type within each optic nerve sample was determined from the electron microscopic studies, and the total number of glial nuclei per cross section was determined from plastic section light microscopic counts. Calculations of projected numbers of each glial cell type per optic nerve cross section were based on the percentage of each cell type and the number of all glial nuclei per cross section.

The full three-trimester–equivalency alcohol exposure resulted in fewer glial cell nuclei per cross section on G20–P90 (Phillips 1992; Phillips and Krueger 1992), which generally paralleled a reduction in optic nerve cross-sectional area. Thus, the number of glial cells per unit area was relatively unchanged. No evidence of alcohol-induced cellular degeneration was found in axons, myelin, or any of the glial cell types.

Microglia are generally considered to be the actively phagocytic cells of the CNS and are most typically described as arising early in development from exogenous mesodermal or vascular sources (Boya et al. 1987; Polak et al. 1982), although some studies have suggested a neuroectodermal origin (Kitamura et al. 1984). The number of microglia varies from a scattered few in white matter tracts like the optic nerve (Ling and Leblond 1973; Skoff et al. 1976*a*) to dramatic numbers arising in response to cellular degeneration or pathology in the CNS (Streit et al. 1988).

Microglia were identified by their morphological characteristics: a relatively dense, small, and angular or elongate nucleus; a relatively electron-dense cytoplasm; elongated, thin strands of rough endoplasmic reticulum (RER); and the frequent presence of dense bodies or fat droplets in the cytoplasm (Phillips 1973; Phillips and Krueger 1990; Vaughn 1969). We found few microglial cells in the optic nerve between G20 and P90. The proportion of microglia was generally from 0.5 to 2.0 percent of the total number of glial cells characterized and was never more than 5 percent of the cells in any alcohol-exposed or control animal. The small numbers of microglia verify that there was no obvious alcohol-induced pathology or degeneration.

Glioblasts, characterized by relatively large, pale nuclei; a thin rim of cytoplasm; and only scattered ribosomes and mitochondria (Skoff et al. 1976*b*; Vaughn 1969), were present in small numbers in the tissues examined on P5–P20. Compared with control animals, there were generally fewer glioblasts identified at P5 in experimental animals and more from P10 to P20, as one would expect if there were a generalized delay in one or more of the glial lineages. Glioblasts were not found at P90.

Immature oligodendroglia (light oligodendroglia) had a somewhat pale staining nucleus with scattered chromatin, a relatively small amount of electron-lucid cytoplasm, obvious ribosomes, numerous microtubules, and obvious aggregates of Golgi apparatus. Short cisternae of RER contained material similar in density to the cytoplasm. Immature oligodendroglia were

present in similar proportions in the exposed and control tissues from P5 to P20.

Active oligodendroglia, the cells generally considered to be involved in the early development of myelin (Tennekoon et al. 1980; Vaughn 1969), were characterized by dense cytoplasm with obvious stacks of RER containing electron-lucid material; obvious microtubules, especially at the base of processes; numerous ribosomes; and usually several, easily distinguished cell processes per cell body, which occasionally could be traced to continuity with developing myelin. Active oligodendroglia were most common at P10 and P15 when they constituted the majority of the cells in the oligodendroglial lineage. Because of a continuous spectrum of transitional cells, it was impossible to always distinguish active oligodendroglia from mature oligodendroglia. Consequently, the two categories were grouped together for statistical analysis.

The mature oligodendroglia had many morphological features similar to the active cells, except that the stacks of RER were far less complex or apparent and the cell processes were not easily visualized. Obvious mature oligodendroglia were not present at P10, were only occasionally present at P15, were common at P20, and were the only type of oligodendroglia consistently encountered at P90. Active and mature oligodendroglia, as a combined group, were generally fewer in the alcohol-exposed animals, especially at P10–P20 (figure 3), but by P90 the numbers of oligodendroglia were similar to control values. It is noteworthy that in some alcohol-exposed

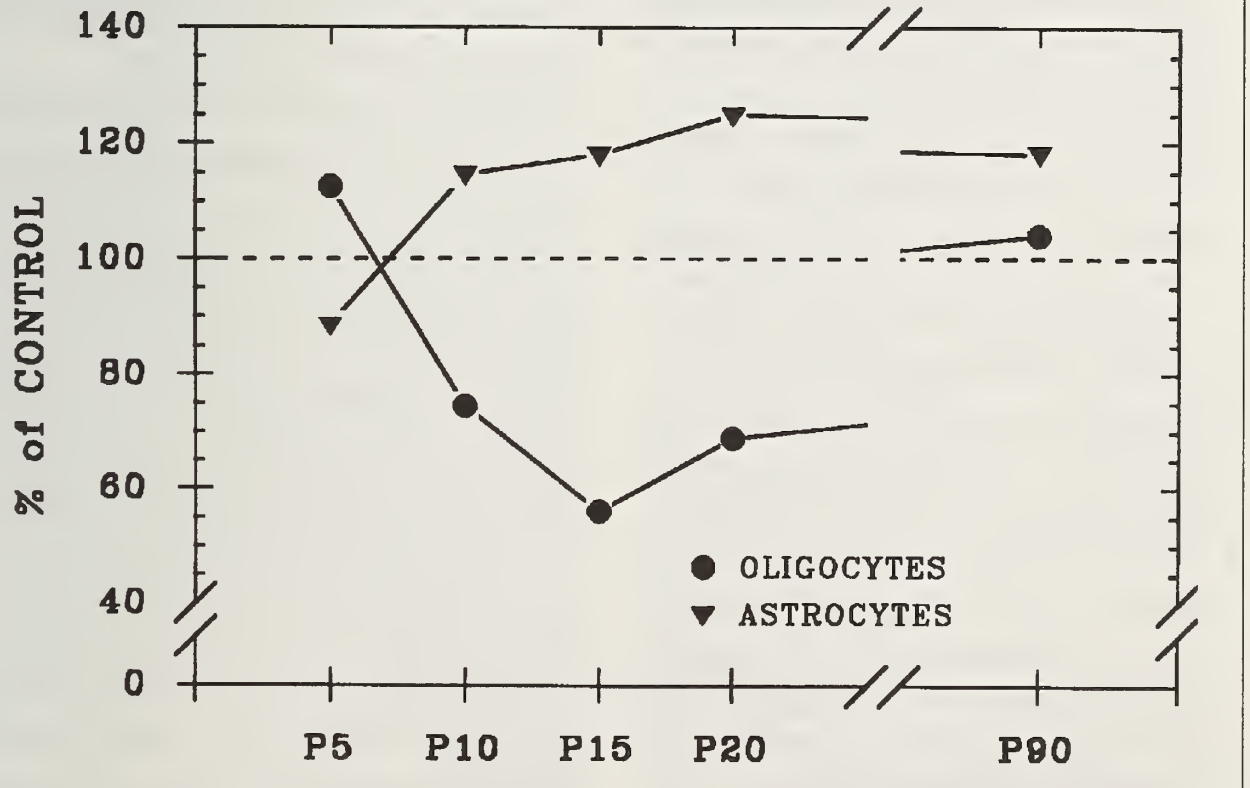

Figure 3. Effects of a three-trimester–equivalency alcohol exposure on the number of glial cells per optic nerve cross section at various ages, based on electron microscopic studies. There was a marked alcohol-induced decrease in the mean number of all types of oligodendroglia in the lineage at P10–P20, which was compensated for at P90. There was also an alcohol-induced increase in the mean number of astrocytes.

animals there were reductions of 70 percent or more on P10 and P15. Thus, the full three-trimester–equivalency alcohol exposure caused a delay in the maturation of cells within the oligodendroglial lineage and a reduction in the number of cells present in that lineage during the times of most active myelin formation.

A study using only a more limited postnatal exposure to alcohol (on P5–9) produced similar findings in terms of oligodendroglial development in the optic nerve (Phillips and Krueger 1990), indicating that, as with myelin, the third-trimester equivalency was the most crucial time for the effects on oligodendroglia.

There are obviously numerous potential mechanisms by which alcohol could affect oligodendroglial development; for example:

- Alcohol could exert toxic effects on developing or differentiating oligodendroglia that would cause oligodendroglial degeneration and thus affect myelin formation or maturation.
- Alcohol could affect progenitor cells, either by delaying proliferation or by altering their long-term metabolic potential, thus delaying the acquisition of oligodendroglia and minimizing their ability to produce normal amounts of myelin.
- Alcohol could exert effects on the cellular metabolism of oligodendroglia essential for maturation and normal myelination.
- Alcohol could exert effects on axons that could in turn alter the signals between axons and oligodendroglia necessary for normal oligodendroglial formation, differentiation, migration, or metabolism required for normal myelination.

Our findings (Phillips and Krueger 1990, 1992) argue against any toxic effects dramatic enough to produce degeneration. Because the effects of prenatal exposure in the full three-trimester–equivalency exposure add little to the effects from an isolated third-trimester–equivalency exposure, it appears that the greatest effects of alcohol are on the postnatally existing oligodendroglia or their immediate precursors present between P5 and P10, and not on prenatally existing progenitor cells. It appears that the alcohol somehow alters the cells' metabolism and thus their potential to produce enough cells at the appropriate time, to mature at a normal rate, and to produce myelin at the appropriate time and of the appropriate thickness. The exact roles of axon development, in terms of stimulating oligodendroglial proliferation and maturation, are still being defined (Barres and Raff 1993; Goto et al. 1990; McPhilemy et al. 1990), but alterations of axon maturation certainly could be involved, particularly since axon maturation or function can be affected by alcohol exposures (Kjellstrom and Conradi 1993; Miller and Al-Rabiai in press; Phillips et al. 1991; Stephens 1992).

Further studies need to be done to understand what steps of oligodendroglial development are affected by alcohol. Such studies need to examine specific events, including precursor proliferation; oligodendroglial acquisition, migration, maturation, and metabolism; oligodendroglial metabolism required to produce normal myelin; and oligodendroglial-axon interactions. One approach might be to define the specific temporal vulnerabilities of oligodendroglial and myelin development in systems where the exact details of normal development are well defined and then to relate the temporal vulnerabilities to known developmental events occurring at those times. In vivo studies must involve tissues, such as optic nerve or corpus callosum, in which the details of oligodendroglial origin, proliferation, maturation, and aging are well defined and in which the organization of the tissue morphology lends itself to quantification.

Implications of alcohol-induced alterations in oligodendroglial acquisition and maturation are far-reaching, mostly because of the effects on myelin acquisition or myelin thickness. If action potential patterns are altered by delays in myelin development, then secondary neuronal elements in a pathway could be affected, since alterations of action potential input affect neuronal development (Shatz 1990). Alterations in nerve fiber conduction rates due to thinner myelin could alter the communication within the CNS, potentially causing slower response times. Such alterations could indirectly or directly change sensory and motor processing in the CNS and even influence higher functions such as memory and learning.

EFFECTS OF ALCOHOL ON ASTROCYTIC DEVELOPMENT

The first functional astrocytes to differentiate from the primitive neuroectoderm are radial glia (Edwards et al. 1990; Hockfield and McKay 1985), which extend from the neuroectoderm to form the primitive external boundary layer of the developing neural tube (Choi 1988; Edwards et al. 1990; Vanselow et al. 1989). These same radial glia form guides during development for the migration of neurons, a phenomenon best documented in the cerebellar and cerebral cortices (Rakic 1985, 1988).

Astrocytes (type 1) form relatively early in development (G16 in rat optic nerve) from either radial glia (Choi et al. 1983; Hirano and Goldman 1988; Pixley and de Vellis 1984; Skoff 1990) or glioblast precursor cells (Phillips 1973; Raff 1989; Skoff 1990; Skoff et al. 1976*a*; Vaughn 1969). The early-arising astrocytes are considered to be the cells that form end feet at the surface of the CNS and on vessels, as well as filling the interstices of much of the nervous system (Raff 1989). Type 2 astrocytes are characterized as developing at a much later time (P14 in rat optic nerve) from an O-2A progenitor cell that has been described as common to both oligodendroglia and type 2 astrocytes (Raff 1989). Type 2 astrocytes have been localized mostly in myelinated fiber tracts and have end feet associated with the bare areas of myelinated nerve fibers (Raff 1989). The evidence for two types of astrocytes, each forming at different stages of development, is disputed by evidence that almost all astrocytes can form early in development, before oligodendroglia (Skoff 1990; Skoff and Knapp 1991).

We examined the morphological development of astrocytes in rat optic nerve following developmental alcohol exposures, using the same full three-trimester–equivalency exposure (Phillips and Krueger 1992) and the more limited third-trimester–equivalency exposure (Phillips and Krueger 1990) described previously. As described earlier, cells were identified by their electron microscopic characteristics as to specific type, then projected numbers of each cell type per optic nerve cross section were calculated.

Mature astrocytes were characterized in the optic nerve by a round-to-oval nucleus with evenly distributed chromatin; relatively electron-lucid cytoplasm; typical bundles of astrocytic intermediate filaments; some glycogen; and short, slightly dilated profiles of RER with a flocculent, slightly electron-dense content. Immature astrocytes were similar in appearance except that there was slightly less cytoplasm, microtubules were more common, and filaments were less common and more randomly arranged in the cytoplasm, rather than in bundles.

Again, as with the oligodendroglia, there was no consistent indication that the three-trimester–equivalency exposure to alcohol caused any degeneration of astrocytes or their

organelles. Immature astrocytes were more common in less mature animals (P5–P15) in both alcohol-exposed and control animals, with somewhat more immature cells in experimental animals than in control animals (Phillips and Krueger 1992). The proportion of mature astrocytes decreased with aging in control animals, from near 65 percent of all cells at P5 to near 25 percent at P90. The average projected number of all astrocytes per cross section was increased by approximately 15 percent in alcohol-exposed animals compared with control animals. Although the differences were consistent, they were generally only marginally significant statistically ($p \leq 0.10$) (figure 3). When considered in the context of the smaller nerve at those times, alcohol caused an increase of 30–50 percent in the number of astrocytes per unit area.

After the more limited third-trimester–equivalency exposure, the same trend toward increased numbers of astrocytes was present, but it was much more subtle and did not approach statistical significance.

Astrocytic Gliosis as a Feature of Developmental Alcohol Exposures

Reactive gliosis, the hypertrophy and hyperplasia of astrocytes, is the normal response of the CNS to invasive injury, cell death, and degeneration of nerve terminals (Malhotra et al. 1990), or it can occur as a response to the lack of development of a specific nerve cell population (Martinez Garcia et al. 1991). Evidence that developmental alcohol exposures can cause astrogliosis includes, from our studies, increased numbers of astrocytes in optic nerve, as determined by electron microscopic counts (Phillips and Krueger 1990, 1992); increased numbers of astrocytes in the oculomotor nucleus (Burrows 1992), superior colliculus (Wall and Phillips 1993), and spinal cord corticospinal tract (Phillips unpublished data), based on plastic section light microscopic counts; and hypertrophy of GFAP-stained astrocyte processes in both gray and white matter of the developing cerebellum (Shetty and Phillips 1992).

Studies of the developing rat cerebral cortex have shown increased amounts of GFAP-stained astrocytes, both after prenatal exposures (Miller and Robertson 1993) and after binge-like postnatal exposures (Goodlett et al. 1993). Binge-like postnatal exposures can also cause an increased expression of GFAP messenger RNA (mRNA) in the cerebral cortex (Fletcher and Shain 1993). In all the studies looking at GFAP staining or GFAP mRNA expression (Fletcher and Shain 1993; Goodlett et al. 1993; Miller and Robertson 1993; Shetty and Phillips 1992), the increases indicative of gliosis are short term or transient and do not appear to persist.

The mechanisms by which developmental alcohol exposures cause astrogliosis and the role of that gliosis in other developmental disruptions need to be further elucidated. Studies that have examined both radial glia and astrocyte maturation (Gressens et al. 1992; Miller and Robertson 1993) suggest that alcohol accelerates radial glial transformation to astrocytes and may accelerate the generation of astrocytes from stem cells. Such effects have the potential of creating a temporary gliosis, while at the same time contributing to the delayed or altered neuronal migration reported in the cerebral cortex (Miller 1986, 1993). Vascular disruption that occurs after high blood alcohol concentrations has also been implicated as a potential trigger for the gliosis (Goodlett et al. 1993), because such disruptions could allow excessive amounts of circulating mitogens to influence the astrocytes (Norton et al. 1992). The studies of GFAP mRNA suggest that alcohol could have a direct influence on transcription (Fletcher and Shain 1993). Other

possibilities include the concept that signals for gliosis could come from a toxic effect on neurons (Aschner and LoPachin 1993), a delay in the development of neuronal elements (Martinez Garcia et al. 1991), or a direct effect on the astrocytic metabolism (Renau-Piqueras et al. 1989). Further studies are needed to define more carefully the relative roles of hypertrophy and hyperplasia in the gliotic response to alcohol.

Alcohol-induced gliosis could affect the development of neural populations by altering the environment for the maturing neurons. Any effects on the relative size of the astrocyte population or its reactivity are obviously of significance to the normal functioning of the CNS because of astrocyte-neuron interactions in activities such as general metabolism (Aschner and LoPachin 1993) and the maintenance of homeostasis of ions (Goldstein and Betz 1986), neurotransmitters (Hansson and Ronnback 1990), and neurotrophic factors (Lauder and McCarthy 1986). The formation of CNS scars (Goldstein and Betz 1986) could be altered, as could the functions of astrocytes in the formation and maintenance of the glial vascular and external limiting membranes (Goldstein and Betz 1986).

Effects of Alcohol on the Development of Vessels and Related Astrocytic Specializations

It is possible that delays or alterations in the development of vessels or the related blood-brain barrier (BBB) could occur following developmental alcohol exposures. Evidence of such a possibility includes the following:

- Alcohol can induce disruptions of BBB integrity in the adult under certain conditions (Altura and Altura 1984; Oztas 1989; Phillips and Cragg 1982).
- Alterations of astrocytic maturation (possible after alcohol exposures) can delay the induction of a functional barrier in vessels (Goldstein and Betz 1986; Janzer and Raff 1987) or can modulate barrier properties by altering the relationship of astrocytes to the vessel (Hozumi et al. 1990; Krum 1991).
- Postnatal binge-like alcohol exposures can cause some vascular disruption in the cerebral cortex (Goodlett et al. 1993; West and Goodlett 1990).
- Alcohol can cause delays in vascular development in some areas of the nervous system, including the cerebral cortex (Al-Rabiai and Miller 1987), the hippocampus (Kelly et al. 1990), and the optic nerve (Phillips et al. 1991).

Any changes in vascular or BBB development could be related to abnormal astrocyte development, since the presence of astrocytes is crucial for the maturation of CNS vessels—particularly the induction of the BBB in endothelial cells (Smith and Miller 1991; Stewart and Coomber 1986)—and since barrier breakdown could allow abnormal concentrations of blood-borne mitogens to influence astrocyte development (Goodlett et al. 1993; Norton et al. 1992). It is important to determine if there are relationships between any BBB effects and the development of the astrocytic end feet that are adjacent to the endothelium and contribute to the induction of the barrier properties unique to CNS endothelium (Janzer and Raff 1987; Smith and Miller 1991).

We did some studies of the effects of alcohol on the fine structure of developing vessels and related astrocyte processes in the G20 and P5 rat optic nerve following a full three-trimester–equivalency exposure to alcohol (Phillips et al. 1993). There was dramatically less area occupied by blood vessels in

alcohol-exposed animals at G20 (43 percent less), as well as a less dramatic reduction (that was not statistically significant) at most other time points studied, including P90 (23 percent reduction, $p = 0.09$). These findings indicate the potential for a developmental delay and/or a long-term change in the vasculature and point to the need for additional study.

There were no differences in the mean diameter of the capillaries randomly selected at either G20 or P5 in alcohol-exposed or control optic nerve tissues. There was no obvious alcohol-induced pathology or hypertrophy of the astrocytic end feet that form an essentially continuous layer surrounding the basal lamina of vessels (the vascular limiting membrane, or VLM). There was no evidence of pathology in the endothelial cells that would indicate disruption of the vessels, and endothelial tight junctions were intact and similar in number and extent in both alcohol-exposed and control animals. The vessels in both alcohol-exposed and control animals generally had a complete astrocytic ensheathment and only rare vessels, present in both experimental and control animals in similar proportions, had small extents of the basal lamina that were not covered by astrocytic processes. The extent of the incomplete ensheathment was less than 12 percent of the perimeter of the basal lamina of the involved vessels at G20 and less than 1 percent at P5. There were no consistent, statistically significant differences in the numbers of dense bodies, myelin figures, or vacuoles in endothelial cells and astrocytic end feet.

Thus, this specific developmental alcohol exposure appears to have no obvious disruptive effect on the development of white matter vasculature in the rat optic nerve, and any specific effects of alcohol on developing endothelial cells or related astrocytic end feet, if present, must be subtle. There is a need to do similar detailed studies of vessels in other locations in the CNS and after higher doses of alcohol, particularly in the cerebral cortex where vascular disruption has been shown (Goodlett et al. 1993).

EFFECTS OF ALCOHOL ON THE DEVELOPMENT OF RADIAL GLIA

Radial glia arise early in development as part of the neuroectoderm (Edwards et al. 1990; Hockfield and McKay 1985) and form the structural skeleton of the early CNS, extending from the ventricle to form the primitive external boundary layer of the developing neural tube (Choi 1988; Edwards et al. 1990; Vanselow et al. 1989). During and after neurogenesis, the radial glia are involved as guides for the translocation/migration of neurons (Rakic 1985; Shults et al. 1990). The radial glia in the cerebellum, which are known as Bergmann glia and which mature early, are involved in the outside-inward migration of external granule neurons, after which they persist as support structures within the molecular layer (Das 1976; Rakic 1985).

We used light microscopic immunocytochemical methods to examine the cerebellar vermis from P15 and P22 rats that were exposed to alcohol during gestation (Shetty and Phillips 1992). Antibody to GFAP was used to label developing Bergmann glia and astrocytes in sections of the cerebellar vermis, then three lobules of cortex were studied in detail, as well as the central core of white matter.

On P15, compared with control animals, alcohol-exposed animals had fewer Bergmann glial fibers per unit length of molecular layer (15–20 percent fewer) and 2–3 times more morphologically mature Bergmann fibers in the molecular layer (figure 4). They also had a significantly greater GFAP-positive astrocytic area

per unit area of internal granular layer (4–12 percent greater) and per unit area of central white matter (36 percent greater) (figure 5). GFAP-positive astrocyte processes were wider, more intensely stained, and more closely packed in both the internal granular layer and in the white matter of alcohol-exposed animals on P15. These glial changes were associated with a significantly thicker external granular layer in all three lobules examined at P15, indicating a probable delay in granule cell migration. No significant differences were seen between the alcohol-exposed and control animals on P22, indicating "catchup" in the alcohol-exposed animals during the third postnatal week.

These results suggest that prenatal alcohol exposure causes a delay in the maturation of Bergmann glia, which in turn could be associated with a delay in granule cell migration. Additionally, the alcohol exposure can cause alterations in the normal postnatal development of astrocytes, which includes some transitory component of overgrowth or developmental gliosis.

Similar studies of P15 animals following a full three-trimester–equivalency exposure (Shetty et al. unpublished manuscript) indicated that the exposure caused a marked delay in Bergmann glial maturation, as with the prenatal exposure. There were 16–20 percent fewer Bergmann fibers per unit length of the folia.

Electron microscopic studies of P15 cerebellum from full three-trimester–equivalency exposed animals revealed that alcohol-induced differences in the fine structure of Bergmann glial fibers in the alcohol-exposed animals were minimal and mostly related to the shape of the

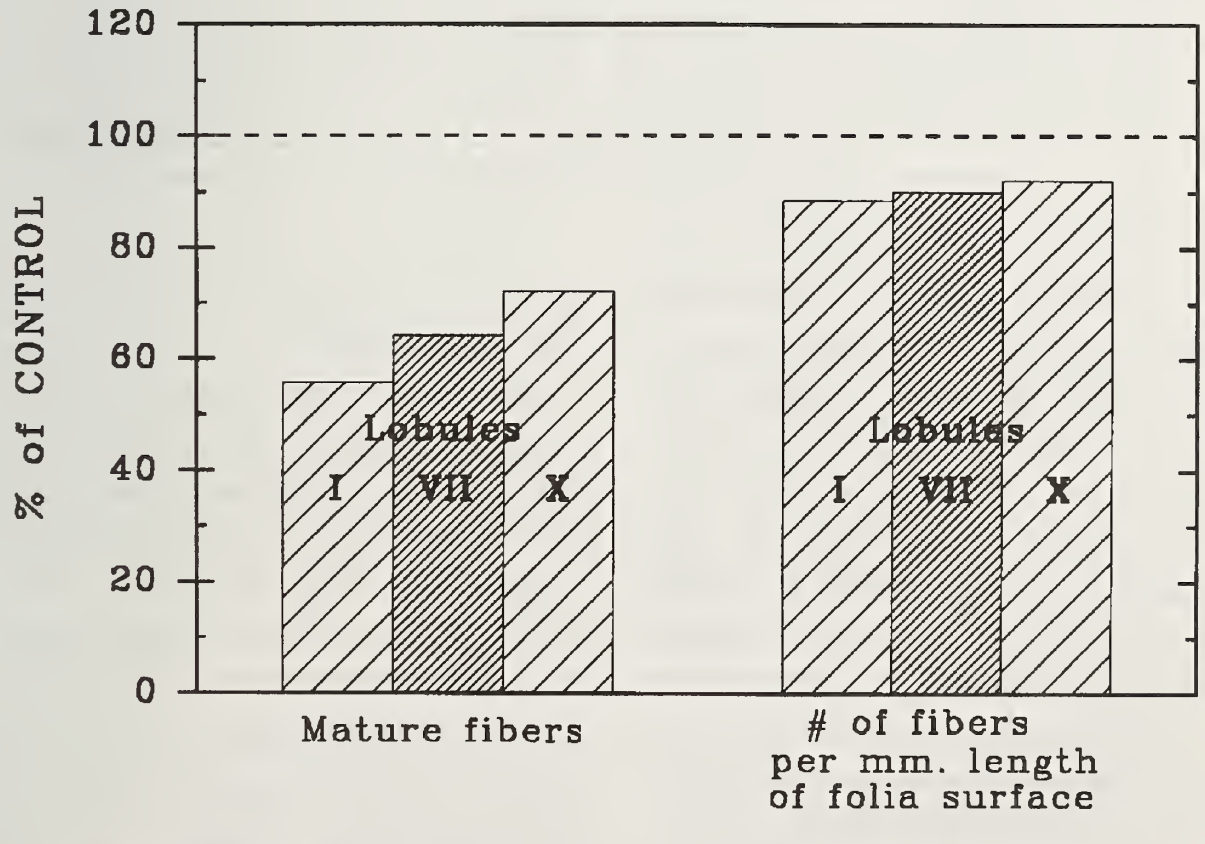

Figure 4. Effects of a prenatal ethanol exposure on GFAP-stained Bergmann glial fibers on P15. The relative proportion of mature fibers was less in alcohol-exposed animals in all three lobules of the vermis that were studied, as was the number of GFAP-positive Bergmann fibers per unit length of the folia surface. Such effects were no longer apparent at P22.

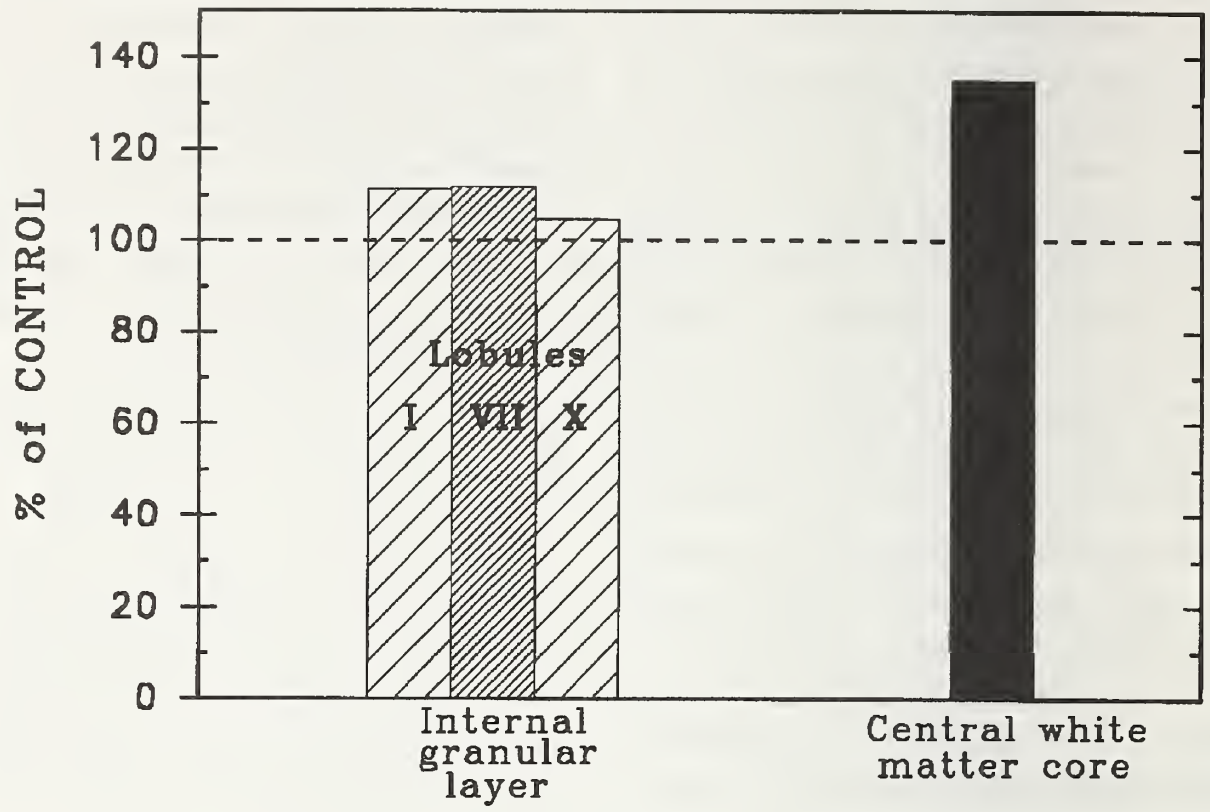

Figure 5. Effects of a prenatal ethanol exposure on the fractional area occupied by GFAP-positive astrocytes and astrocyte processes in the internal granular layer of three lobules of the rat cerebellar vermis and in the central core of white matter, on P15. There were statistically significant alcohol-induced increases in two of the three lobules in terms of the proportion of GFAP-stained area per unit area. The effect of alcohol in the central white matter core was more dramatic, causing over a 35 percent increase in the relative area of GFAP-stained astrocytes. Such effects were no longer present at P22.

process (Phillips and Krueger unpublished data). The Bergmann processes appeared as shorter, thinner, more irregular (wavier) profiles in alcohol-exposed animals compared with control animals, where the processes were longer, thicker, more regular in shape (straighter) and could be traced for considerable distance across the depth of the molecular layer. The relationship of Bergmann fibers to the migrating granular neurons and the related puncta adherens–like junctional complexes appeared unaffected by the alcohol exposure. The morphology of the external granule cells appeared similar in experimental and control animals, and there was no indication of ectopic granule cells, which might be expected with pathology or if migration were interrupted (Berciano et al. 1990).

These slight alcohol-induced changes in morphology are indicative of relative immaturity, based on descriptions of Golgi stained tissues (Das 1976) and on our immunocytochemical work (Shetty and Phillips 1992). It appears that the alcohol exposure causes a delay in the overall maturation of the cerebellar cortex, but does not cause any major alteration of Bergmann glial structure, other than that related to a delayed rate of development, which might cause delayed granule cell migration.

Radial glia in the cerebrum, contrasted to Bergmann glia, develop early and are involved

in the inside-outward migration of immature cortical neurons, after which they become much less prominent and many develop into astrocytes (Miller and Robertson 1993; Pixley and de Vellis 1984). Prenatal alcohol exposures in the rat caused alterations in the shape of radial glial processes (as labeled by the antibody Rat-401 and examined on P1–P45), accelerated the transformation of radial glia to more typical GFAP-positive astrocytes (Miller and Robertson 1993), and caused an apparent increase in gliogenesis. Such effects on radial glia could cause the earlier, more intense GFAP staining of astrocytes (astrogliosis) in alcohol-exposed CNS, while having a potential effect in terms of delaying or aborting some neuronal migration (Miller 1993; Miller and Robertson 1993). A similar acceleration of radial glia transformation to astrocytes has also been suggested from an examination of radial glia stained by antibody to RC2 in the P0 mouse cerebral cortex (Gressens et al. 1992), following prenatal alcohol exposure.

Studies of the effects of alcohol on radial glia in other CNS regions have not been reported. There is a need to do such studies to determine if either the delays reported in cerebellum (Shetty and Phillips 1992) or the accelerated transformations reported in the cerebral cortex (Miller and Robertson 1993) are regular features of radial glial development after alcohol exposures.

SUMMARY

Developmental alcohol exposures delay the maturation of oligodendroglia and related myelin in vivo, produce transient astrogliosis, and alter radial glial maturation and transformation to astrocytes, but do not alter the development of the morphological components of the endothelial cells or astrocytes associated with the BBB. Further studies are needed to define the consistency of these changes in other parts of the CNS, to define temporal and regional vulnerability, to further define the mechanisms by which alcohol produces these changes, and to define how these effects contribute to alcohol-induced alterations of CNS functions.

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Chapter 5

Effects of Ethanol on Substrate Uptake and Incorporation in Primary Astrocyte Cultures

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Although both the developing and the mature brain are highly vulnerable to the effects of ethanol, the mechanisms underlying these impairments remain to be elucidated. Only recently has attention been focused on the effects of ethanol on astrocytes and the possible role of these effects in its action on the central nervous system (CNS). It is now recognized that astrocytes play a key role in the regulation of the neuronal microenvironment and thus participate in the control of CNS development and function. Astrocytes have been shown to synthesize neuropeptides (Intebi et al. 1990; Schwartz and Simantov 1988; Shinoda et al. 1989) and other neurotrophic factors (Rudge 1993), to buffer extracellular ionic concentrations (Kimelberg et al. 1993), and to take up excitatory amino acids and other neurotransmitters (Kimelberg et al. 1993). In the developing brain, radial astrocytes direct the migration of neurons, and normal astrocyte function is critical to axonogenesis and dendridogenesis. In the mature brain, astrocyte function influences neuronal physiology and survival. The presence of astrocytes has been shown to protect neurons in culture from injury induced by exogenous glutamate (Rosenberg and Aizenman 1989) or anoxia (Vibulsreth et al. 1987).

ADVANTAGES OF ASTROCYTE CULTURES

Primary cultures have proven to be useful systems for the characterization of those effects of ethanol on astrocytes that may play a role in its impairment of brain growth and development or in its neurotoxicity. Utilization of astrocyte cultures permits the control of nutrients, growth factors, and ethanol concentrations, which cannot be controlled independently in vivo and allows their interactions to be analyzed. For example, glial development is impaired by undernutrition (Clos et al. 1977), which is commonly associated with alcoholism. In rats, introduction of ethanol into the diet generally results in reduced total caloric intake and necessitates the use of pair-fed controls. Moreover, trophic factors such as the insulin-like growth factors (IGF's), which are

regulated by nutritional status, are altered under these conditions as well.

Astrocyte cultures are of particular value in identifying effects of ethanol during development, as the outcome seen in vivo reflects the summation of direct cellular effects with those secondary to factors such as altered placental nutrient and gas transfer, impaired fuel homeostasis, and litter size. In comparison with other in vitro systems such as tissue slices or cell suspensions, astrocyte cultures, which grow as monolayers, are technically more easily employed and often provide more valid results for studies of uptake and metabolism. Additional advantages and limitations of the use of astrocyte cultures in alcohol and drug research have been reviewed by Rönnbäck and colleagues (1988).

METHODOLOGICAL CONSIDERATIONS

Astrocyte Cultures

Various procedures have been employed for culturing astrocytes, including those of Frangakis and Kimelberg (1984), Hertz and colleagues (1978), and McCarthy and de Vellis (1980). Our laboratory has utilized the single-pass method of Clarke and colleagues (1984). Cells from the cortical hemispheres of term fetal or early neonatal rats are dissociated with trypsin, and grown for 6 days in Dulbecco modified Eagle medium (DMEM) containing 10 percent fetal bovine serum at 37 °C in a humidified atmosphere of 5 percent CO_2 and 95 percent air. The cultures are then replated, and 5 days later the monolayers are composed of nearly 95 percent astrocytes, as demonstrated by positive immunostaining with antiserum to glial fibrillary acid protein and negative immunostaining with neuron-specific enolase (Raju et al. 1981). The ethanol concentration of the culture medium is maintained by incubation in an atmosphere saturated by constant exposure to an aqueous ethanol solution of the same concentration, which prevents ethanol loss from exceeding 5 percent during an 18-hour interval. Serum-free medium can be used for intervals of at least 18 hours if the effects of trophic factors are to be examined.

Uptake and Incorporation Studies

Astrocyte monolayers are washed with a physiological buffer solution, such as Krebs-Ringer phosphate buffer, pH 7.4, and then incubated at 37 °C in buffer containing substrates (thymidine, uridine, amino acids, or hexose) labeled with tracer amounts of ^{3}H or ^{14}C for various intervals. The cultures are then rapidly and extensively washed with cold buffered saline, after which cold 10 percent trichloroacetic acid (TCA) is added. After 2 hours at 4 °C, the supernatant is removed, and the TCA-insoluble cell residue is dissolved in NaOH. Radioactivity is determined in aliquots of the TCA-soluble fraction, containing free intracellular radiolabel, and of the TCA-insoluble fraction, containing radiolabel incorporated into nucleic acids or proteins.

EFFECTS OF ETHANOL ON NUCLEOSIDE UPTAKE AND INCORPORATION BY CULTURED ASTROCYTES

A number of studies have reported the effects of ethanol on growth and DNA synthesis of astrocyte cultures. In our laboratory (Snyder et al. 1992), astrocyte monolayers incubated for 18 hours in serum-free DMEM containing 25, 50, 100, and 200 mM ethanol showed linear responses of cell number ($r = -0.959$, $p < 0.01$) and of protein content ($r = -0.970$, $p < 0.01$) to

ethanol dose when studied 5 days after replating. Figure 1 shows the dose-dependent effect of ethanol on ^{3}H-thymidine incorporation into DNA, normalized to the protein content of these cultures. A minor increase in thymidine incorporation was found in response to 50 mM ethanol, but decreases of 15 percent and 30 percent were seen at higher ethanol concentrations. The increase seen with 50 mM ethanol paralleled an increase in total thymidine uptake. However, thymidine uptake was not significantly altered by higher ethanol concentrations, and thus the fraction of the total thymidine uptake that was incorporated into DNA was reduced at higher doses.

Nucleoside transport is mediated by a facilitated transport process, with an equilibration half-life in cultured astrocytes of < 1 minute at 100 µM substrate (Wu et al. 1986). Thus, parallels between uptake and incorporation do not necessarily indicate that uptake is rate limiting for incorporation.

Kennedy and Mukerji (1986) also showed a biphasic effect on the DNA content of mouse astrocyte cultures exposed to 11 or 22 mM ethanol for 11 days, with a return to near control levels at 44 mM ethanol. Guerri and colleagues (1990) exposed growing astrocyte cultures to 100 mM ethanol for up to 28 days and found decreased thymidine incorporation at each time point studied. However, not all studies have reported these changes. Bass and Volpe (1989) found no effect of 17 to 86 mM ethanol on DNA synthesis in synchronized mixed rat glial primary cultures, and Davies and Vernadakis (1984) reported decreases in the DNA content of chick glial cell cultures only at ethanol concentrations greater than 0.5 percent (109 mM).

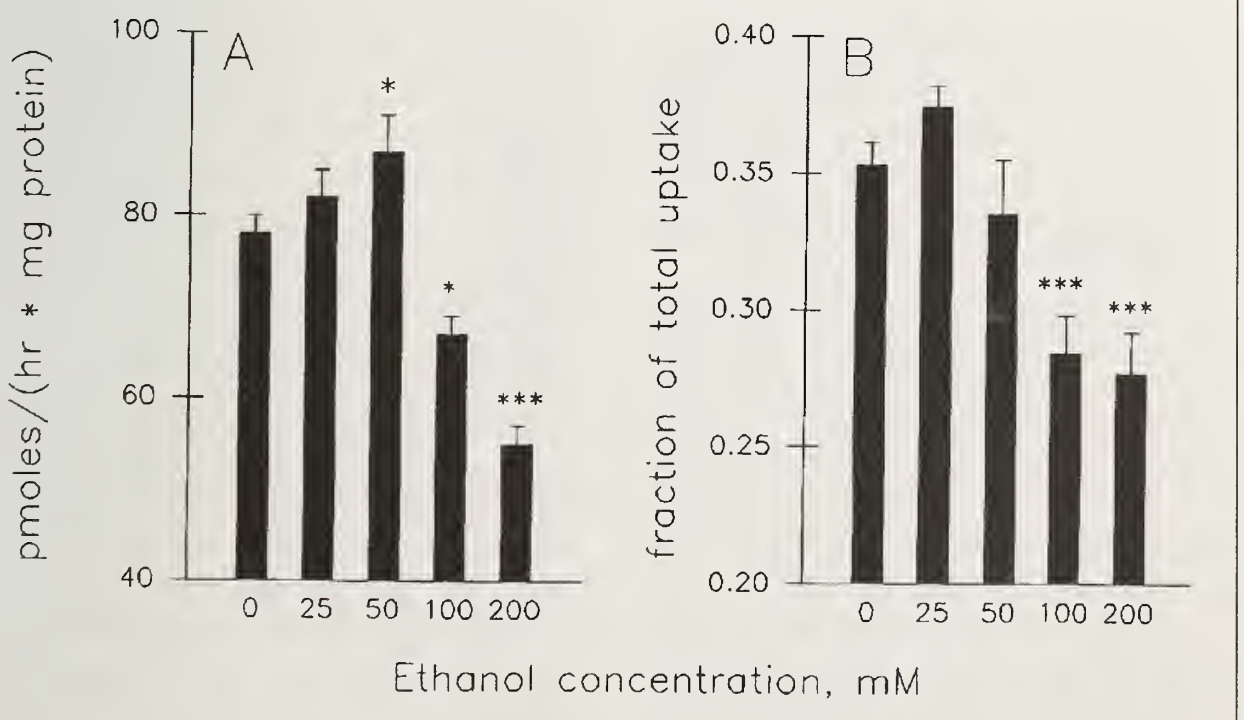

Figure 1. Effect of ethanol on incorporation of tritiated thymidine by rat astrocyte cultures. (A) Incorporation (TCA precipitable radioactivity) following a 1-hour incubation in Krebs-Ringer phosphate buffer containing 1 µCi [methyl-^{3}H]thymidine and 10 µM unlabeled thymidine at 37 °C. (B) Ratio of thymidine incorporation to total thymidine uptake. Values given are mean ± SEM of six or more replicates. $*p < 0.05$ and $***p < 0.001$ versus control (0 mM ethanol).

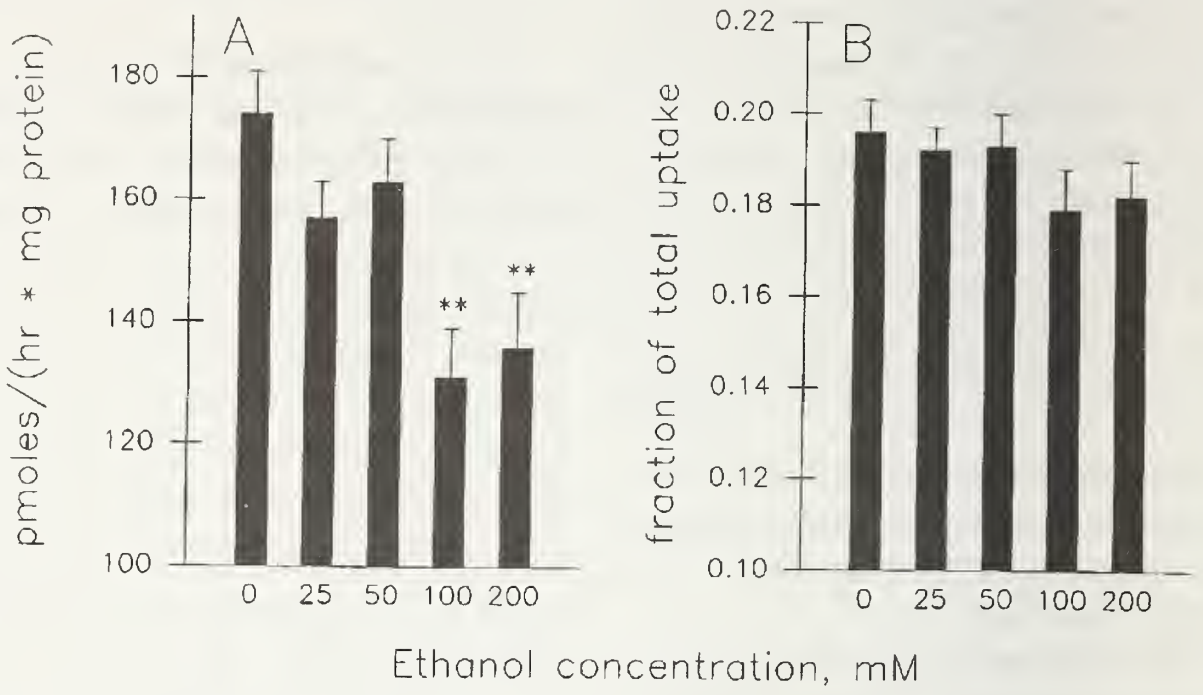

Figure 2. Effect of ethanol on incorporation of tritiated uridine by rat astrocyte cultures. Conditions were the same as in figure 1, but using [5-^{3}H]uridine. (A) Incorporation. (B) Ratio of incorporation to total uridine uptake. Values given are mean ± SEM of six or more replicates. $^{**}p < 0.01$ versus control.

There are few studies of the effects of ethanol on astrocyte RNA synthesis. Fleming and colleagues (1981) found reduced RNA synthesis in astrocytoma cultures and Kennedy and Mukerji (1986) found reductions of the RNA content of primary astrocyte cultures, exposed to 22 or 44 mM ethanol for 11 days. We found parallel reductions in ^{3}H-uridine uptake and incorporation, also normalized to culture protein content, when astrocyte cultures were exposed to ethanol concentrations of 100 mM or higher for 18 hours (figure 2) (Snyder et al. 1992).

Astrocytes express receptors for various growth factors, including insulin (Clarke et al. 1984; Lowe et al. 1986), epidermal growth factor (EGF) (Wang et al. 1989), IGF-I (Han et al. 1987; Shermer et al. 1987), and IGF-II (Han et al. 1987). Physiological concentrations of insulin stimulate RNA and DNA synthesis in rat (Clarke et al. 1985) and mouse (Kum et al. 1987) astrocyte cultures. IGF-I and IGF-II, mitogenic polypeptides with structural homology to proinsulin (Daughaday and Rotwein 1989), are also synthesized by astrocytes (Rotwein et al. 1988) and are believed to play important roles in CNS development. Shermer and colleagues (1987) showed stimulation of DNA synthesis in rat astrocyte cultures at IGF-I concentrations as low as 13 pM, and Han and colleagues (1987) also showed rat astrocyte culture DNA synthesis to be stimulated by IGF-I, IGF-II, and insulin, in order of decreasing sensitivity. It has been suggested that abnormal IGF regulation may mediate the effects of teratogenic agents on the developing brain (Sara and Carlsson-Skwirut 1988), and reductions of IGF-I, but not IGF-II, have been observed in rats following exposure to ethanol in utero (Breese et al. 1993), but few studies have addressed this question.

Ethanol (25–200 mM) inhibits thymidine and uridine incorporation by astrocytes in the presence of insulin (10^{-6} M) or IGF-I (10^{-9} M) (Snyder et al. 1992). Analysis of variance indicates significant interaction between the effects of ethanol dose and insulin or IGF-I on DNA and RNA synthesis. In the presence of 200 mM ethanol, neither insulin nor IGF-I significantly stimulates these synthetic processes.

EFFECTS OF ETHANOL ON PROTEIN SYNTHESIS BY CULTURED ASTROCYTES

Valine uptake by astrocyte monolayers shows a biphasic response to ethanol dose, with reductions of 20 percent and 13 percent following an 18-hour exposure to 50 and 100 mM and dose-dependent increases at higher concentrations (Snyder et al. 1992). Studies with the nonmetabolized alanine analog, α-aminoisobutyric acid, show a similar biphasic effect (Singh et al. 1989), with a significant decrease in response to 50 mM ethanol appearing after exposure for 6 hours and becoming more pronounced after 18 hours. In the first study, valine incorporation paralleled the changes in its uptake (figure 3), and the fraction of the total uptake that was incorporated into protein was not affected at ethanol concentrations ranging from 25 to 200 mM ethanol. Despite increased valine incorporation at high ethanol concentrations, total culture protein content was decreased, which may indicate increased protein turnover.

In the study in which Guerri and colleagues (1990) noted decreased thymidine incorporation by astrocyte cultures exposed to 100 mM ethanol at each time point studied for up to 28 days, ^{3}H-leucine incorporation was significantly decreased only during the peak period of leucine incorporation, which occurred after approximately a week in culture. Rönnbäck and

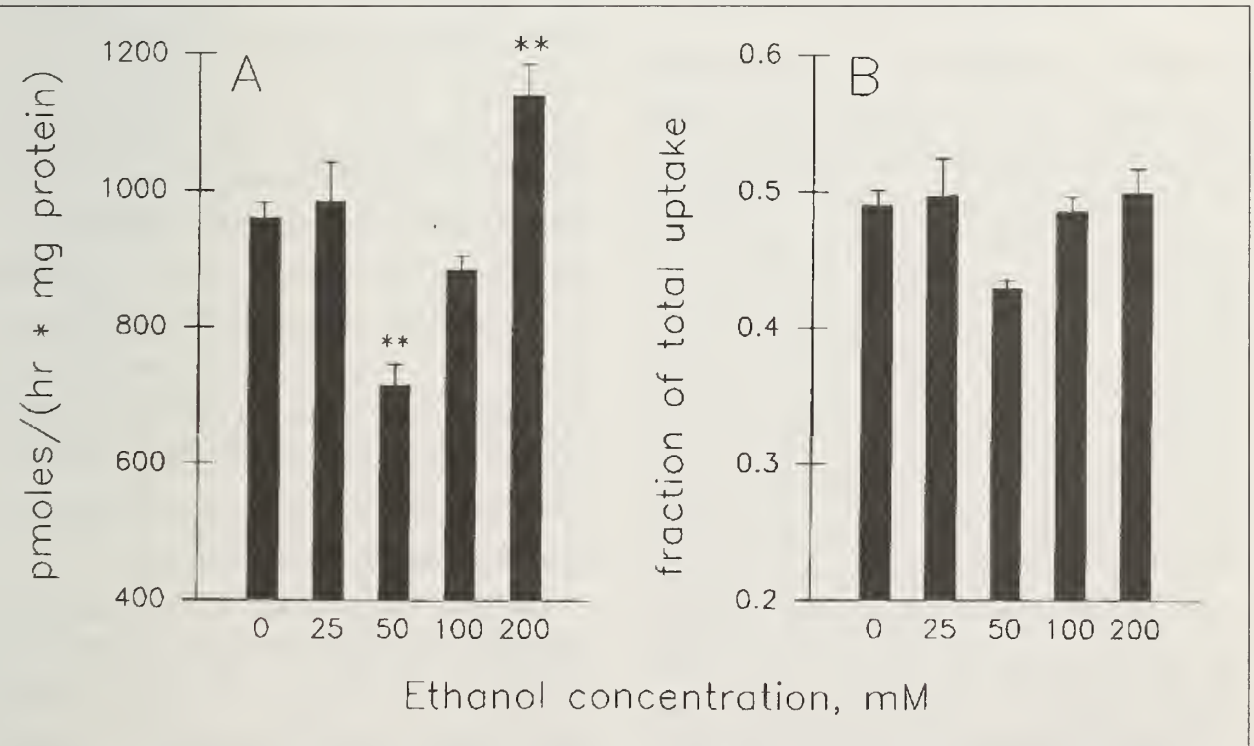

Figure 3. Effect of ethanol on incorporation of tritiated valine by rat astrocyte cultures. Conditions were the same as in figure 1, but using [2,3-^{3}H]valine. (A) Incorporation. (B) Ratio of incorporation to total valine uptake. Values given are mean ± SEM of six or more replicates. $^{**}p < 0.01$ versus control.

colleagues (1988) found increases or decreases in ^{3}H-valine incorporation by cultured astrocytes from differing brain regions exposed to ethanol for 2 or 4 hours. The dose-response curves were nonlinear, with astrocytes from cortex, hippocampus, and striatum showing peaks between 75 and 125 mM and those from brain stem showing a nadir at 100 mM ethanol.

Similar to the effects on DNA and RNA synthesis, significant interactions are observed between the effects of ethanol and insulin or IGF-I stimulation of protein synthesis (Snyder et al. 1992). Ethanol inhibits amino acid uptake and incorporation in the presence of insulin or IGF-I, and also inhibits the stimulatory effect of insulin or IGF-I on culture protein content.

EFFECTS OF ETHANOL ON HEXOSE UPTAKE AND METABOLISM BY CULTURED ASTROCYTES

The plasma membranes of most mammalian cells contain one or more carrier proteins which allow glucose to cross the otherwise impermeable hydrophobic core by facilitated diffusion (Pessin and Bell 1992; Silverman 1991). Seven distinct facilitative transporter isoforms (GLUT-1 through GLUT-7) have been described. The expression of these proteins, which show considerable sequence homology, is tissue specific and developmentally regulated. GLUT-1 is found in embryonic and fetal tissues and is highly expressed in the adult brain and in cultured brain cells, including astrocytes (Sadiq et al. 1990). It is the major glucose transporter of the blood-brain barrier and is relatively insensitive to insulin. GLUT-1 is a high-affinity transporter and, for this reason, its membrane concentration is the primary determinant of the rate of glucose uptake in the tissues in which it is expressed (Pessin and Bell 1992). Specific regulation of glucose transporter gene expression in the developing brain (Devaskar et al. 1991; Sadiq et al. 1990) suggests that glucose transporters play a critical role in meeting the changing metabolic demands of that organ.

Astrocyte hexose uptake has been shown to be regulated by ambient glucose levels (Hara et al. 1989), thyroid hormone (Roeder et al. 1988), and insulin (Clarke et al. 1984), although the response to insulin is low compared with that of tissues which express the insulin-responsive glucose transporter. In primary cultures 5 days after replating, hexose uptake—measured with tritiated 2-deoxy-D-glucose, a glucose analog that is not metabolized beyond phosphorylation—is reduced by exposure to 25, 50, or 100 mM ethanol for 4 hours (figure 4) (Singh et al. 1990). With higher concentrations of ethanol (200 and 300 mM), hexose uptake returns to near the control value. When the period of exposure is increased to 18 hours, 50 mM ethanol reduces 2-deoxy-D-glucose uptake by 36 percent compared with control cultures incubated for the same period. 2-deoxy-D-glucose uptake kinetics show a 20-percent decrease in V_{max} after 4 hours in the presence of 50 mM ethanol. Insulin (10^{-6}M) stimulates hexose uptake to equivalent rates in control cultures and those exposed to 50 mM ethanol for 4 hours, and no significant interaction is observed between ethanol and insulin on this parameter.

Glucose transport can be characterized directly by the binding of cytochalasin B, which competes with D-glucose for specific binding sites on the glucose transporter molecule (Wardzala et al. 1978). Exposure of rat astrocyte cultures to 50 mM ethanol for 18 hours reduces the number of glucose transporters by approximately 40 percent, despite an increase

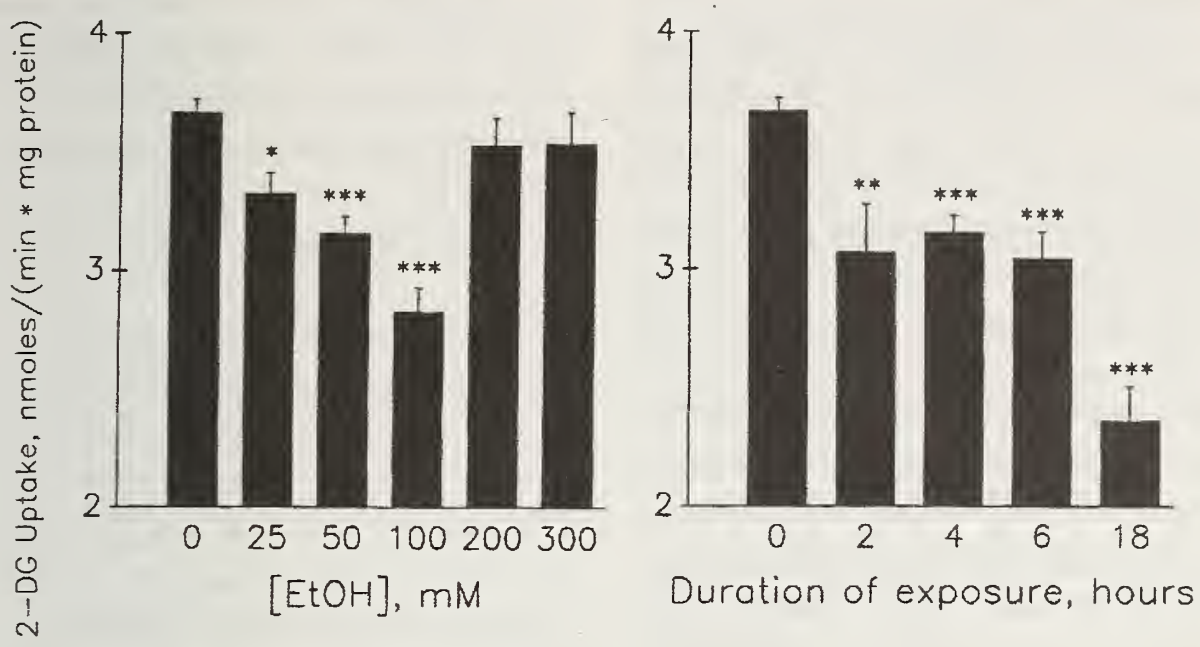

Figure 4. Effect of ethanol on specific 2 deoxy-D-glucose (2-DG) uptake by rat astrocyte cultures. The cell monolayers were washed with Dulbecco's phosphate-buffered saline (PBS), pH 7.4, and incubated in 2 mL PBS containing 0.5 mM 2-DG and 1 μCi ^{3}H-2-DG for 5 minutes at 37 °C. 2-DG uptake was linear during this period. The monolayers were then washed four times with cold PBS, and dissolved in 0.5 mL 0.2 percent sodium dodecyl sulfate in 2 N NaOH. Nonspecific 2-DG uptake was estimated in the presence of 200 mM 2-DG. (Left) Dose response during exposure to ethanol for 4 hours prior to uptake assay. (Right) Time course for exposure to 50 mM ethanol. Values given are mean ± SEM of at least 10 replicates at each point for the dose response and at least 5 replicates at each point for the time course. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus control.

in steady-state GLUT-1 messenger RNA (mRNA) levels (Singh et al. 1992).

Astrocytes use glucose both for energy metabolism and as a substrate for synthetic processes. In various tissues, factors that increase or decrease glucose uptake have been shown to produce corresponding changes in the activity of the pentose phosphate pathway, which provides ribose phosphate precursors for nucleotide synthesis and NADPH for lipid synthesis. Brain pentose phosphate pathway activity has been shown to peak in the developing rat brain in synchrony with maximal rates of RNA synthesis and glial proliferation (Hakim et al. 1980), and astrocytes in culture show significant pentose phosphate flux (Loreck et al. 1987). Pentose phosphate pathway activity is determined as the difference between oxidation of glucose specifically labeled on carbons 1 and 6 (Larrabee 1990), since C-1 and C-6 are utilized equally by the TCA cycle, whereas the latter pathway oxidizes C-1 only. We have measured significant pentose phosphate pathway activity in cortical astrocyte cultures within 3 days of redissociation. When glucose oxidation rates were normalized to protein content, exposure to 50 mM ethanol for 4 hours reduced pentose phosphate pathway activity by approximately 25 percent; a significant reduction of the incorporation of uniformly labeled glucose into DNA was also observed with exposure to 50 mM ethanol for 18 hours (Snyder et al. unpublished observations).

Effects of ethanol on astrocyte glucose uptake and utilization may have significant functional impact. Reduced flux of glucose carbon through the pentose phosphate pathway, by

restricting nucleotide or lipid synthesis, could contribute to the mechanism by which ethanol impairs CNS development. In the adult brain, 90 percent of the energy requirement is supplied by glucose, and reductions of less than 20 percent below normal fasting glucose levels significantly impair cognitive function. The maintenance of astrocyte membrane potential and K^+ and glutamate gradients has been shown to be dependent on glycolysis, and it has been suggested that glucose deprivation–induced excitotoxic damage results from a failure of astrocyte function, which leads to an accumulation of K^+ and glutamate in the extracellular space, initiating a cascade of further extracellular glutamate accumulation (Kauppinen et al. 1988). Restricted astrocyte glucose uptake in the presence of ethanol could have a similar effect on astrocyte function, thus contributing to ethanol-induced toxicity.

SUMMARY

Ethanol, at physiologically relevant and at high concentrations, has been shown to produce various effects on the synthesis of DNA, RNA, and protein in primary astrocyte cultures. The dose-response curves for these effects are frequently nonlinear, and the direction of the effect may vary with the brain region from which the cultures are derived. At concentrations as low as 25 mM, ethanol inhibits the stimulatory effects of insulin and IGF-I on nucleic acid and protein synthesis. Thus, interference with the action of neurotrophic factors may be a significant factor in the effects of ethanol on the developing brain.

Hexose uptake, which also shows a nonlinear response to ethanol dose, is reduced by concentrations of 100 mM and lower, and this effect appears to result from a decrease in the number of membrane transporters. Ethanol does not interact with the stimulation of hexose transport by insulin. Restriction of glucose uptake by ethanol may have a significant effect on glucose utilization and on astrocyte function.

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Chapter 6

Alcohol-Induced Changes in Astrocyte Gene Expression During Central Nervous System Development: Methods and Mechanisms

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In humans, exposure to ethanol in utero has been associated with a number of clinical manifestations including microcephaly, craniofacial dysmorphogenesis, glial and neuronal heterotopias, gyral malformations, enlarged ventricles, agenesis of the corpus callosum, and midline cerebral dysgenesis (Clarren 1986; Clarren et al. 1978; Coulter et al. 1993; Jones 1975; Peiffer et al. 1979). This devastating spectrum of clinical pathology has inspired a concerted effort to delineate both the sites of action and the relevant neuropathological processes that underlie the effects of ethanol on the developing fetus. It has been a working postulate that multiple pathological actions of ethanol may be involved given the broad spectrum of its cellular and neurodevelopmental manifestations. Nevertheless, an enhanced understanding of even one of these effects could provide the basis to greatly augment our ability to identify and treat individuals with ethanol-related brain damage.

The limited availability of neuropathological data in humans has encouraged the development of animal models of both fetal alcohol syndrome (FAS) and fetal alcohol effects (FAE). These models have demonstrated analogous consequences of ethanol on the developing rodent brain (Goodlett and West 1992; Miller 1992; Streissguth et al. 1980; West 1987). Primary cultures of central nervous system (CNS) cells, which hold the promise of elucidating basic pathological mechanisms, have also been developed to demonstrate the effects of ethanol on brain cells under more readily accessible conditions. Indeed, the ability to experimentally control the dose, duration, pattern, and timing of ethanol treatment in relation to CNS development and cellular differentiation has led to a number of compelling

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findings. One intriguing hypothesis, consistent with many CNS abnormalities linked to ethanol-based teratogenesis, proposes an alcohol-induced disruption of astrocyte-mediated developmental events.

In humans and other mammals, astrocytes appear early in embryonic development and undergo proliferation and differentiation throughout gestation and for several weeks after birth. Astrocytes participate in a wide variety of developmental processes in the CNS, including boundary formation during neural morphogenesis, cerebral compartmentalization, neuronal migration, axon outgrowth and guidance, formation of the blood-brain barrier, and production of trophic support molecules (Hatten et al. 1990; Kimelberg and Norenberg 1989; Silver et al. 1993; Steindler 1993; see also chapter 1). Even a transient disruption in astrocyte function in the developing brain might have significant developmental consequences. However, experiments to directly assess the effects of ethanol on astrocytes are constrained by our limited understanding of the molecular substrates involved in specific astrocyte functions and by significant obstacles to the study of glial-mediated events within a complex developing nervous system. Nevertheless, recent studies utilizing in vivo and in vitro animal studies raise the possibility that changes in the expression of certain genes, including astrocyte-specific genes, are associated with, and may mediate, some of the effects of ethanol on the developing brain.

In this chapter, we review recent evidence that implicates astrocytes as a CNS cell type whose gene expression may be influenced by ethanol. Specifically, we review evidence identifying glial fibrillary acidic protein (GFAP) as an ethanol-responsive gene, and we consider possible approaches to determining the mechanism(s) underlying ethanol-related changes in GFAP expression. These investigations may help to identify previously obscure cellular targets of ethanol action in the developing CNS (e.g., regulatory factors, second messenger pathways, growth factors, or receptors) and may suggest common pathways by which ethanol disrupts diverse developmental events. In addition, we discuss the potential implications of alterations in GFAP and phenotypic changes in astrocytes on the etiology of FAS and FAE.

ETHANOL MODULATES THE EXPRESSION OF SPECIFIC GENES

There is a growing body of evidence that ethanol alters the expression of a variety of genes in diverse cell types. For example, one recent report revealed that in response to ethanol there is an increase in messenger RNA (mRNA) of GFAP (Fletcher and Shain 1993). GFAP, the principal constituent of astrocytic intermediate filaments, has been used as a marker of astrocyte development and reactivity (Bignami et al. 1972; Eng 1985; Hatten et al. 1991). This large transient increase in GFAP mRNA occurs in cerebral cortex of neonatal rats exposed to ethanol on postnatal days 5, 6, and 7, demonstrating that brief ethanol exposure can alter gene expression in astrocytes (Fletcher and Shain 1993).

To evaluate the expression of specific genes, investigators often utilize in situ hybridization, immunocytochemical staining, and Northern blot analysis. Localization of mRNA by in situ hybridization (Ausubel 1989; Conn 1989), and of proteins by immunocytochemical staining (Ausubel 1989; Sternberger 1986), can provide important information about the distribution of specific gene products in complex tissues, but these methods are only semiquantitative. By

contrast, Northern blot analysis provides a quantitative measure of steady-state levels of specific mRNA's in tissues or cell cultures. In this technique, RNA from control and experimental tissues or cells is isolated, bound to a membrane, hybridized with a radiolabeled DNA probe complementary to the specific mRNA of interest, and analyzed by densitometry of autoradiograms of hybridized membranes. Quantitative levels of specific mRNA's can then be expressed relative to an internal control RNA (Ausubel 1989).

Table 1 presents a representative list of genes determined by Northern blot analysis to be ethanol sensitive. All of the studies included in this table report changes in gene expression in response to concentrations of ethanol that have been measured in the blood of humans after consuming alcoholic beverages, and that are below the levels thought to be overtly cytotoxic or fatal (Ritchie 1985).

Given the variety of genes whose expression is altered by ethanol exposure, the question arises, "How selective are the effects of ethanol on the expression of specific genes?" There are several lines of evidence suggesting that ethanol affects only certain genes and does not simply alter the overall capacity of the cell to transcribe DNA. In many of the studies that identify ethanol-sensitive genes, β-actin and α-tubulin mRNA levels are not altered (Aldo-Benson et al. 1992; Datta et al. 1990; Gayer et al. 1991; Kharbanda et al. 1993; Mochly-Rosen et al. 1988; Montpied et al. 1991). Even closely related genes can be differentially affected by ethanol. For example, in the rat cerebral cortex prolonged ethanol treatment reduces the levels of $GABA_A$ receptor α_1 and α_2 subunit mRNA, but has no effect on α_3 subunit mRNA levels (Montpied et al. 1991). In astrocytes, genes for vimentin and GFAP, whose expression is coordinately regulated during normal differentiation and development (Lazarides 1982; Steinert et al. 1985), are differentially altered by brief ethanol exposure both in vivo and in vitro (Fletcher and Shain 1993). Thus, both acute and prolonged ethanol treatment in various cell types can alter the levels of specific mRNA's, apparently without overt cellular toxicity or general effects on cellular transcription.

What could a change in the level of mRNA (and protein product) of a particular gene in response to ethanol tell us about the mechanism of ethanol action on the cell? The differential sensitivity of particular genes to a given stimulus is thought to depend on regulatory factors that influence the interaction of 5'-untranslated *cis*-acting DNA sequences with promoters and DNA polymerases that initiate transcription, or that interact with 3'-untranslated regions containing mRNA degradation determinants (He and Rosenfeld 1991; Nielsen and Shapiro 1990; Shyu et al. 1989). Thus, changes in the level of a particular mRNA species can reflect a change in transcriptional rate or a change in mRNA stability after transcription, or both. When quantitative changes in mRNA levels are detected by Northern blot analysis, a first step in determining the more proximal effects of ethanol involves evaluation of transcriptional and posttranscriptional processes. Knowing the molecular pathway(s) involved in the modulation of such "ethanol-sensitive" genes may lead to the identification of other biochemical consequences of ethanol's action on the cell, and might also suggest cellular functions altered by ethanol. Although the ultimate goal of these studies is to identify the mechanisms by which ethanol alters the expression of particular genes in vivo, significant contributions to this goal can be made by the use of well-characterized in vitro models of altered gene expression in response to ethanol.

Table 1. Messenger RNA's Found by Northern Blot Analysis To Be Altered by Ethanol Treatment

Gene	Cell Type	Direction of Change	Reference
Glial fibrillary acidic protein (GFAP)	Cerebral cortex astrocytes	↗	Fletcher and Shain 1993
Class I major histocompatibility complex	Various cell lines[a]	↗	Parent et al. 1987
Tumor necrosis factor	HL-60 myeloid leukemia cells	↗	Datta et al. 1990
Tyrosine hydroxylase	N1E-115 neuroblastoma cells	↗	Gayer et al. 1991
Hsc70, heat shock (constitutive)	NG108-15 neuroblastoma cells	↗	Miles et al. 1991
Cytochrome P450 CYP 2E1	Liver	↗	Ronis et al. 1993
c-*jun*, *jun*-B, c-*fos*	Keratinocytes	↗	Kharbanda et al. 1993
G-protein subunit, $G_o\alpha$	NG108-15 neuroblastoma cells	↗	Williams et al. 1993
G-protein subunit, $G_s\alpha$	Lymphocytes	↗	Waltman et al. 1993
	NG108-15 neuroblastoma cells	↘	Mochly-Rosen et al. 1988 Williams et al. 1993
β–Luteinizing hormone (β-LH)	Pituitary	↘[b]	Emanuele et al. 1991
		↗[c]	Salonen et al. 1992
Proopiomelanocortin (POMC)	Pituitary	↘	Dave et al. 1986
β–Follicle-stimulating hormone (β-FSH)	Pituitary	↘	Salonen et al. 1992
$GABA_A$ receptor subunit, α_1	Cerebral cortex	↘	Montpied et al. 1991
Acetylcholine receptor, α subunit	Soleus muscle	↘	Held et al. 1991
Immunoglobulin, κ-chain	B-lymphocytes	↘	Aldo-Benson et al. 1992

[a]OVTC, teratocarcinoma; K562, lymphoma; L cells, fibroblastoma; E14, lymphoma; Lan5, neuroblastoma; F9, teratocarcinoma.
[b]Acute ethanol exposure.
[c]Chronic ethanol exposure.

A number of well-established methods may be used to elucidate the molecular mechanism(s) that account for quantitative changes in the steady-state levels of mRNA species. Nuclear run-on assays (in vitro transcription), in which preinitiated mRNA transcripts are synthesized in isolated nuclei and detected by hybridization to specific cDNA's, can be performed to determine whether changes in mRNA levels reflect changes in the rates of gene transcription (Ausubel 1989). Nuclear run-on assays have been used to show that increased transcription is in part responsible for ethanol-induced increases in cytochrome P-450 CYP 2E1 in rat liver (Badger et al. 1993) and Hsc70 in neuroblastoma cells (Miles et al. 1991).

While nuclear-run on analysis can reveal whether ethanol treatment results in changes in transcriptional rate, both in vivo and in vitro, other molecular approaches that examine the factors involved in transcriptional and post-transcriptional regulation can best be performed using cells in culture. In transient or stable transfection analysis, sequences from the promoter region of the gene of interest are attached to reporter genes and introduced into cells in vitro where their influence on the rate of transcription is assessed by quantitative measurement of the reporter gene product being expressed (Berger and Kimmel 1987). By systematically eliminating portions of the promoter, sequences involved in transcription of the gene can be identified under control and experimental conditions. The identity of these 5' DNA sequences can suggest what *trans*-acting factors (regulatory DNA binding proteins) and biochemical pathways are involved in the response of a cell to given stimuli. This approach has been used to identify *cis*-acting DNA sequences involved in the induction of Hsc70 (Miles et al. 1991) and in tyrosine hydroxylase (Gayer et al. 1991) transcription in neuroblastoma cells after ethanol treatment in vitro, though the exact molecular identity of these sequences has not yet been determined.

Altered stability of mRNA can also contribute to changes in mRNA levels observed after ethanol exposure. For example, some evidence suggests that β–luteinizing hormone (β-LH) mRNA levels may be modulated by ethanol-induced changes in mRNA stability. In a study by Emanuele and colleagues (1991), β-LH mRNA was transiently decreased to 50 percent of control levels in pituitary of adult male rats 1.5 hours following a single injection of ethanol. Since the half-life of β-LH mRNA is greater than 8 hours, the decreased β-LH mRNA levels may reflect increased mRNA degradation rather than decreased transcription, which would not be expected to significantly change the level of β-LH mRNA detected until considerably later. One approach used to directly determine the turnover rate of specific mRNA's under various conditions is the use of kinetic labeling or decay following administration of inhibitors of transcription such as actinomycin D (Harrold et al. 1991). These studies can be performed most effectively in vitro, where precise concentrations of inhibitors can be applied directly to the cells.

Comparing which specific genes are altered in cells exposed to ethanol in vivo and in culture may suggest the potential usefulness of a given in vitro model for mechanistic studies. For example, treatment of confluent primary astrocyte cultures with ethanol at a concentration and a duration that approximate those previously shown to increase GFAP mRNA and protein in newborn rat cortex results in comparable changes in GFAP expression. In both models, ethanol increases GFAP expression greater than twofold without altering the expression of α-tubulin, vimentin, or β-actin

(Fletcher unpublished observations; Fletcher and Shain 1993). Taken together, these findings suggest similarities in the effects, and potentially the mechanisms, underlying ethanol induction of GFAP in these model systems. A comparison of dose dependence and time course of changes in GFAP mRNA and protein in vivo and in vitro could provide important information regarding the congruity of these models, and the mechanism underlying altered gene expression following ethanol exposure.

Interpretation of results from studies of ethanol-treated astrocytes in vitro must take into consideration some potentially important differences between astrocytes in developing brain and astrocytes in culture. Astrocytes in culture are isolated from interactions with neurons. Such cell-cell interactions influence the morphology and physiology of both astrocytes and neurons (Hatten 1989; Lauder and McCarthy 1986). The presence of microglia in cultures of astrocytes may affect results, because microglia are known to respond to injury and insult by secreting cytokines and other biologically active molecules (Giulian and Baker 1985; Giulian et al. 1989; see also chapter 3). The implications of microglial responses to ethanol and the potential effects of their humoral interactions with astrocytes are not known. In addition to the possible influences of nonastrocytic cell types, serum and other components of culture medium may also influence the response of astrocytes to ethanol in culture.

An additional consideration when astrocytes in culture are treated with ethanol-containing medium is the possible influence of osmolarity on changes in gene expression. Isosmotic ethanol-containing medium stimulates both cell swelling and prolactin secretion in a pituitary-derived cell line (Sato et al. 1990), and swelling and excitatory amino acid release in primary astrocyte cultures (Kimelberg et al. 1993). By contrast, hyperosmotic ethanol-containing medium has no effect on swelling or release in either system. Thus, changes in cell volume induced by changes in osmolarity due to ethanol may be responsible for some cellular responses to ethanol treatment. It is not known whether astrocytes in vivo swell after ethanol exposure, or whether cell swelling may influence transcriptional processes in astrocytes.

Although experiments that examine the effects of ethanol on astrocytes in vitro offer unique opportunities for molecular analyses, the results of these studies must be considered in light of the unique environments of astrocytes in the brain versus in culture. Ultimately, it will be important to verify that mechanisms of ethanol action discovered in vitro operate in the intact animal.

ALTERED GFAP EXPRESSION AND FAS

We have discussed several methods for determining the mechanisms of ethanol-responsive gene expression in the developing nervous system, and we have described the compelling evidence that GFAP is an ethanol-responsive gene in the neonatal rat CNS. We now consider the possible role of altered GFAP expression in the pathogenesis induced by ethanol during brain development.

GFAP is a glial-specific intermediate filament protein whose function, like that of other intermediate filament proteins, is unknown. Evidence suggests that GFAP functions during normal astrocyte differentiation and in astrocytes responding to injury. A possible role for GFAP in differentiated astrocytes is suggested by the observation that GFAP expression increases when astrocytes undergo a transition from flat, polygonal, or radial glia to multipolar, process-bearing astrocytes (Bovolenta et al.

1984; Eng 1985; Trimmer et al. 1982). Elevated GFAP expression is also a hallmark of astrogliosis, a common reaction of astrocytes to injury in the mature and developing nervous system. Other reactions of astrocytes to injury include cellular hypertrophy, formation of thick astrocytic processes, and up-regulation of numerous molecules. Reactive astrogliosis in the CNS can be induced by diverse stimuli, such as stab wound (Bignami and Dahl 1976), ischemia (Petito et al. 1990), spreading depression (Kraig et al. 1991), excitotoxins (Burtrum and Silverstein 1993), triethyltin (O'Callaghan and Miller 1988), and intense neuronal activity (Steward et al. 1991) (see also reviews by Eddleston and Mucke 1993; Hatten et al. 1991). Elevated levels of GFAP are also associated with numerous neurological diseases (Eng and DeArmond 1982).

Ethanol-induced changes in GFAP expression may reflect perturbation of astroglial differentiation or activation of astrogliosis. Some evidence suggests that prolonged ethanol exposure during gestation in the rat may alter the postnatal maturation of Bergmann glia of the cerebellum (Shetty and Phillips 1992; see also chapter 4) and the timing of the phenotypic transition of radial glia to astrocytes in the cerebral cortex (Miller and Robertson 1993). Alterations in the timing of astrocyte differentiation induced by ethanol could disrupt the migration of neurons that are guided via Bergmann and radial glia, resulting in neuronal ectopias that characterize FAS (Miller and Robertson 1993; Rakic 1988). It is not clear how alterations in the expression of GFAP per se might be involved in ethanol-induced changes in the timing of astrocyte differentiation or the ability of glia to function as guides for migrating neurons. However, experimental evidence that GFAP is required for the formation of stable astrocytic processes in response to neurons (Weinstein et al. 1991) is consistent with the idea that ethanol disruption of GFAP could affect neuronal-astroglial interactions. Studies designed to directly test the effect of ethanol on glial-guided neuronal migration and the involvement of GFAP and other developmentally regulated molecules on this process would be of considerable interest.

Prolonged ethanol exposure during gestation also results in a transient hypertrophy of astrocytes and increased density of GFAP immunoreactivity in the internal granular layer and central white matter of the rat cerebellum on postnatal day 15, criteria characteristic of reactive astrogliosis (Shetty and Phillips 1992). Brief *postnatal* ethanol exposure can also cause transient reactive astrogliosis in the rat (Goodlett et al. 1993). Treatment of neonatal rats with ethanol on postnatal days 4 through 9 causes a dramatic increase in immunoreactive GFAP primarily in layer V of the cerebral cortex in rats with mean blood alcohol levels as low as 50 mg/dL (Goodlett et al. 1993). It is interesting that the distribution of reactive astrocytes is apparently different in animals exposed to ethanol postnatally and prenatally. In contrast to prenatal ethanol exposure, which results in reactive gliosis in the cerebellum, reactive gliosis is not observed in the cerebellum of animals treated with ethanol postnatally. This region-specific difference in sensitivity to postnatal ethanol is mimicked by the localization of changes in gene expression: GFAP mRNA levels are elevated in the cerebral cortex, but not in the hippocampus, cerebellum, or brain stem of 7-day-old rats exposed to ethanol on postnatal days 5 through 7 (Fletcher and Shain 1993).

Differences observed in the distribution of reactive astrocytes in animals exposed to ethanol prenatally or postnatally may result from either variability in the duration of ethanol

exposure or the timing of ethanol exposure relative to particular stages in astrocyte differentiation. Changes in the expression of certain genes upon prolonged ethanol exposure may reflect compensatory activities of cells aimed at maintaining homeostasis. In contrast, changes in gene expression that correlate with acute or brief ethanol treatments may reflect primary cellular responses to ethanol. In the case of GFAP, for example, mRNA and protein both increase in the neonatal rat cortex after only 3 days of postnatal ethanol exposure, suggesting that transcriptional changes in GFAP expression may be a relatively rapid response to ethanol in astrocytes (Fletcher and Shain 1993).

Differences in the localization of astrocytes with elevated GFAP expression after prenatal and postnatal ethanol exposure may also be related to the timing of ethanol exposure relative to particular stages of astrocyte differentiation or other developmental events. In primary cultures of astrocytes, ethanol influences different morphological and biochemical markers of glial cell differentiation depending on whether it is present during cell division of glial cell progenitors or during the proliferation of astroglial cells in culture (Guerri et al. 1990; Kennedy and Mukerji 1986*a*, 1986*b*). The finding that astrocytes in different brain regions respond differently to ethanol with changes in GFAP expression could reflect stage-dependent vulnerability, since astrocytes in different brain regions perform specific developmental functions asynchronously. For example, radial glia undergo the transition to astrocytes (and express higher levels of GFAP) after the completion of neuronal migration, which occurs at different times in different brain regions (Altman 1969, 1972; Bayer 1980; Hicks and D'Amato 1968). Regional differences in the effect of ethanol on astrocytes could also reflect endogenous heterogeneity of astrocytes. Experiments designed to examine the temporal coincidence of ethanol-sensitive gene expression and particular developmental events will help to determine whether cellular sensitivity to ethanol's effects on gene expression are linked to specific biochemical or physiological conditions.

What are the potential functional consequences of ethanol-induced reactive gliosis during development? Although recent evidence suggests that reactive astrocytes, with high levels of GFAP expression, can inhibit axon outgrowth in regenerating CNS (Liuzzi and Lasek 1987; McKeon et al. 1991), reactive astrocytes are the preferred substrate for the outgrowth of nerve growth factor (NGF)–responsive axons in the adult CNS (Kawaja and Gage 1991). Reactive astrocytes have been shown to produce neurotrophic factors, like insulin-like growth factor I (IGF-I) (Gluckman et al. 1992) and NGF (Bakhit et al. 1991), which could protect neurons from ethanol-induced cellular toxicity. Reactive astrocytes can also produce substances such as tumor necrosis factor α, which may be cytotoxic to oligodendrocytes (Robbins et al. 1987; Selmaj et al. 1991); this cytotoxicity could disrupt developmental processes including neuronal migration, myelination, and programmed cell death. Reactive gliosis, therefore, could provide a desirable defense against ethanol-induced damage or, alternatively, could result in destructive changes in astrocyte biochemistry that could interfere with normal developmental events.

The induction of reactive gliosis by ethanol may result from a direct effect on astrocytes or as a secondary response of astrocytes to injury of neurons or other cell types in the CNS. Although neuronal death is thought to trigger astrocytic reaction under a variety of circumstances (Lindsay 1986), it is not known whether cortical neurons are lost in the regions where GFAP expression is induced in neonatal rats ex-

posed to ethanol (Goodlett et al. 1993). The demonstration that GFAP expression can be induced in astrocyte cultures that lack neurons argues against the hypothesis that ethanol-induced increases in GFAP are necessarily linked to neuronal damage or death, although it would be difficult to entirely rule out since it is not yet known whether induction of GFAP in vitro and induction of GFAP in vivo occur via the same cellular mechanism(s). It is also possible that ethanol induces GFAP expression via a direct effect on astrocytes, rather than an effect secondary to neuronal death or damage.

Disruption of the blood-brain barrier is also thought to lead to the activation of astrocytes via the extravasation of blood products into a site of injury (Oehmichen 1983). Reactive astrocytes are commonly associated with the microvasculature, specifically in vessels of cortical layers I–IV, in animals in which high blood alcohol concentrations are produced (300 mg/dL) (Goodlett et al. 1993).

More information is needed to determine the mechanisms of ethanol-induced reactive changes in astrocytes. Additional information regarding mechanisms by which ethanol may cause astrocytes to become reactive may come from comparisons of reactive gliosis that occurs in response to ethanol and in response to other forms of brain injury. For example, GFAP levels affected by ethanol exposure return to normal within 2 weeks of the last day of ethanol treatment (Fletcher and Shain 1993; Goodlett et al. 1993), in contrast to the lasting elevation of GFAP levels in areas of permanent tissue injury resulting from ischemia (Petito et al. 1990). Ethanol-induced reactive changes in astrocytes may be unique in other ways as well. Hsp70 heat shock mRNA is elevated severalfold within hours of stab wound brain injury and peaks within 1 day of the peak for GFAP mRNA levels (Brown et al. 1989; Hozumi et al. 1990*a*, 1990*b*). In contrast, Hsp70 mRNA is not induced with GFAP in neonatal rats exposed to ethanol (Fletcher unpublished observations). Such quantitative differences in the response of astrocytes to ethanol and other inducers of reactive gliosis may reflect differences in mechanisms for induction of reactive gliosis.

It is likely that the response of astrocytes to ethanol involves changes in the expression of genes other than GFAP. Other genes whose expression could be altered by ethanol might be linked to astrocyte functions during development (e.g., trophic factors and cell adhesion molecules). Examination of the response to ethanol of other genes in astrocytes will help to define a molecular profile of ethanol's effects on astrocytes that may be useful to predict possible functional consequences.

CONCLUSION

Because of advances in our understanding of the pathological cellular and molecular responses to ethanol by the developing nervous system, we may greatly enhance our ability to identify individuals with FAS and FAE and we may be able to develop innovative medical interventions for patients. However, this will require a more complete understanding of the relationship between ethanol, alterations in gene expression, and abnormal development.

The critical role that astrocytes play during CNS development and the recent evidence that astrocytes react to ethanol exposure suggest that further studies of the underlying mechanisms of action of ethanol on astrocytes are of great importance. For these studies, consideration should be given to the likelihood that chronic ethanol exposure influences numerous developmental events. Therefore, brief ethanol treatments may provide the best experimental resolution of ethanol's primary effects on

astrocytes and other cells of the developing CNS. The hypothesis guiding our work is that ethanol disrupts CNS development by altering the normal expression of specific genes, resulting in the cascade of developmental consequences that characterize FAS. Testing this hypothesis will require the identification of "ethanol-sensitive" genes, the elucidation of mechanisms by which ethanol alters their expression, and the demonstration that these changes are responsible for the abnormalities of CNS development attributed to ethanol.

In this chapter we have described experimental evidence that ethanol can alter gene expression in a variety of cell types, and we have explored the recent finding that expression of GFAP, an astrocyte-specific intermediate filament protein, is up-regulated after brief ethanol exposure in vivo and in primary cultures of astrocytes. This observation provides the basis for a model system that can be used to investigate possible mechanisms by which ethanol alters gene expression in astrocytes. This model exploits a potential congruity of astrocyte response to ethanol in in vivo and in vitro systems in order to complement the molecular investigation of cells in culture with whole-animal studies in which the complexity of intact cellular relationships is retained. It is, however, premature to assume that the mechanisms of action of ethanol on astrocytes in rat cortex are comparable to those in primary astrocyte cultures. Regional specificity, time-course, and dose-response studies of ethanol-induced changes in GFAP expression in astrocytes in intact animals and from astrocytes in culture must be systematically correlated before such a conclusion can be accepted.

Further refinement of model systems for studying the primary response of astrocytes in the developing CNS to ethanol may enhance these investigations. Moreover, the role of altered gene expression in the etiology of FAS- and FAE-related neuropathology eventually may be evaluated directly with techniques such as homologous recombination or the expression of antisense mRNA's, which could advance studies beyond correlative observations to a direct assessment of the functional effects of altered gene expression on developmental events in the CNS.

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Chapter 7

Fetal Alcohol Exposure and Glial Cell Development

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Many effects of parental alcohol abuse can be studied in humans, but ethical considerations do not allow research on the mechanisms underlying these effects. Therefore, experimental models are necessary to understand how alcohol acts in the body. Such models must allow the study of the mechanisms of action of alcohol at the cellular or subcellular level. Because nervous tissue contains different cell types, it is necessary to study the effect of alcohol on these various species. Cells in culture can provide these features.

The numerous functions of glial cells in brain metabolism and development were first shown more than 20 years ago when Bignami and colleagues (1972) discovered the glial fibrillary acidic protein (GFAP) used to characterize astrocytes. Since then, new culture techniques have helped to elucidate the participation of astrocytes in nervous tissue activity. Deficiencies in glial cell function are responsible for disturbances in normal brain function and development.

Alcohol is one of the compounds producing deficiencies, mainly in glial cells. The effect of alcohol may be investigated either by adding alcohol at various concentrations directly to the cell culture medium or by culturing glial cells from the brains of animals exposed to alcohol during embryogenesis.

EFFECT OF ALCOHOL ON GLIAL CELLS COMPARED WITH NEURONS

Differences between neuronal and glial cell types in regard to alcohol effect were first observed by Syapin and colleagues (1976, 1980), working with cell lines either acutely or chronically exposed to 100 mM ethanol. They measured enzymatic activities in plasma membranes (Mg^{2+} ATPase, (Na^+, K^+)ATPase, and 5' nucleotidase). Modifications of these enzymes result from the direct effect of alcohol on the fluidity of the membrane. Differences between neuronal and glial cell lines seem to be related to the specific structure of each cell type.

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Neurons also behave differently from glial cells in primary cultures. We showed that superoxide dismutase activity involved in the elimination of free radicals formed during alcohol degradation is not modified when neurons or neuronal cell lines are treated with 100 mM ethanol (Ledig et al. 1980), whereas the activity is significantly decreased in glial cells and glial cell lines (Mandel et al. 1980). Primary cultures of neurons or neuronal cell lines and primary cultures of glial cells or glial cell lines treated with 100 mM ethanol showed opposite effects for enolase activity, a marker of nerve cell development, and for various species of ATPase activity (Mg^{++}, Na^{+}, K^{+}, ecto Mg^{++}, and ecto Ca^{++}). Trypsinization of the cells before seeding showed that the response to ethanol depended mainly on the specific lipoproteins in the membranes of each cell type (Ledig et al. 1985).

Davies and Vernadakis (1984, 1986), using glutamine synthetase as a marker of cell proliferation, showed in the glioma C6 cell line that the growth kinetics of cultures influence the susceptibility of cells to perturbations by alcohol. Guerri and colleagues (1989, 1990) also demonstrated that the effect of alcohol on membrane-bound enzymes in primary cultures of astrocytes depends on the age of the cultures. The effect of alcohol is more important during the proliferative period than during the maturation period. As shown by Rönnbäck and colleagues (1988), primary astroglial cultures prepared from various brain areas demonstrate specific responses to alcohol treatment concerning RNA, DNA, and protein synthesis according to the specific area of the brain.

Microscopic observations (Davies and Cox 1991) also showed that astrocyte cultures are vulnerable to alcohol exposure even at moderate levels (0.2 percent) for a short time. Compared to control cultures with complex stratified cellular layers, the alcohol-exposed cultures present a dose-dependent alteration in both depth and complexity of the cell layer. A quantitative immunofluorescence and immunogold electron microscopy study performed by Renau-Piqueras and colleagues (1989) on astrocyte cultures from rat embryos exposed to alcohol in utero demonstrated failure to develop processes or to acquire filamentous intermediate filament distribution pattern during the proliferative period, suggesting an initial damage to astrocyte precursor cells. In the same conditions, alcohol also decreased the amount of cytoskeletal proteins such as α-tubulin, vimentin, and GFAP (Saez et al. 1991).

The proliferation period of astrocytes until 2 weeks of culture seems to be particularly sensitive to either in vitro or in utero alcohol effect as shown by the decrease in both DNA and protein synthesis of astrocytes cultured until 21 days, either exposed to 100 mM ethanol in vitro or cultured from pups born to mothers who have consumed an alcohol-containing liquid diet before and during pregnancy. Using an astrocytoma cell line, Tewari and colleagues (1988) reported also an inhibition of protein synthesis in the presence of 100 mM ethanol, especially during the exponential phase of growth.

It seems now well established that astrocytes have a greater sensitivity to alcohol than neurons, mainly during the period of intense multiplication. This sensitivity has been shown by in vitro studies and also by in utero alcohol exposure that induces astrocytic damage in the offspring.

EFFECT OF PARENTAL ALCOHOL EXPOSURE ON GLIAL CELLS IN THE OFFSPRING: AN EXPERIMENTAL MODEL

We developed an experimental model that parallels human situations resulting in morphological and behavioral abnormalities of the offspring

following either maternal or paternal alcohol abuse, called fetal alcohol syndrome in humans.

Animal Treatment

Female rats from an Italian Wistar strain adapted for alcohol consumption received increasing concentrations of alcohol in the drinking water (10 percent vol/vol for 1 week, then 20 percent vol/vol for 4 weeks) before mating, while control animals received only tap water. Alcohol treatment was either continued during pregnancy or stopped at mating. During the 20 percent alcohol feeding, the mother consumed about 10 g ethanol/kg/d and had blood alcohol levels of about 0.6 g/L at 9:00 a.m. The animals had no sign of undernutrition (e.g., weight loss or dull hair) and no withdrawal syndrome when alcohol was removed at mating. Control animals and alcohol-treated animals consumed laboratory chow ad libitum. Male rats were exposed to ethanol under the same conditions for at least 3 months before mating with control females.

The litter size (8–12 pups) was similar for treated animals and control animals. The pup weights were about 8 g for the controls, 6.3 g for the mothers who were treated with alcohol until delivery, and 6.4 g for the mothers who were treated with alcohol until mating or after only paternal alcohol exposure.

Cell Culture

Glial cells were grown for 4 weeks in cultures of cortical brain cells from newborn pups in 10 percent fetal bovine serum–supplemented Dulbecco modified Eagle medium (DMEM). The 6-mm-diameter petri dishes (Falcon) were incubated in 5 percent CO_2-95 percent humidified atmospheric air at 37 °C (Sensenbrenner 1977).

Biochemical Measurements

For biochemical determinations, the culture medium was removed by aspiration. The cells were washed twice with ice-cold 0.9 percent wt/vol NaCl and harvested in 1 mL of imidazole 50 mM, SO_4Mg 2 mM, KCl 40 mM, pH 6.8 buffer. The cells were disrupted by sonication and centrifuged for 1 hour at 100,000 x g. The supernatant was used for the measurement of the enzymatic activities.

Maturation of the cells was evaluated by the level of enzymatic markers: enolase isoenzymes (nonneuronal enolase, neuron-specific enolase, and the hybrid form) and glutamine synthetase. Enolase isoenzymes were separated by ion exchange chromatography on small (0.5 x 1 cm) DEAE-cellulose columns (DE52 Whatmann) eluted with the imidazole buffer containing 40 mM KCl for the nonneuronal form, 130 mM KCl for the hybrid form, and 240 mM KCl for the neuron-specific form. The enzymatic activity was measured in the presence of 2 mM 2-glycerophosphate by monitoring the initial velocity at 240 nm (Ledig et al. 1985). Glutamine synthetase activity was estimated according to the method described by Tholey and colleagues (1983).

Because ethanol oxidation produces free radicals (Nordmann et al. 1988), the antioxidant defense system was also investigated. The superoxide dismutase activity involved in superoxide radical elimination was measured using a colorimetric technique described by Fried (1975). The superoxide dismutase activity was found in the fraction eluted from the DEAE-cellulose columns containing the nonneuronal enolase.

RESULTS

Cell Morphology

Phase-contrast microscopy of 2-week-old glial cells showed a large proportion of flat undifferentiated cells in both cell types, alcohol exposed before and during pregnancy (B) or only

before pregnancy (C) (figure 1). The same shape was also observed when only the male was exposed to alcohol.

Ultrastructural observations of 4-week-old glial cells demonstrated alterations of the mitochondria, the Golgi apparatus, and the endoplasmic reticulum. The mitochondria were swollen and contained myelin-like formations initiated from the endoplasmic reticulum (figure 2).

Biochemical Parameters

In the control cells (A), the protein content per dish increased linearly by about 3 times until 3 weeks of culture, then reached a plateau. Compared with the control cells, the protein content per dish of the B cells was significantly decreased up to 4 weeks of culture, whereas for the C cells a significant decrease was only observed after 2 weeks of culture, during the maturation phase (figure 3).

The control activity (A) of enolase increased linearly by about 2.5-fold between 1 and 4 weeks of culture. Compared with the controls, the activity of the B cells was significantly decreased up to 4 weeks of culture. The same reduction was found for the C cells (figure 3). Decreased enolase activity was even found when astrocytes were exposed to alcohol in vitro. Since enolase is a marker of nerve cell maturation, its decrease may be a biochemical basis for the mental retardation observed in fetal alcohol syndrome.

The glutamine synthetase activity of the control cells (A) increased linearly by about four times until 4 weeks of culture. The activity of the B cells as well as the C cells was significantly lower than the control values up to 3 weeks of culture. At 4 weeks, only the activity of the B cells remained decreased significantly (figure 3). Inhibition of the glutamine synthetase activity by

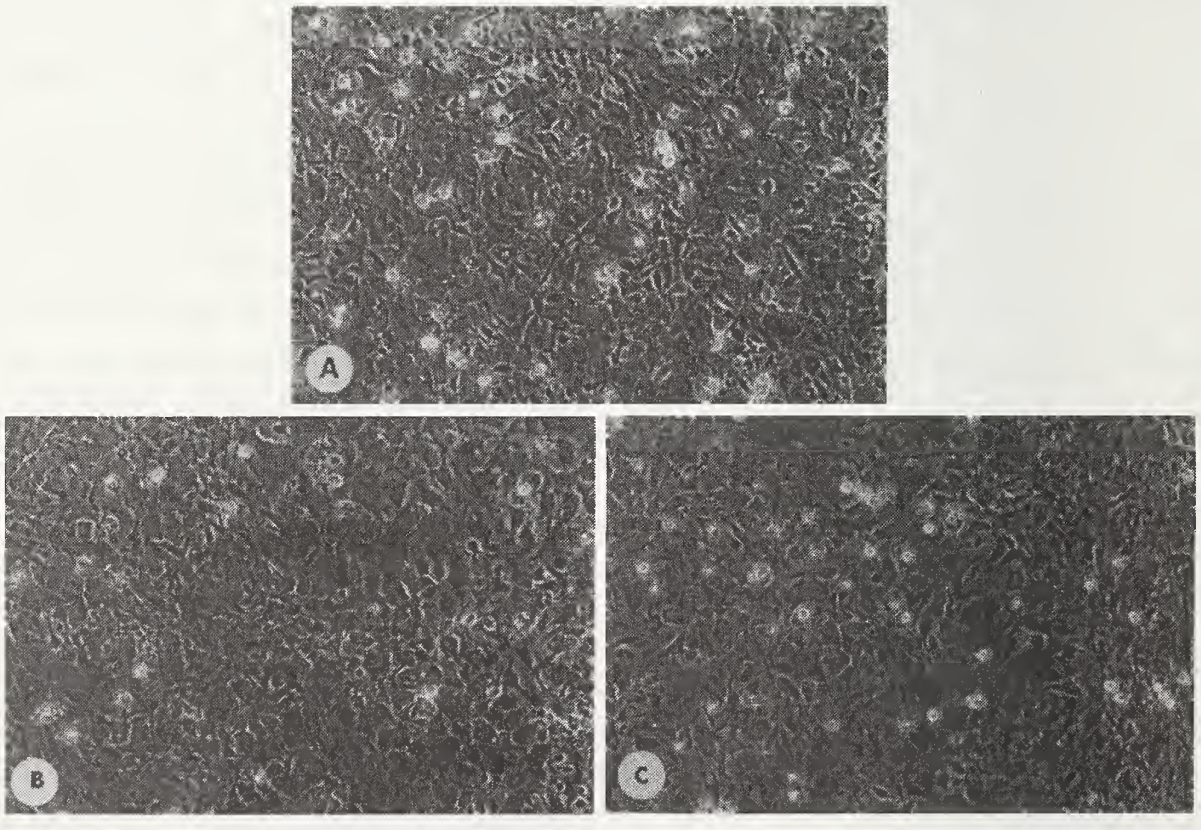

Figure 1. Phase-contrast microscopy of 2-week-old rat glial cells. (A) Control. (B) Maternal alcohol until delivery. (C) Maternal alcohol withdrawn at mating.

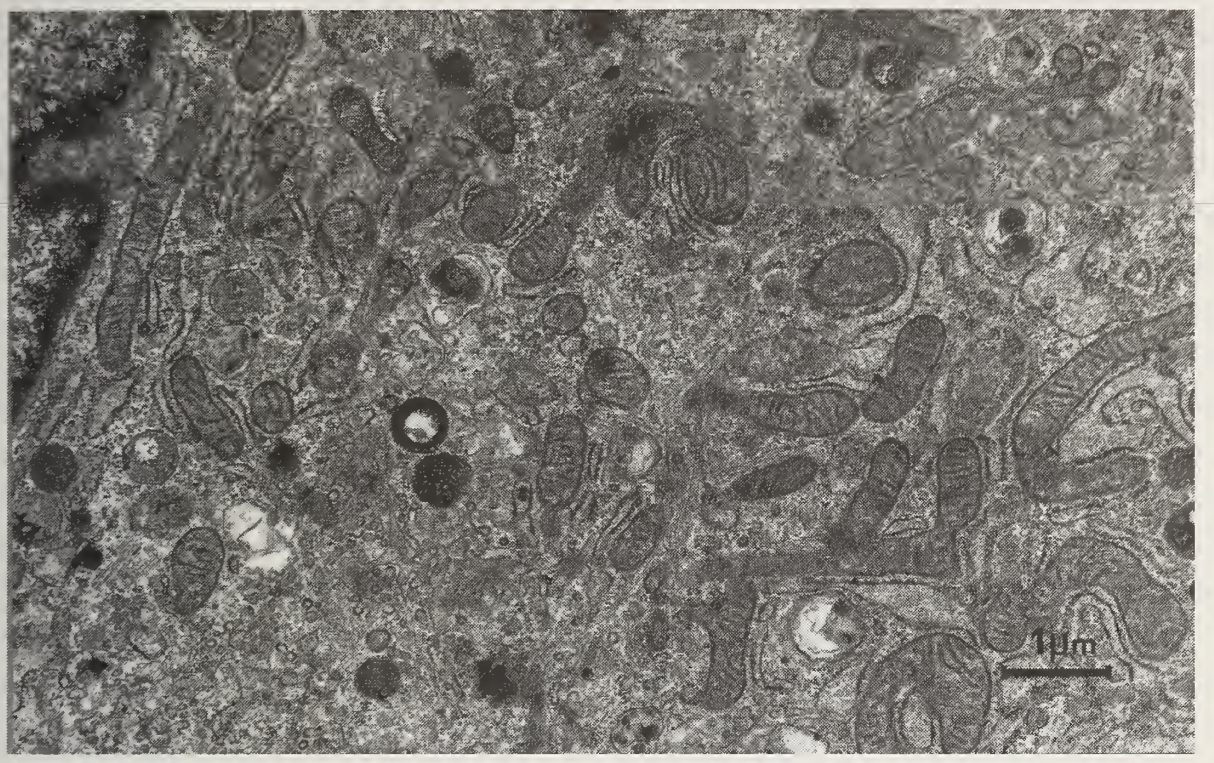

Figure 2. Electron microscopy of 4-week-old glial cells. Maternal alcohol until delivery.

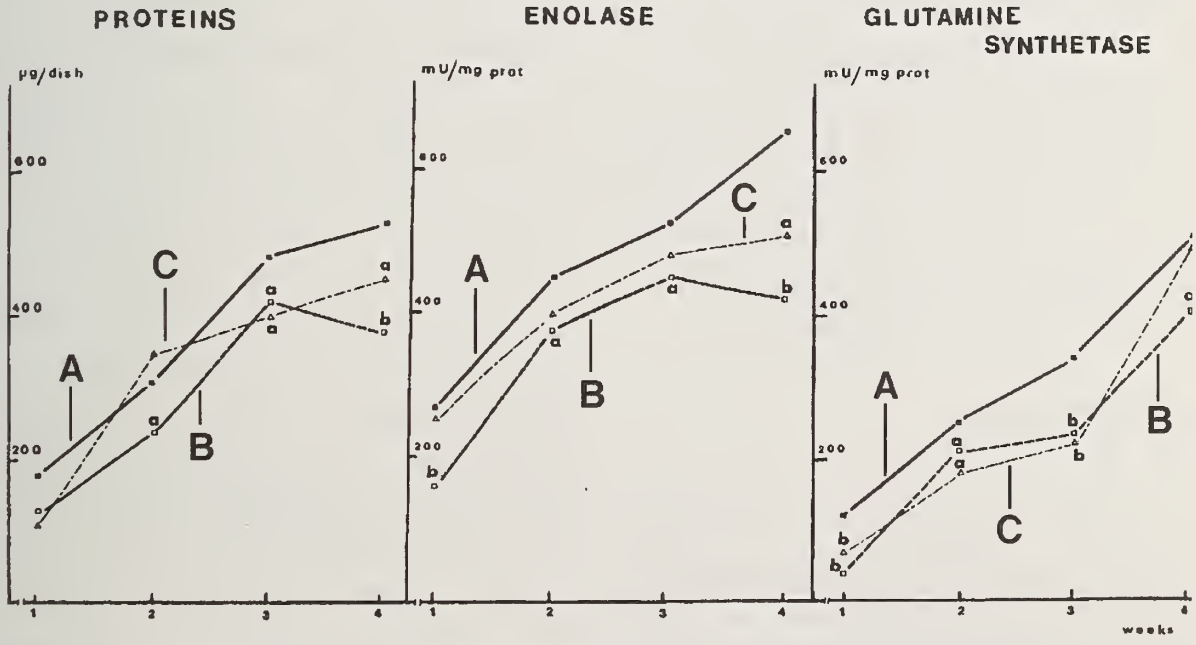

Figure 3. Protein, enolase, and glutamine synthetase activity of glial cells cultured for 4 weeks. A = control; B = maternal alcohol until delivery; C = maternal alcohol withdrawn at mating. a = $p < 0.05$; b = $p < 0.001$.

alcohol was also found in vitro (Davies and Vernadakis 1984). Since glutamine synthetase is also involved in ammonia elimination, a decrease of its activity produces an increase of ammonia which may add to the toxicity induced by alcohol and its degradation products.

Superoxide dismutase develops slowly in astrocytes. The peak of activity is only reached after 3 weeks of culture. Compared with control values (A), there was a significant decrease at 3 weeks of culture for the B and C cells and until 4 weeks only for the C cells (figure 4). That observation suggests that superoxide dismutase is not related to the maturation process but that prenatal or prepregnancy alcohol exposure affects the synthesis of the enzyme (Ledig et al. 1991*a*, 1991*b*). Low superoxide dismutase activity means a less effective elimination of free superoxide radicals formed during alcohol degradation.

Superoxide radicals may reduce nucleic acid and protein synthesis and also produce breaks in DNA. Snyder and colleagues (1992) showed that insulin-like growth factors I and II may antagonize the inhibitory effect of alcohol on DNA, RNA, and protein synthesis. The effect of alcohol depends on the cell type, the alcohol dose, and the duration of the treatment. These authors found that a relatively low dose of ethanol (50 mM) inhibited protein synthesis and amino acid uptake while thymidine uptake and incorporation into DNA were significantly increased. On the other hand, high concentrations of ethanol (100 mM) decreased RNA and DNA synthesis as well as protein content.

DISCUSSION

Opposite effects depending on the alcohol dose is a general feature found in alcohol research. A general mechanism for alcohol-induced deficit in protein synthesis involving transport and intracellular release of ions has been suggested by West and colleagues (1990). Recently Kimelberg and colleagues (1993) showed an alcohol-induced release of taurine and aspartate, two amino acids involved in neurotransmission, from primary astrocytic cultures. A similar phenomenon may be produced by hypotonicity or high potassium concentration in the culture medium.

Recent ultrastructural analysis of astrocytes cultured from rats exposed to alcohol prenatally have shown alterations of the plasma membrane glycoproteins, depending on the age of the culture. Prenatal exposure to alcohol decreased the ability of astrocytes to bind concanavalin A, altered the surface distribution of the receptors for lectin, and decreased the activity of 5' nucleotidase activity. The effects were more marked in proliferating than in mature cells (Renau Piqueras et al. 1992).

Using the same cytochemical and stereological analysis of astrocytes cultured from rats exposed to alcohol prenatally, Mayordomo and colleagues (1992) found that subcellular particles such as mitochondria, rough endoplasmic reticulum, and lysosomes exhibit qualitative and quantitative ultrastructural changes during the process of astrocyte maturation. These morphological changes were accompanied by variations of the cytochemical activity of enzymes located in these organelles, suggesting that these enzymes and the functional state of these organelles are modulated during astrocyte development. These results suggest that prenatal exposure to alcohol could affect astrocytes during development in two different but probably complementary ways, by causing a delay in astrocyte maturation and by inducing a direct toxic effect on these cells.

EFFECT OF ALCOHOL-ANTAGONIZING COMPOUNDS

Various experiments have been performed to antagonize some of the effects of alcohol on

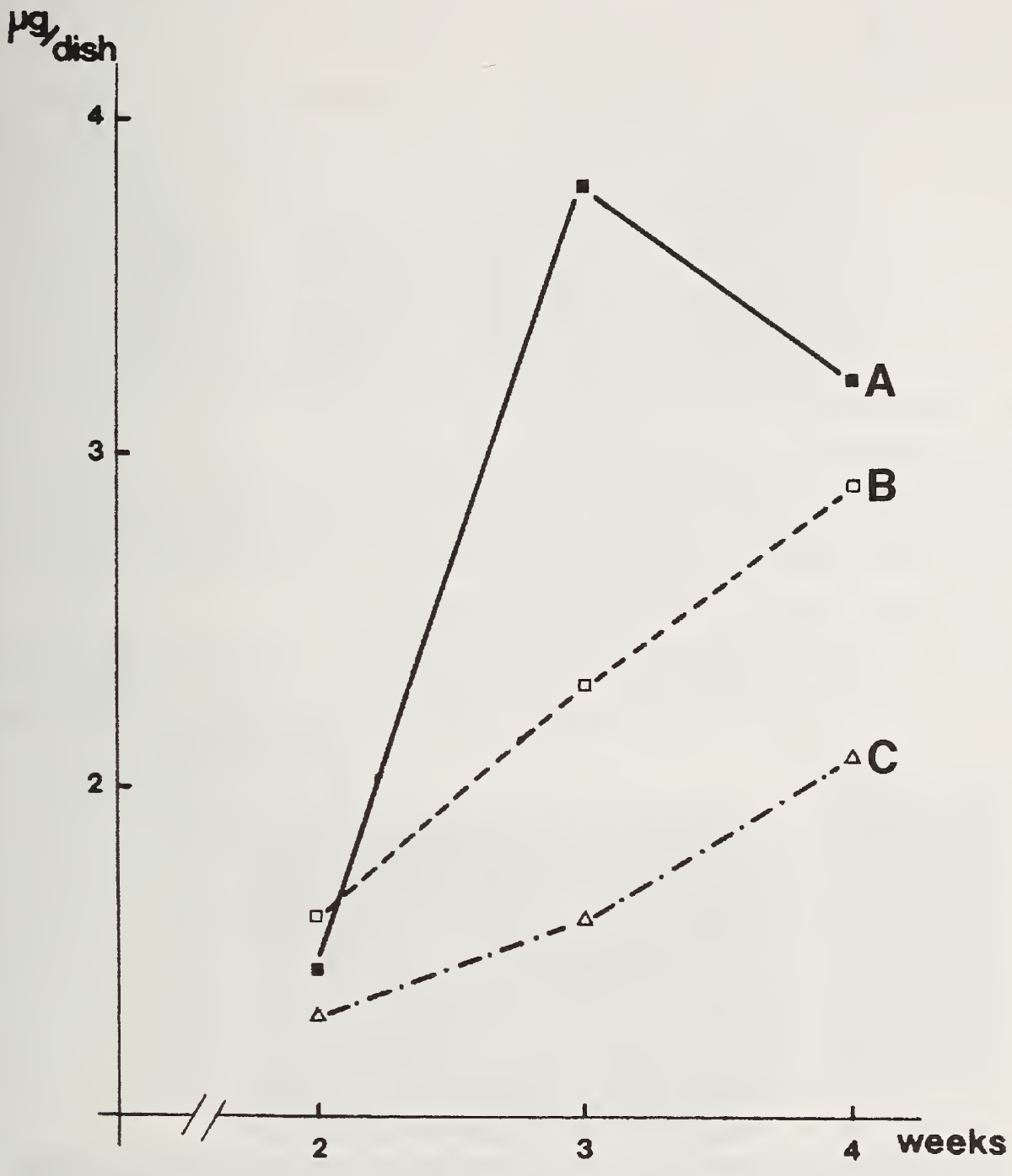

Figure 4. Superoxide dismutase of glial cells cultured for 4 weeks. A = control; B = maternal alcohol until delivery; C = maternal alcohol withdrawn at mating.

development. Klemm and colleagues (1988) found that gangliosides, or sialic acid, may be able to antagonize the effects of ethanol, specifically in glial cells. Gottesfeld and Silvermann (1990) showed that abnormalities of righting reflex, dental eruption, and eye opening could be reversed in infant rats treated with thyroid hormones during postnatal days 1–10, the period corresponding to glial cell maturation. Rezvani and colleagues (1990) demonstrated an alcohol-antagonizing effect of verapamil on physiological and behavioral parameters.

We studied the effect of manganese (Mn) on neuronal and glial cells cultured in the

presence of alcohol (Ledig et al. 1991*c*). Manganese is involved in numerous biological functions altered by alcohol intoxication, mainly protein synthesis and defense against free radical toxicity. Manganese is also a cofactor or an activator of various enzymes such as pyruvate carboxylase, pseudocatalase, peroxidase, mitochondrial superoxide dismutase, a specific ATPase, glutamine synthetase, and enolase (two markers of nerve cell development).

Chronic alcohol exposure may cause changes in the distribution of Mn. The levels of Mn were found to be increased in the brain and serum of alcoholics (Khan et al. 1984; Zarski et al. 1985). Manganese uptake in cultured nerve cells is also modified by alcohol (Tholey et al. 1988, 1990).

Morphological observation showed that in the presence of Mn (2 µM) added to the culture medium, the undifferentiated glial cells observed in the presence of 30 mM ethanol switched to more differentiated cells, close in development to the control cultures (figure 5). The same effect was found in neuronal cultures.

Biochemical parameters confirmed the morphological modifications in 2-week-old primary cultures of glial cells and also in C6 cells. Compared with controls, protein levels were increased when Mn was added alone. Compared with cells treated with alcohol only (figure 6), the levels were also increased when alcohol was supplemented with Mn. Also, enolase (figure 7) and glutamine synthetase activity (figure 8) approached control values when alcohol-treated glial cells were supplemented with Mn. Alcohol alone decreased glutamine synthetase activity. This effect of Mn was not found for neurons.

Manganese also activated the antioxidant defense system, cytosolic and mitochondrial superoxide dismutase, when 3-week-old primary

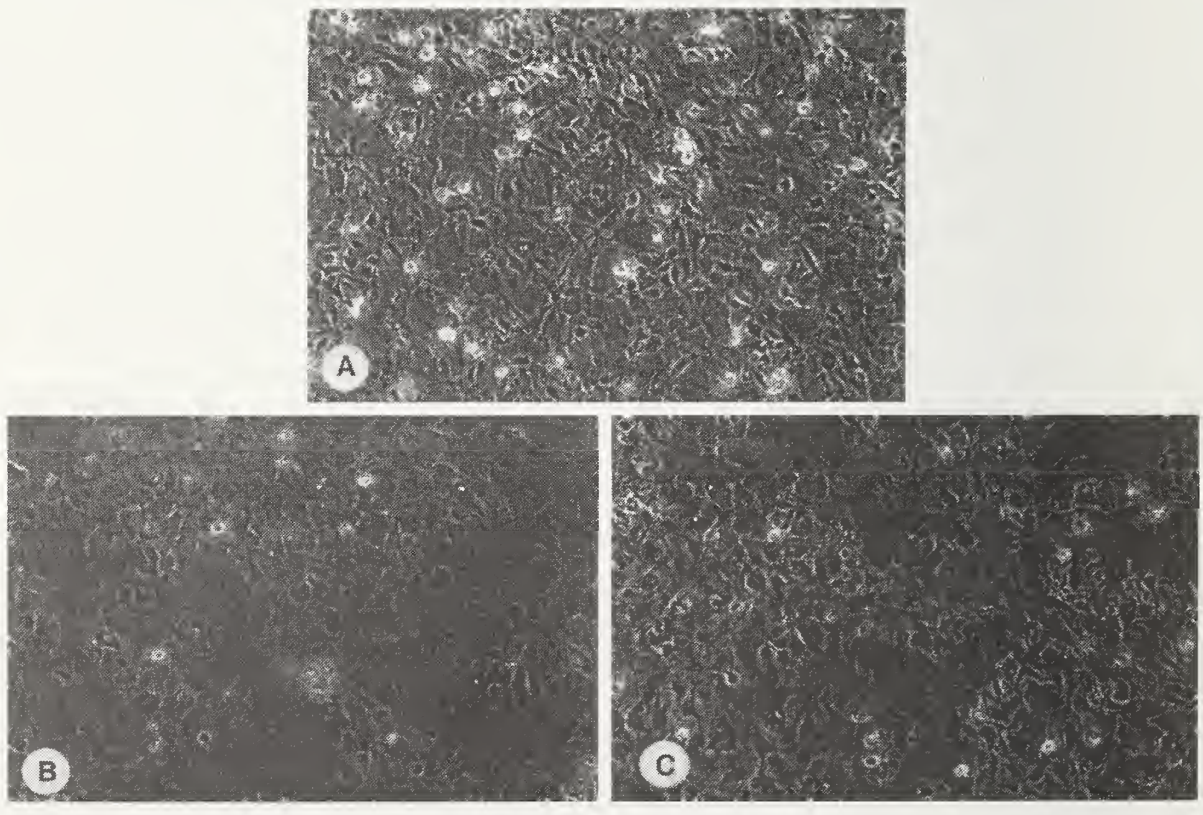

Figure 5. Phase-contrast microscopy of rat glial cells cultured for 15 days. (A) Control. (B) 30 mM ethanol added in vitro. (C) 30 mM ethanol + 2 µM Mn added in vitro.

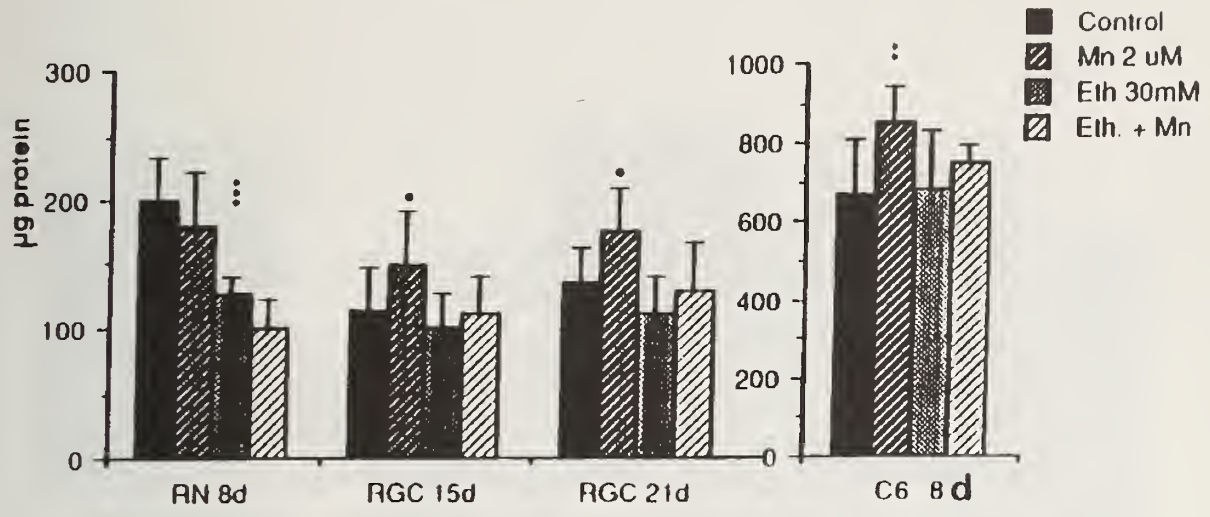

Figure 6. Protein level (µg/dish) of 8-day-old rat neurons (RN 8d), 15-day-old rat glial cells (RGC 15d), 21-day-old rat glial cells (RGC 21d), 8-day-old C6 glioma cells (C6 8d). Cells treated with 30 mM ethanol only and 30 mM ethanol + 2 µM Mn were compared with controls and those to which only 2 µM Mn had been added in vitro. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

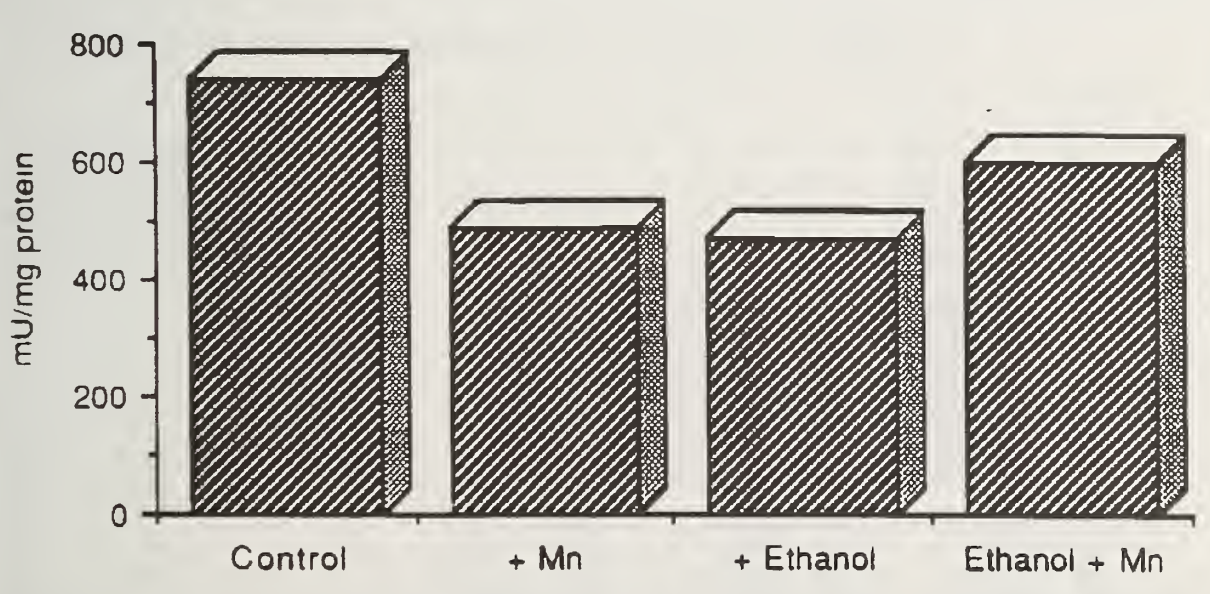

Figure 7. Enolase activity of 15-day-old rat glial cells. Cells treated with 30 mM ethanol only and 30 mM ethanol + 2 µM Mn were compared with controls and those to which only 2 µM Mn had been added in vitro.

cultures of glial cells as well as C6 cells were treated with alcohol combined with Mn (figure 9). Since both the cytosolic (Cu^{2+}, Zn^{2+} form) and the mitochondrial activities (Mn^{2+} form) are stimulated, it seems likely that the mechanism of activation is not exclusively related to a direct effect on Mn-dependent enzymes.

We were able to reproduce in vivo the alcohol-antagonizing effect of Mn by adding $MnCl_2$ (25 mg/L) to the 20 percent (vol/vol) ethanol consumed by female rats during pregnancy. When Mn was given with alcohol, we observed that the pup weights were no longer significantly different from the control values,

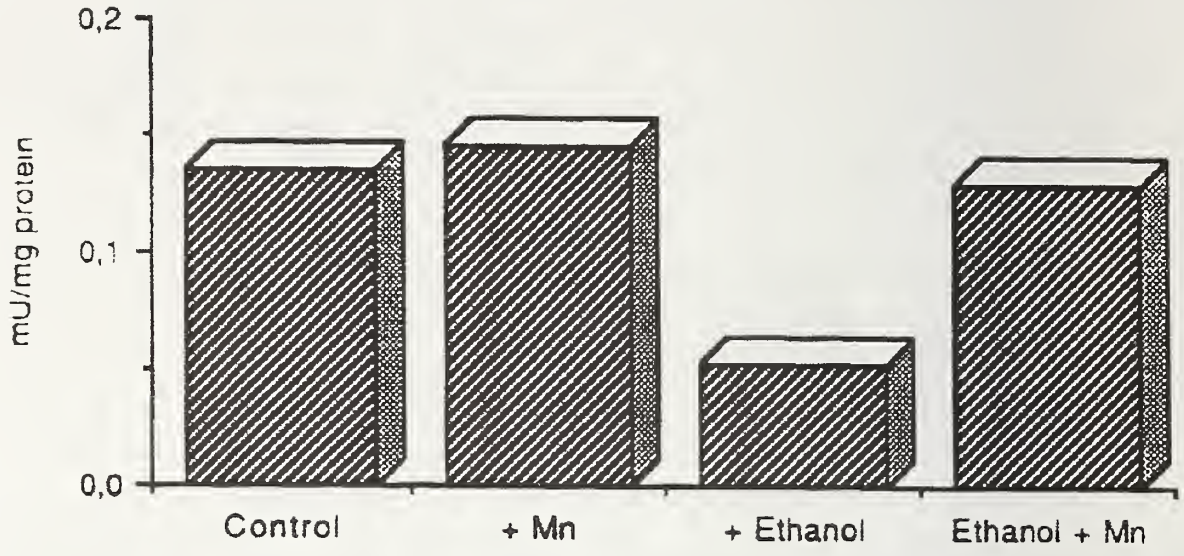

Figure 8. Glutamine synthetase activity of 15-day-old rat glial cells. Cells treated with 30 mM ethanol only and 30 mM ethanol + 2 µM Mn were compared with controls and those to which only 2 µM Mn had been added in vitro.

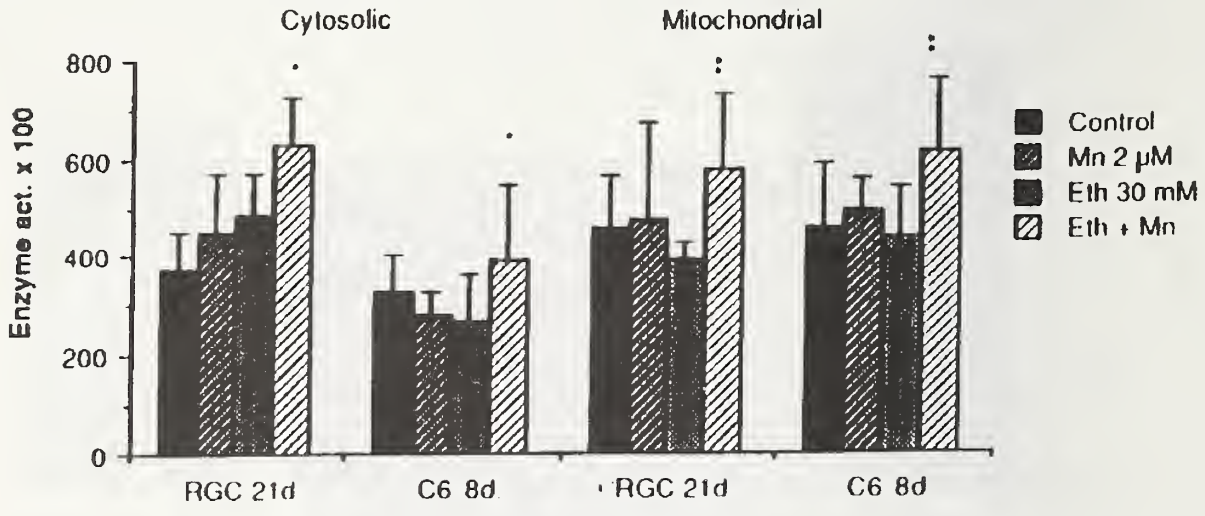

Figure 9. Superoxide dismutase activity (µg/mg protein) of 21-day-old rat glial cells (RGC 21d) and 8-day-old C6 glioma cells (C6 8d). Cells treated with 30 mM ethanol only and 30 mM ethanol + 2 µM Mn were compared with controls and those to which only 2 µM Mn had been added in vitro. $^{*}p < 0.05$; $^{**}p < 0.01$.

compared with decreased pup weights found for mothers consuming alcohol only (figure 10).

Glial cells were cultured for 4 weeks from cortical brain cells of these pups. Compared with the animals treated with alcohol only, the protein levels of the cells treated with alcohol + Mn were significantly increased during the proliferation period (figure 11). The same effect was observed for enolase (figure 12) and glutamine synthetase activity (figure 13). When the alcohol + Mn treatment lasted for a longer period (3 months), only the effect on the protein level was observed. There was no longer an effect on enolase and glutamine synthetase.

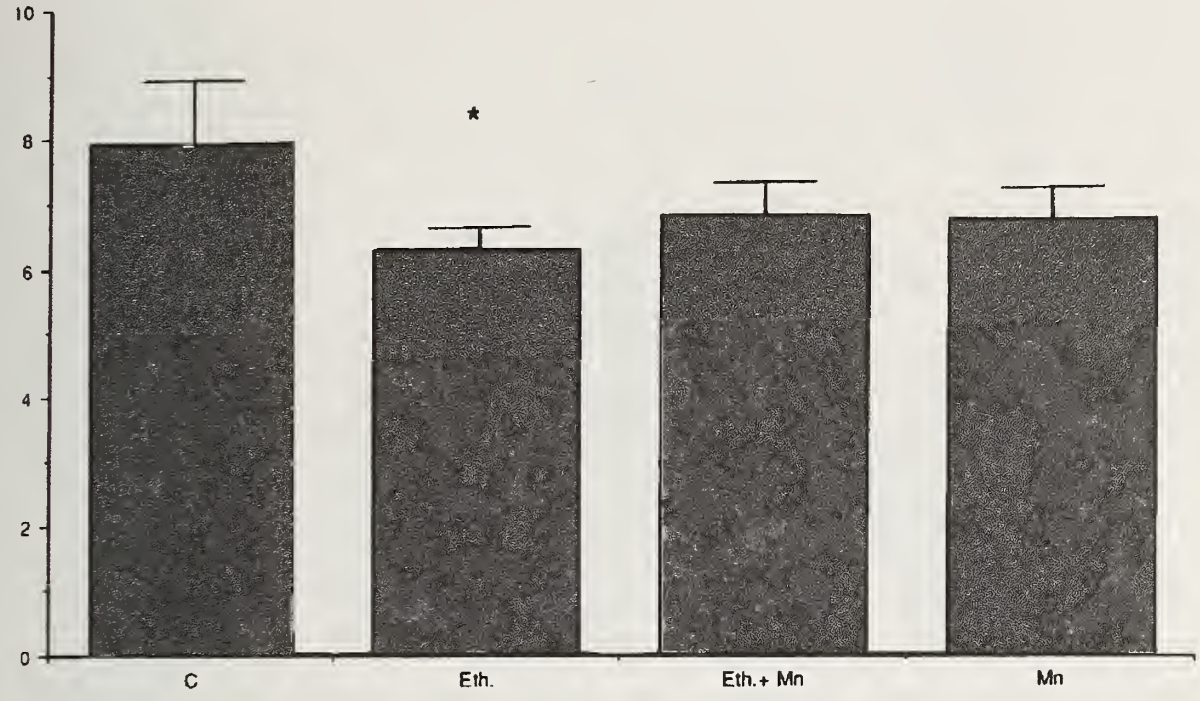

Figure 10. Pup weights at birth (in grams) of mothers treated prenatally with 20 percent ethanol and mothers treated prenatally with 20 percent (vol/vol) ethanol + $MnCl_2$ (25 mg/L) compared with pup weights of controls and mothers treated only with Mn. *$p < 0.001$.

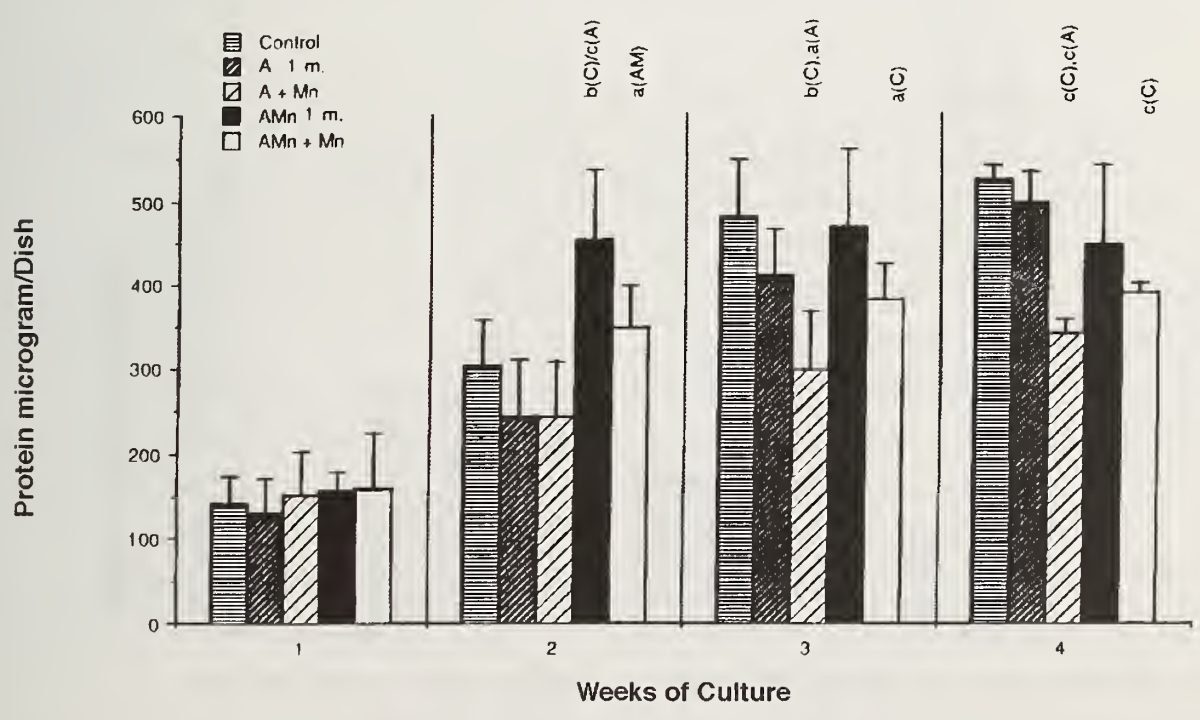

Figure 11. Protein of rat glial cells cultured for 4 weeks from pups born to alcohol-treated and control mothers. A = prenatal alcohol; AMn = prenatal alcohol + Mn; A+Mn = prenatal alcohol + 2 µM Mn in vitro; AMn + Mn = prenatal alcohol + Mn, and + 2 µM Mn in vitro. (C) = versus control; (A) = versus alcohol; (AM) = versus alcohol and Mn in vivo. a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$.

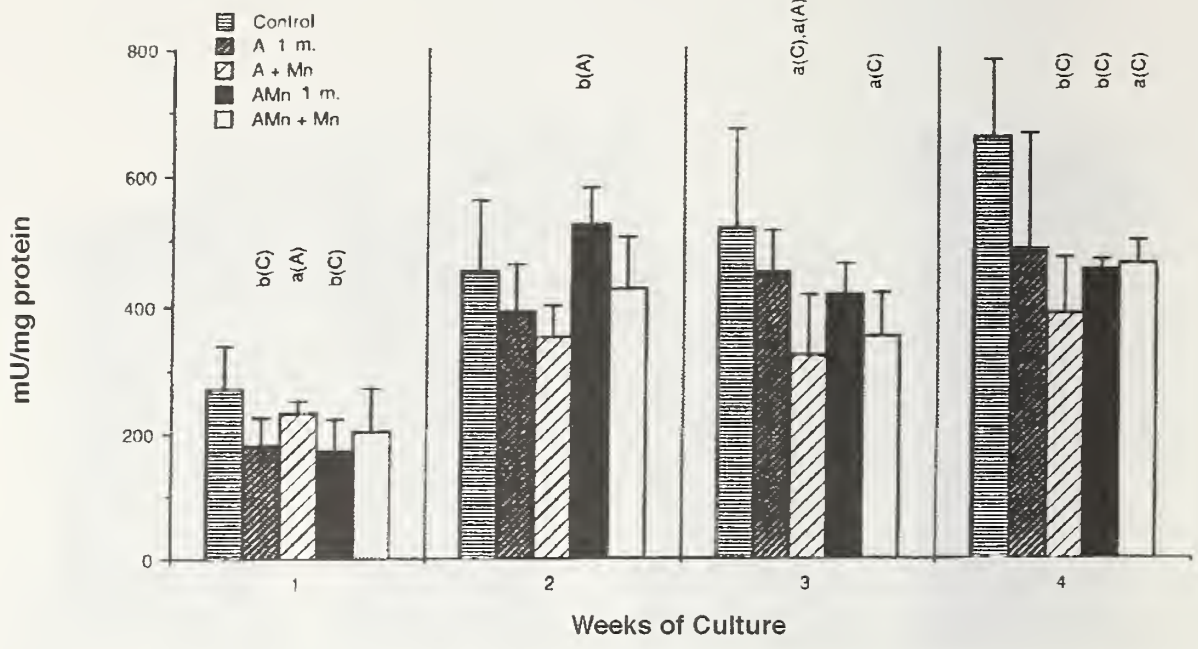

Figure 12. Enolase activity of rat glial cells cultured for 4 weeks from pups born to alcohol-treated and control mothers. A = prenatal alcohol; AMn = prenatal alcohol + Mn; A+Mn = prenatal alcohol + 2 µM Mn in vitro; AMn + Mn = prenatal alcohol + Mn, and + 2 µM Mn in vitro. (C) = versus control; (A) = versus alcohol; (AM) = versus alcohol and Mn in vivo. a = $p < 0.05$; b = $p < 0.01$.

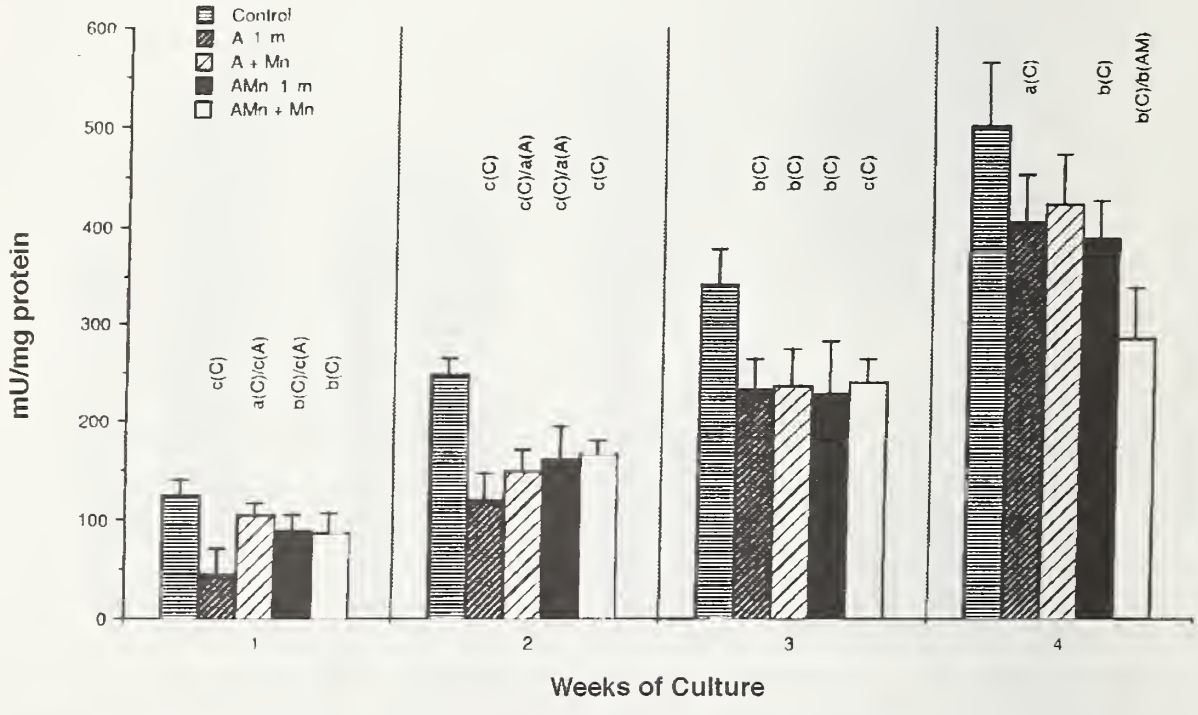

Figure 13. Glutamine synthetase activity of rat glial cells cultured for 4 weeks from pups born to alcohol-treated and control mothers. A = prenatal alcohol; AMn = prenatal alcohol + Mn; A+Mn = prenatal alcohol + 2 µM Mn in vitro; AMn + Mn = prenatal alcohol + Mn, and + 2 µM Mn in vitro. (C) = versus control; (A) = versus alcohol; (AM) = versus alcohol and Mn in vivo. a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$.

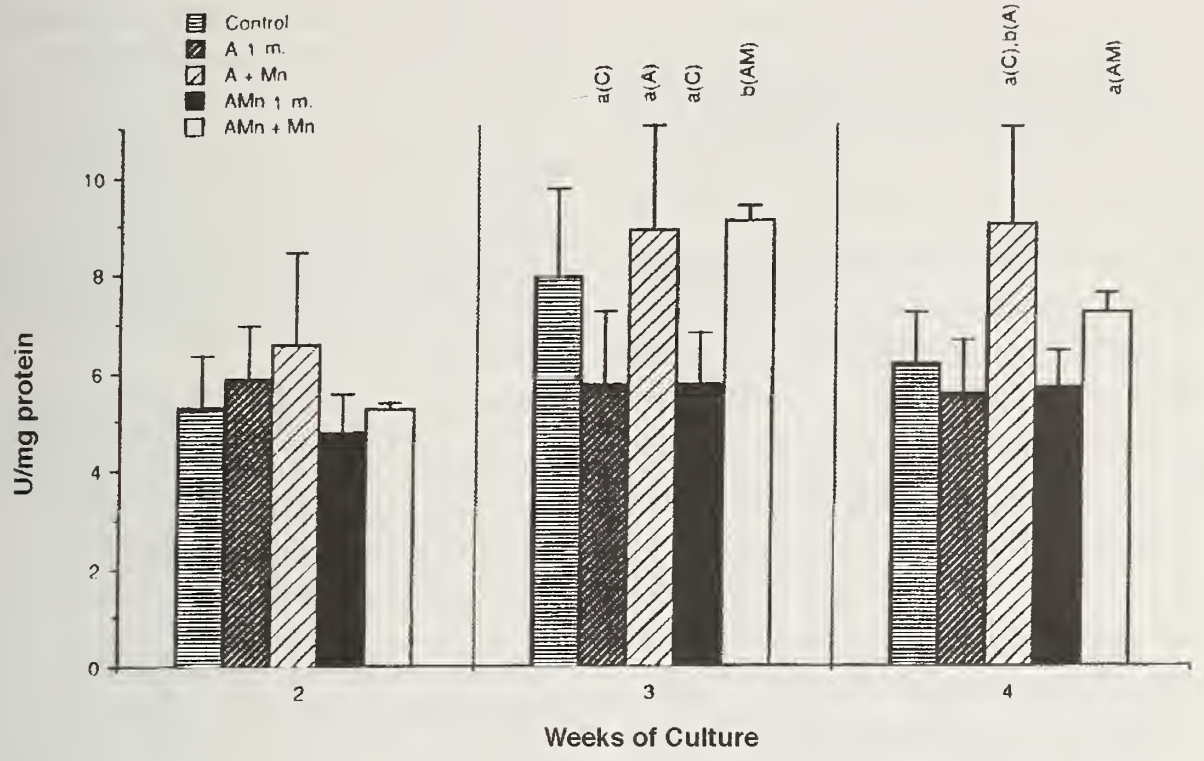

Figure 14. Superoxide dismutase activity of rat glial cells cultured for 4 weeks from pups born to alcohol-treated and control mothers. A = prenatal alcohol; AMn = prenatal alcohol + Mn; A+Mn = prenatal alcohol + 2 µM Mn in vitro; AMn + Mn = prenatal alcohol + Mn, and + 2 µM Mn in vitro. (C) = versus control; (A) = versus alcohol; (AM) = versus alcohol and Mn in vivo. a = $p < 0.05$; b = $p < 0.01$.

For superoxide dismutase activity, we found no action of Mn when added to the alcohol diet of the mothers. We only observed a stimulation of superoxide dismutase activity when Mn was added in vitro to these cultures (figure 14). These results are similar to our previous reports of increased superoxide dismutase activity when Mn was added in vitro to alcohol-treated glial cells of control animals (Ledig et al. 1991*c*).

We conclude that Mn at micromolar concentrations is able to antagonize some alcohol effects on glial cell development.

Recently, we experimented with another alcohol-antagonizing substance found to have an effect on alcohol-induced disorders of neurotransmitter metabolism (Anokhina et al. 1990). This substance is a plant extract from grapes. When added in vitro to alcohol-treated glial cells, there was an antagonizing effect on superoxide dismutase activity. When added in vivo to the alcohol diet of pregnant mothers, there was some positive effect on the development of the glial cells cultured from the brain of the offspring. Protein levels returned to control values. Enolase activity was even increased compared with the control values when the treatment lasted 2 months. Glutamine synthetase activity was also increased over the control values, but only when the plant extract was administered together with alcohol during pregnancy. Superoxide dismutase was stimulated, reaching control values when the treatment lasted no longer than 2 months. Our data suggest that a grape extract probably containing polyphenol compounds may act as an antioxidant, as well as a development-stimulating factor on glial cells.

CONCLUSION

It seems now well established that astroglial cells are more sensitive to alcohol than neurons, considering developmental parameters and morphological observations. The effect occurs mainly during the period of cell multiplication, as shown by in vitro and in vivo alcohol treatment. Other glial cell types like oligodendrocytes are affected in the same manner as astrocytes. Exposure to alcohol during prepregnancy only as well as paternal alcohol exposure only were also found to have some effect on glial cell development. These observations demonstrate that alcohol produces alterations at the level of the genome. Experimental models may help us to understand the mechanisms underlying the neurological dysfunctions appearing in fetal alcohol syndrome. Experimental models also allow us to test the effect of alcohol-antagonizing compounds such as oligoelements, plant extracts, growth factors, or antioxidants in order to elaborate a therapy for fetal alcohol syndrome.

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Chapter 8

Alcohol and Neuroimmune Dysfunctions of the Central Nervous System

Sujata Tewari, Ph.D., and Virginia Carson, Ph.D.

The major goals of this chapter are to examine the possible roles of alcohol (ethanol) in causing neuroimmunological dysfunctions of the central nervous system (CNS), specifically focusing on the developing brain.

The CNS is composed of the cerebrum, the cerebellum, the brain stem, and the spinal cord. It is bathed both internally and externally by the cerebrospinal fluid (CSF), which circulates through the ventricular and leptomeningeal spaces. The white matter in the CNS consists of axons and their myelin sheaths and two types of glial cells (oligodendrocytes and astrocytes). The CNS parenchyma also contains microglial cells, macrophages, and blood vessels. Both the astrocytes and oligodendrocytes are ectodermal; the smaller microglia are mesodermal in origin. The microglial cells can become very mobile and active macrophages when necessary. For example, microglia will phagocytize debris (1) when some CNS elements degenerate during normal wear and tear or (2) under adverse conditions, such as trauma, when they can become activated and migrate to the area of injury. Glial cells, unlike neurons, lack synaptic contacts and have the ability to divide or replicate throughout their lifespan, specifically in response to injury. In the CNS, virtually nothing can penetrate or enter the CNS parenchyma without being confronted by an astrocytic interphase (Raine 1976).

A review of the literature demonstrates an increasing recognition of the role that the CNS plays in the regulation of the immune system, possibly by acting as the modulator of the immunological apparatus (Calabrese et al. 1987). The cerebral tissue within the CNS has been recognized as an immunologically privileged site, with the astrocytes having the ability to initiate immune responses (Massa and ter Meulen 1987). Furthermore, astrocytes are thought to secrete factors that are necessary for neuronal growth and to act to expedite neuronal recovery from injury (Frohman et al. 1988; Kim et al. 1985). The ability of astrocytes to respond to biological effectors is thus an important tool in designing neuroimmunological studies. Finally, astrocytes have been identified as one of the

process-bearing cells containing proteins made by the acquired immunodeficiency syndrome (AIDS) virus (Barnes 1986).

A major response of astrocytes is the ability to secrete cytokines, which are critically involved in generating inflammatory responses. Alcohol is now recognized for its adverse effects on the immune system, affecting the release of cytokines in both human and animal models. Taking into consideration all of this information, in this chapter we examine the possibility of whether alcohol-induced alterations in the neuroimmunological properties of astrocytes could affect their infectibility and susceptibility to retroviral infection. Attention is paid to alcohol exposure conditions, human versus animal studies, and the involvement of astrocytes and/or microglia in clarifying the role of alcohol as a possible cofactor in the transmission of viral infection in the CNS. We also assess the relationship between the nervous system and the immune system and how it may be influenced by alcohol exposure. This includes the possibility that alcohol may act by interfering with astrocytes' ability to secrete cytokines, like tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6), or to express immune-associated (Ia) antigens following exposure to various biological effectors.

This review should be helpful in understanding the diverse effects of alcohol in mammalian CNS and in generating leads that will be significant in the development of new research directions.

ALCOHOL AND IMMUNE FUNCTION

Chronic alcohol use over a long period of time leads to a variety of clinical disorders of the CNS, including neurological dysfunctions, affective disorders, encephalopathies, dementia, cognitive dysfunctions, mental retardation, and problems with memory, learning, and conceptual abilities in children born with fetal alcohol syndrome (FAS) or fetal alcohol effects (FAE) and in adults. Alcohol is now recognized as a teratogen and the primary agent involved in producing the symptoms of FAS/FAE (Clarren and Smith 1981). This is not surprising since, within the CNS, the cerebral tissue is one of the most affected organs in fetuses or newborns afflicted with congenital malformations and birth defects. In addition, some of the primary consequences of chronic alcohol ingestion are the development of tolerance and physical dependence as manifested by heightened CNS excitability following alcohol withdrawal, the development of cross-tolerance to other similarly acting drugs, and teratogenic effects.

Although it exerts its primary effects on the CNS, alcohol has also been identified for its powerful immunosuppressive effects (Kaplan 1986). Both adult alcoholics and FAS-affected children are known to suffer from increased incidences of life-threatening infections. Although the exact relationship of the immune system and exposure to alcohol remains unclear, evidence points to an alcohol-induced interference of the normal immune defense mechanisms. Excessive alcohol consumption has been found to alter host defense mechanisms. Clinical and experimental studies show that alcohol impairs cell-mediated immunity, which is important for the organisms' defense against viral and fungal infections and tuberculosis (MacGregor 1987). In recent years, alcohol consumption has been associated with rising incidences of malignancies and is considered

Table 1. Clinical Consequences of Chronic Alcohol Use

- Liver disease
- Diseases of the gastrointestinal system and the pancreas
- Cardiomyopathy and endocrine system disorders
- Cancer, infectious diseases (e.g., head and neck cancer, pulmonary infection)
- Immunosuppressive effects/dysfunctions of the immune system
- CNS damage and dysfunctions
  - Development of tolerance and physical dependence
  - Development of cross-tolerance
  - Cerebellar degeneration and damage to brain cells
  - Wernicke-Korsakoff syndrome
  - Fetal alcohol effects/fetal alcohol syndrome
  - Learning, memory, and cognitive deficiencies
  - Dementia, encephalopathy, depression
- Cofactor in transmission/rapid progression of AIDS?
- Role in viral infection/replication in the brain?

to be an increased risk factor for certain types of cancer. Therefore, long-term use of alcohol has profound effects in chronic alcoholics.

Table 1 summarizes the clinical consequences of chronic alcohol exposure.

Clinical observations on the effects of alcohol have been supported by experimental studies (Tewari and Sytinsky 1985). The vulnerability of the developing brain to prenatal alcohol exposure can be assessed from observations in our laboratory (Tewari et al. 1989, 1992) that alcohol induced interferences with the translational control mechanisms of brain messenger RNA (mRNA). The in vitro translation of hippocampal mRNA in a message-dependent reticulocyte lysate system was significantly inhibited in 9-day-old neonatal rat brain when alcohol exposure occurred in utero during the third trimester of pregnancy. The observed inhibition was found to be irreversible, continuing throughout maturity as demonstrated by reduced in vitro translation of poly(A)+ mRNA in the cerebral cortex, the hippocampus, and the cerebellar regions of the mature brain of offspring. However, the degree of sensitivity varied among the three regions tested, with the hippocampal region being the most affected region (figure 1). It should be noted that under similar experimental conditions, polysomes from the hippocampal and the cerebellar regions of the control group demonstrated the highest and the lowest mRNA translational activities, respectively, among all the regions tested.

These data are consistent with clinical findings and may explain the learning problems and intellectual deficits experienced by FAS children. Experimental studies by West and colleagues (1981) demonstrating behavioral deficits using in utero alcohol–exposed rats lesioned in the septal hippocampal region also confirm this notion. Furthermore, the presence of reactive astrocytes in the hippocampus following triethyltin injury or in aging demonstrates the high sensitivity of this region to chemical injury or developmental/maturational changes (Brock and

O'Callaghan 1987). It was concluded from these data that alcohol-induced inhibition of protein synthesis would be a major factor in causing interference with growth, proliferation, and secretory activities of astrocytes in various brain regions, particularly in the hippocampus (Tewari et al. 1992*a*). The consequences of astrocytic inability to perform at optimum levels could have long-term effects on several aspects of neuronal growth and recovery from injury.

Children born with FAS or FAE exhibit pre- and postnatal growth deficiencies, motor dysfunctions, physical malformations, mental retardation, high susceptibility to bacterial infections, and a propensity to minor infections (Rosett and Weiner 1982; Streissguth et al. 1984). Clinically, prenatal alcohol exposure is considered to be responsible for the development of malignancy or autoimmune processes, including defects in the immune system playing a role in the pathogenesis of alcoholic liver disease. Also established are the subnormal responses of human and murine lymphocytes to alcohol exposure in vitro (Fernandez et al. 1982;

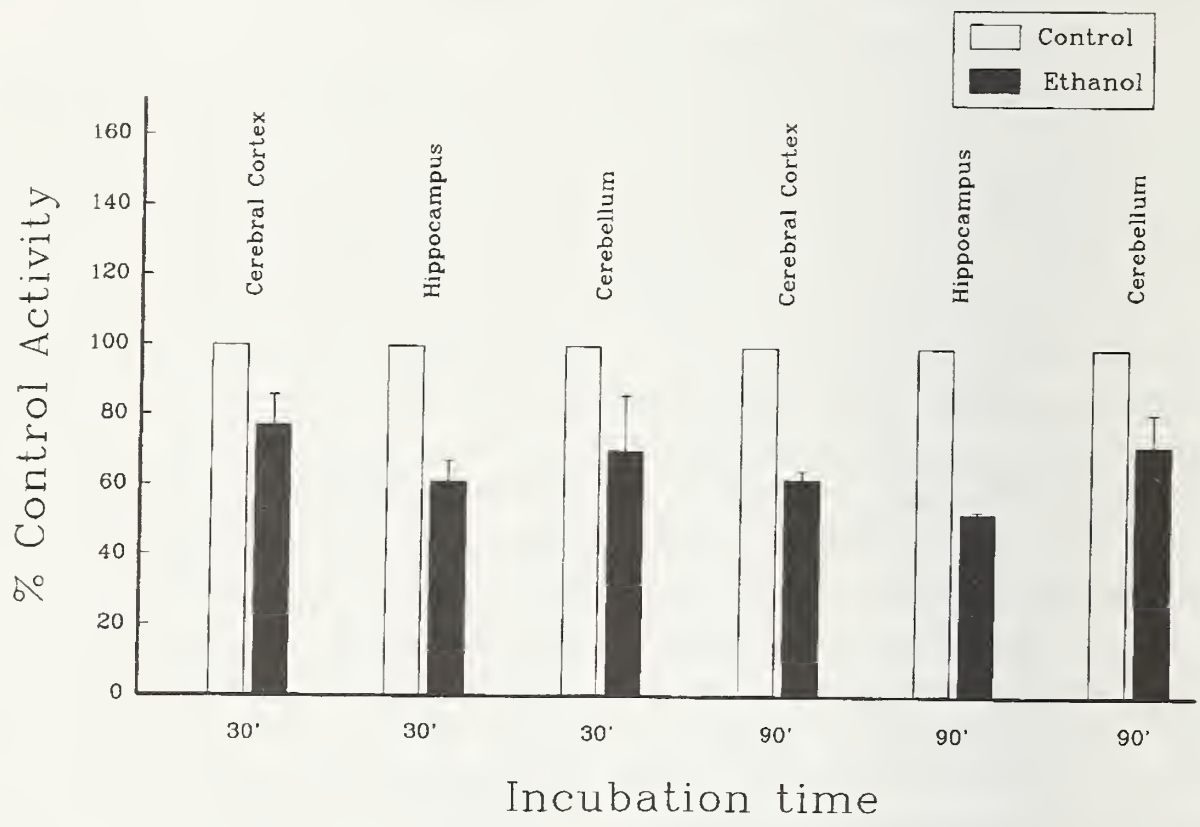

Figure 1. Ethanol effects on the translational rate of polysomal RNA's in separate brain regions. Experimental conditions are similar to those described elsewhere (Tewari et al. 1992). Average values are expressed as mean ± SEM. The 100 percent control activity is expressed as ^{35}[S]-methionine (cpm x 10^{-3}) incorporated/mg polysomal protein. The ^{35}[S]-methionine–incorporating activities are (1) at 30 minutes, 50 x 10^{-3} cpm for cerebral cortex, 110 x 10^{-3} cpm for hippocampus, and 25 x 10^{-3} cpm for cerebellum; and (2) at 90 minutes, 275 x 10^{-3} cpm for cerebral cortex, 350 x 10^{-3} cpm for hippocampus, and 75 x 10^{-3} cpm for cerebellum. Rats were 45 days old in this study. Reprinted from Tewari, S.; Diano, M.; Bera, R.; Nguyen, Q.; and Parekh, H. Alterations in brain polyribosomal RNA translation and lymphocyte proliferation in prenatal ethanol-exposed rats. *Alcohol Clin Exp Res* 16(3):436–442, 1992.

Glassman et al. 1985; Johnson and Williams 1986; Johnson et al. 1981; Walia et al. 1987; Wards et al. 1981). The immunological studies have further reported that children born with FAS exhibit decreased E rosette–forming lymphocytes, have inhibited colony-stimulating factor activity from T lymphocytes, and respond negatively to a variety of mitogens such as phytohemagglutinin (PHA), pokeweed, and concanavalin A (Con A) (Imperia et al. 1984). These clinical observations gained support from experimental studies utilizing the mouse or rat model (Abel et al. 1983; Randall and Taylor 1979; Sulik et al. 1981).

Chang and Norman (1992) studied the effects of feeding 3- to 4-month-old C57BL/6 mice a 5 percent vol/vol ethanol liquid diet for 28–36 days. The mouse splenic cells were cultured in the presence of plant lectins to assess T-cell proliferation, interleukin-2 (IL-2) production, and the expression of T-cell subset markers (CD4+/CD8+). T-cell proliferation was suppressed, but ethanol had no effect on IL-2 production. The authors proposed that this suppression was due to selective interference with events following the IL-2–IL-2 receptor interaction. Blank and colleagues (1991) gave female C57BL mice various diets—unrestricted (ad libitum), moderately restricted (2.2 g/d), and severely restricted (1.8 g/d)—with water or 20 percent wt/vol ethanol. Baseline natural killer cell activity was suppressed 50–90 percent in all ethanol and food-restricted groups.

Mitogenic stimulation of T-cell and B-cell proliferation was used to study immunological suppression in adult Sprague-Dawley rat pups exposed in utero to ethanol (Tewari et al. 1992*a*). In this study, ethanol was administered to pregnant rats in a high-protein, vitamin-fortified Lieber-DeCarli liquid diet where 35 percent of the calories were ethanol derived (35 percent EDC diet containing 6.6 percent vol/vol ethanol). Controls were *pair fed* and postnatal fostering conditions were used to avoid transfer of maternal factors to the newborn. The immunological functions of the pups were examined by measuring the mitogenic responses of lymphocytes cultured from spleen. Lymphocytes were first exposed for 48 hours to the following mitogens: PHA, which acts on both T cells and B cells; Con A, which is specific for T cells; and lipopolysaccharide (LPS), which is specific for B cells. The cells were then labeled and incubated for 16 hours in the presence of ^{3}[H]-thymidine. Data in table 2 show that in lymphocytes isolated from control pups, ^{3}[H]-thymidine uptake into cold trichloroacetic acid (TCA)–insoluble residue was stimulated by as much as 581 percent with PHA, 700 percent with Con A, and 400 percent with LPS. However, under the same conditions, the mitogenic responses of lymphocytes prepared from ethanol-exposed pups were substantially reduced.

These findings suggest that prenatal ethanol exposure, by affecting the functions of both the T-cell and B-cell lymphocyte populations, does have the ability for long-lasting interference with the proper functioning of the immune system of the offspring. Data further suggest that IL-2 activity may be affected by ethanol, because in order to proliferate, the T cell requires the synthesis and secretion of IL-2, the acquisition of IL-2 surface receptors, and the interaction of IL-2 with these receptors. Therefore, the inhibition of IL-2 production and its interactions may be a major mechanism by which ethanol exerts its

immunosuppressive effects. The involvement of IL-2 is in agreement with earlier studies of Kaplan (1986), who also proposed an ethanol-induced inhibition of IL-2 binding with its receptor.

In summary, these studies establish that the outcome of prenatal ethanol exposure is extremely serious. The effects are irreversible and diverse, as seen by the damages sustained by the CNS and the immune systems of the developing offspring.

THE CNS AND THE IMMUNE SYSTEM

Immune-Response Properties of Astrocytes

The mammalian brain is recognized for its heterogeneity, extensive postnatal development, and high sensitivity to pharmacological agents and environmental alterations. Ethanol-induced inhibition of fetal and neonatal brain RNA and protein synthesis has been reported at the transcription level (Rawat 1985, 1989; Tewari and Crain 1980).

The brain is distinguished from other organs by (1) the presence of the blood-brain barrier, which restricts and regulates the access of cells, chemicals, and other macromolecules to the brain; (2) the absence of lymphatic drainage; and (3) the capability of expression of molecules encoded by the major histocompatibility complex (MHC) genes, which function to both initiate and modulate immune responses in the brain (Wong et al. 1985). The MHC genes are a tightly linked complex of genes located on chromosome 17 in the mouse and chromosome 6 in the human. These genes encode a series of cell surface antigens, the MHC antigens, that play critical roles in generating immune responses and are responsible for the rapid rejection of tissue grafts from one member of a species to another (Klein et al. 1981).

Table 2. Ethanol Effects on the Mitogenic Responses of Lymphocytes

Experimental Conditions	^{3}[H]-Thymidine Incorporation cpm x 10^{-3}	Percent Activity
Control cells		
Baseline activity	9.055 ± 9.96	100
+PHA	52.633 ± 2.22	581
+Con A	63.385 ± 5.3	700
+LPS	36.230 ± 4.4	400
Ethanol cells		
Baseline activity	7.543 ± 0.5	100
+PHA	18.12 ± 0.4	240
+Con A	5.66 ± 0.80	75
+LPS	4.90 ± 0.12	65

Mitogens were added at 10 µg/mL concentrations. Prior to mitogen exposure, there were 0.2 x 10^6 cells. Forty-eight hours after adding the mitogens, cultures were labeled for 16 hours with ^{3}[H]-thymidine. The incorporation of ^{3}[H]-thymidine into DNA was determined by TCA extraction procedure as described in the text. Results are expressed as mean ± SEM. Reprinted from Tewari, S.; Diano, M.; Bera, R.; Nguyen, Q.; and Parekh, H. Alterations in brain polyribosomal RNA translation and lymphocyte proliferation in prenatal ethanol-exposed rats. *Alcohol Clin Exp Res* 16(3):436–442, 1992.

The class I MHC antigens (H-2, K, D, and L in the mouse) are transplantation antigens and, among these, the H-2 antigens are the best studied. These antigens are present on most cells of the body and have been reported in adult but not young mouse brains (Schachner and Sidman 1973). The class II MHC antigens (Ia) are cell surface glycoproteins, which are found on a more restricted range of cell types than are H-2 antigens. The cell types are B lymphocytes, T4 cells, macrophages, and dendritic cells. Ruhland and Peterson (1989) showed that the induction of class I and class II antigens on human fetal islets following exposure to gamma interferon (γ-IFN) can take place as early as 18 weeks of gestation.

The primary function of Ia antigen is to present foreign antigens to regulatory T cells. This is modulated by the antigens' repertoire of receptive T cells and the antigens' variable expression on certain antigen-presenting cells. Fierz and colleagues (1985) proposed this second property of Ia antigens to be a powerful regulatory mechanism for the local immune reaction, particularly in the CNS, in which Ia antigens are not normally expressed. The Ia-inducing activity of T cells can be replaced by γ-IFN, a lymphokine, which is produced by activated T lymphocytes. The lymphokines are non–antigen-specific factors released by activated T lymphocytes, affecting the functions of both lymphoid and nonlymphoid cells. Other lymphokines are IL-2 (T-cell growth factor), B-cell stimulating factors, and cell granulocyte-macrophage colony-stimulating factor (GM-CSF). Interleukin-1 (IL-1) participates in the activation of effector T cells such as the T-helper cells to induce IL-2 production and express IL-2 receptors.

Ethanol in vitro has been shown to enhance the expression of class I and class II MHC products on various cell types (Kolber et al. 1988; Parent et al. 1987; Singer et al. 1989). The incubation of human fetal pancreatic cells with 1.5 percent ethanol for 48 hours resulted in significant increases in class I and class II antigen expression (Ruhland et al. 1991). The authors concluded that ethanol might play a role in autoimmune diabetes by enhancing the expression of MHC products in the pancreas during early gestation.

The existence of an immunoregulatory circuit linking neuroendocrine structures and the immune system is now widely accepted (Besedovsky et al. 1985; Gillis et al. 1979). It was suggested that the immune system is a receptor organ which, by detecting external or modified self-antigens, can evoke in the CNS discriminative information about various types of stimuli. Following a CNS response intervention, the regulation outcome will be the result of external neuroendocrine signals and autoregulatory immunological signals. Observations of decreases in the activity of brain noradrenergic neurons after incubation with supernatant from Con A–stimulated spleen cells, and increased slow wave sleep and pyrogenic effects of IL-1 when introduced into the lateral ventricles of the brain, further strengthen this hypothesis (Besedovsky et al. 1985; Krueger et al. 1984).

Exploring the possibility that the brain may be an immunologically privileged site, Fontana and colleagues (1986) showed that when exposed to γ-IFN, 85–90 percent of astrocytes in culture expressed cell-surface class II MHC antigens. The ability to present class II MHC antigens reflects the ability of astrocytes, like other accessory cells such as macrophages, to initiate immune

responses of both the cellular and the humoral types. The validation of this finding was achieved by observations that class II positive astrocytes have the ability to activate T cells in vitro, and upon activation these astrocytes can release IL-I, an important lymphokine (Fontana et al. 1983, 1984; Frei et al. 1985). Similar to macrophages and T lymphocytes, astrocytes exhibit many important properties such as expression of membrane potassium channels and activation by viral infection, metabolic disturbances, or reaction to various disease processes of the CNS (Lieber-man et al. 1989; Roessmann and Gambetti 1986). They can react to injury by increasing in size and number, producing larger amounts of glial fibrillary acidic protein (GFAP). Astrocytes are the major scar-forming cells of the CNS (Weller et al. 1983). The consequences of alcohol exposure on the astrocyte functions are evaluated in the following sections.

Alcohol's Effects on the Properties of Astrocytes

The information presented in the preceding section establishes that the CNS and especially its astrocytes can initiate immunological responses. Virtually indestructible, astrocytes are the most resilient cells of the nervous system, with very few disorders causing their degeneration. Unfortunately, alcoholism is one such disorder. Recent years have seen a surge in interest in investigating the effects of alcohol exposure on astrocyte properties, though most of these studies are concerned with astrocyte growth and proliferation.

Several studies have examined ethanol effects on the growth of fetal brain, using various exposure conditions. Goodlett and colleagues (1993) examined the effects of ethanol exposure during the neonatal period on the rat brain growth spurt and reported an ethanol-induced transient cortical astrogliosis under these conditions. Shetty and Phillips (1992) observed the effects of feeding a 6.7 percent vol/vol ethanol liquid diet to pregnant rats throughout gestation on the postnatal cerebellar development of Bergmann glia and astrocytes in the pups. Their results suggested that prenatal ethanol exposure led to delayed maturation of Bergmann glia (which contributed to delayed migration of granule cells) and alterations in the postnatal development of astrocytes.

A model of whole embryo culture was used to study the effects of ethanol on murine brain development by Gressens and colleagues (1992). Ethanol enhanced cell death in premature neuroepithelium and also inhibited late gliogenesis. These authors concluded that neuropathological and clinical features of FAS could be explained by these glial-neuronal disturbances.

Primary astrocyte cultures from term fetal rats were used by Snyder and colleagues (1992) to study the effects of ethanol and/or insulin or insulin-like growth factors on incorporation of labeled precursors into DNA, RNA, and protein. Ethanol inhibited, while growth factors promoted, the incorporation of precursors into all three types of macromolecules. When both ethanol and growth factors were present, ethanol interfered with the growth factors' effect. The authors postulated that fetal brain growth retardation associated with maternal ethanol ingestion may be due to ethanol's interference with neurotrophic factors (see chapter 5).

Davies and Cox (1991) found a dose-dependent inhibition of neonatal rat brain astrocyte culture growth during the logarithmic phase following 48 and 96 hours of

ethanol exposure at concentrations of 0.2 percent, 0.5 percent, or 1.0 percent (wt/vol). Their data showed an ethanol-induced impairment of growth along with a retention of an extensive cell profile area, suggesting restrained morphological development. The authors attributed these findings to both ethanol cytotoxicity and a deprivation of cellular interaction resulting from the restricted population size.

Guerri and colleagues (1990), using primary cultures of astrocytes prepared from 21-day-old rat fetuses, observed adverse effects of prenatal ethanol consumption on DNA and protein synthesis. These authors further examined control astrocytes exposed in vitro to ethanol for 48 hours during two time points of astrocytic development (8 and 15 days representing the developmental periods of proliferation and differentiation, respectively). Both the synthesis and astrocytic DNA content were more affected by ethanol exposure during the proliferation period than during the differentiation period.

Tewari and colleagues (1988) examined translational regulation in human Cox astrocytoma cells grown in culture in the presence of 100 mM ethanol. Because astrocytoma cells are transformed astrocytes and have close similarities in the sequence complexities of total poly(A)+ RNA of the brain (Kaplan et al. 1978), they represent an ideal system for examining alcohol effects in a single homogenous cell type. Tewari and colleagues (1988) observed that during the transition from exponential to stationary phase of astrocytoma cell growth, both whole-cell protein synthetic activities and the translational activities of poly(A)+ mRNA's declined sharply. The decline was accentuated when the cells were grown in the presence of 100 mM ethanol (figure 2). The inhibition continued even when cells were withdrawn from ethanol.

A major effect of ethanol in this study was the inhibition of the polypeptide chain initiation reaction process of protein synthesis, which is known to play a major regulatory role in cellular maturational processes. These data suggest an interference by ethanol of brain maturation processes.

When Bass and Volpe (1989) incubated newborn rat cerebral glial cultures in the presence of ethanol concentrations ranging from 17 mM to 86 mM (less than the 100 mM concentration used by Guerri et al. 1990), they did not find any decrease in DNA synthesis or effect on astrocytic differentiation as determined by using glutamine synthetase (GS) as a marker. In contrast, oligodendroglial differentiation, as determined by using 2,3'-cyclic nucleotide 3'-phosphohydrolase as a marker, was stimulated by ethanol.

To summarize, all of the information presented in this section supports the hypothesis that the neuropathological effects of alcoholism are caused at least in part by astrocyte dysfunctions in the CNS. Thus, an understanding of events or mechanisms that regulate the growth of astrocytes is crucial, because it can lead to the elucidation of biochemical mechanisms underlying processes involved in neuronal growth, development, and recovery from CNS injury produced by alcohol.

Astrocyte Response to γ-IFN Following Alcohol Exposure

As mentioned earlier, astrocytes can secrete γ-IFN and express Ia antigens upon activation. Tedeschi and colleagues (1986) have postulated important roles for astrocytic IFN in protecting the brain against viruses and generating immune responses. Currently,

information is singularly lacking on alcohol's effects on the astrocytic response to γ-IFN. The available data, in addition to astrocytes, point to the increasing recognition of the role of microglia in the immune function of the CNS (see chapter 3).

A study by Sasaki and colleagues (1989) showed that the expression of Ia antigen was completely dependent upon the presence of γ-IFN. The Ia antigen expression was observed on ameboid microglia (approximately 80 percent) and type 1 and type 2 astrocytes (approximately 55 percent and 7 percent, respectively), but not on oligodendrocytes cultured from Lewis rat cerebral cortex. While suggesting that the type 1 astrocytes and microglia may play more predominant roles in Ia-related, immune-mediated intracerebral lesions, these data do not completely rule out a role for type 2 astrocytes. In a second study, Sasaki and colleagues (1990) reported that both astrocytes and microglia differed in their responses to increasing intracellular levels of adenosine 3',5'-cyclic monophosphate (cAMP) or protein kinase C (PKC). Reagents, known to increase intracellular cAMP

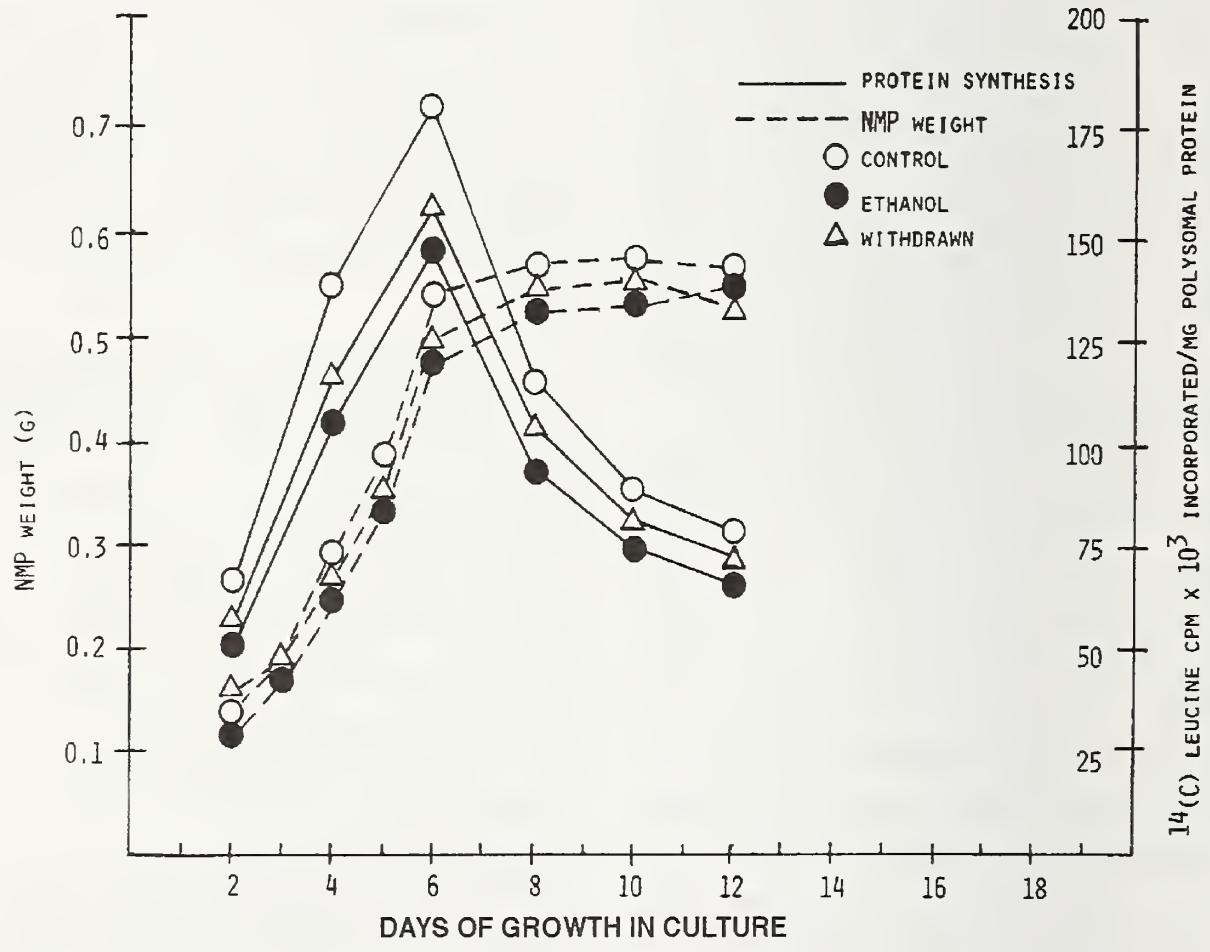

Figure 2. Effect of ethanol on in vivo protein synthesis: nuclear mitochondrial pellet (NMP) weights versus in vivo protein synthesis. Control and ethanol cells on various days of growth were labeled in vivo for 60 minutes with ^{14}[C]-leucine as described by Tewari et al. (1988) (samples for analysis of in vivo protein synthetic activity in intact cells were determined by TCA precipitation and extraction). Values are the average of 3–4 separate determinations. Variations rarely exceed 5 percent of the mean.

or to activate intracellular PKC, reduced γ-IFN–induced Ia antigen expression by astroglia but not by microglia.

The data described in this section suggest that the γ-IFN–induced Ia expression is regulated (1) negatively on astroglia by cAMP and PKC and (2) differentially in astroglia versus microglia. These data may explain the frequent observation of Ia+ microglia (or macrophages) but not astroglia in various neurodegenerative diseases.

The involvement of microglia in the expression of γ-IFN–induced Ia antigen was indicated again by studies conducted by De Groot and colleagues (1991). Using cultured neuroglial cells and brain macrophages from rat spinal cord and cerebrum, type 1 astrocyte–enriched cerebral cultures were found to contain a large number (approximately 46 percent of the cells) of brain macrophages (ameboid microglia), each expressing Ia antigens after treatment with recombinant γ-IFN.

Satoh and colleagues (1991) showed increased expression (greater than 80 percent) of intercellular adhesion molecule-1 (ICAM-1), ICAM-2–like molecule (Lg55), and class I and class II MHC antigens (H-2 and Ia) in murine astrocyte but not oligodendrocyte cultures exposed for 48 hours to γ-IFN (500 U/mL) and supernatant from Con A–activated spleen cells. However, neither ethanol nor TNF alone or in combination with γ-IFN is reported to have significant effects on MHC expression by TM3 and TM4 cells (Tokuda et al. 1990). Using cultured endothelial cells isolated from cerebral microvessels of SJL mice, Tanaka and McCarron (1990) studied the effects of both TNF-α and IL-1 on γ-IFN–induced Ia expression. The observation that Ia induction by IFN was inhibited in endothelial cells but augmented in astrocytes by TNF-α demonstrates that TNF acts on these cells in a disparate manner. Data suggest that both TNF and IL-1 can synergistically down-regulate immune responses involving endothelial cells.

It is clear from the review in this section that information is scarce as it relates to alcohol's effects on astrocytes or microglial response to γ-IFN. Recently data have been gathered on the effects of prenatal ethanol exposure on the γ-IFN–induced proliferation of neonatal rat brain astrocytes, which were cultured from 2- to 4-day-old newborn brains from control pups and in utero ethanol–exposed pups (Tewari et al. 1989, 1992*b*, 1993). Cultures were biochemically identified for the presence of GS and GFAP (specific astrocyte markers) by immunofluorescence techniques and were found to be 95 percent GFAP positive with high GS activity.

Table 3 shows that a 72-hour exposure of control astrocyte cultures to γ-IFN resulted in a pronounced increase of ^{3}[H]-thymidine incorporation (300 percent) and ^{35}[S]-methionine incorporation (275 percent). The increased ^{3}[H]-thymidine incorporation was used as a marker for astrocyte proliferation. This conclusion was based on studies by Barna and colleagues (1985), who showed that rat brain astrocyte cultures can respond to lymphokines produced by mitogen-stimulated human or rat T lymphocytes by exhibiting increased proliferation and enhanced DNA synthesis. Data show that 24-hour exposure to γ-IFN had no effect on ^{3}[H]-thymidine or ^{35}[S]-methionine incorporation in the astrocytic culture. The γ-IFN–induced stimulatory response was markedly reduced by prenatal ethanol exposure (a 135-percent response for ^{3}[H]-thymidine incorporation and a

Table 3. Astrocyte Proliferation Induced by Gamma-Interferon: A Time-Dependent Study

Experimental Conditions	^{3}[H]-Thymidine % Uptake	^{35}[S]-Methionine % Uptake
- γ-IFN	100	100
+ γ-IFN (24 h)	110	115
+ γ-IFN (72 h)	300	275

Control astrocytes were exposed to 10 U/mL γ-IFN for 24 hours or 72 hours before labeling with ^{3}[H]-thymidine and ^{35}[S]-methionine. Cells were labeled with 1.0 µCi of each precursor/mL culture medium in a final volume of 10 mL for 6 hours before TCA processing. Data represent an average of two experiments, with variation not exceeding 10 percent. The 100 percent activity for ^{3}[H]-thymidine = 3.2 x 10^{3} cpm/1,000 cells. The 100 percent activity for ^{35}[S]-methionine = 4.5 x 10^{3}/1,000 cells.

115-percent response for ^{35}[S]-methionine incorporation) (table 4).

The observed increase in astrocytic proliferation probably reflects the induction of Ia antigen by γ-IFN exposure as demonstrated by enhanced DNA synthesis (Barna et al. 1985; Kim et al. 1985). Therefore, ethanol-induced inhibition of γ-IFN response is suggestive of a muted expression of MHC antigens in astrocytes. In the studies conducted in our laboratory, the astrocytic response to γ-IFN was found to be unaffected by norepinephrine (NE). Although both NE and γ-IFN were stimulatory to DNA and protein synthesis in astrocytes, no further increases were observed in the combined presence of NE and γ-IFN (data not shown). The effects of NE are described in the next section. Norepinephrine is known to down-regulate the γ-IFN–induced Ia expression and is involved in neuroimmune regulations (Besedovsky et al. 1985; Frohman et al. 1988). An in-depth review of the currently available observations failed to yield any definitive information on ethanol's effects on microglial response to γ-IFN.

ASTROCYTE PROLIFERATION IN THE PRESENCE OF EFFECTORS: EFFECTS OF ETHANOL

The survey of the literature presented in the preceding section shows that, with a few exceptions, most studies have examined ethanol effects on astrocyte growth and proliferation using in vitro exposure conditions. With the exception of studies from our laboratory, virtually no studies are available on the in vivo effects of ethanol on astrocyte activation by various effectors, or on the neuroimmunological responses. Results of detailed studies on astrocyte responses to various effectors following prenatal ethanol exposure are described in this section (Tewari et al. 1993).

Response to Hydrocortisone, NE, and Soluble Brain Extracts

The relationship between ethanol exposure and astrocyte proliferation was examined (Tewari et al. 1993) following exposure of astrocyte cultures to the following biological agents: (1) hydrocortisone, a glucocorticoid

known to modulate the activity of GS which is located in astrocytes (astrocytes are the target cells for hydrocortisone's action in the brain); (2) NE, a neurotransmitter with ability to activate lymphocytes, monocytes, and astrocytes, among other cells, and to modulate GS activity; and (3) soluble brain extracts, which are known to contain specific differentiation factors (Tardy et al. 1984).

To determine the response to the various effectors, control and in utero ethanol–exposed astrocytes were first exposed to hydrocortisone, NE, or soluble brain extracts for 72 hours, then labeled for 4 hours in the presence of 1 μCi each of ^{35}[S]-methionine and ^{3}[H]-thymidine. Astrocyte proliferation as measured by ^{3}[H]-thymidine and protein synthesis as measured by ^{35}[S]-methionine incorporation were significantly stimulated by all three effectors under these conditions (table 5). The stimulation was more pronounced in the presence of higher, rather than lower, concentrations of the 145,000 x g brain extract, which is known to enhance maturation of astrocytes (Sensenbrenner et al. 1980; Tardy et al. 1984). The measurement of GS activity under these conditions also showed a large stimulation similar to those reported earlier by Tardy and colleagues (1984) and was paralleled by a concomitant increase in astrocyte proliferative activity.

As shown in table 6 (compared with table 5), ethanol administration in utero resulted in reduced NE and hydrocortisone response on all three parameters (Tewari et al. 1993; some findings were presented at the Second International Conference on Alcohol, Drugs of Abuse, and Immunomodulation (AIDS) Symposia in Arizona in 1992). The response of the ethanol-exposed astrocytes to hydrocortisone showed large variability ranging from inhibition to stimulation. Reasons for this large variability are not clear. It is possible that the hydrocortisone response may involve a particular period in the astrocyte differentiation process that may have been influenced by ethanol exposure. Ethanol is thought to result in delayed maturation. Further observation indicated that de novo protein synthesis was required to produce the induction of GS activity by NE (Tewari et al. 1993). Using cycloheximide and actinomycin D (known inhibitors of protein and RNA synthesis), the NE-induced stimulation of GS activity was blocked in both control and ethanol-exposed cultures (table 7).

Response to Con A

The response of control brain astrocytes to the mitogen Con A, a protein with a

Table 4. Effects of Ethanol on Astrocyte Response to γ-IFN

Experimental Conditions	^{3}[H]-Thymidine % Uptake	^{35}[S]-Methionine % Uptake
– γ-IFN	100	100
+ γ-IFN (72 h)	135	115

The 100 percent activity for ^{3}[H]-thymidine = 2.1 x 10^{3} cpm/1,000 cells. The 100 percent activity for ^{35}[S]-methionine = 3.0 x 10^{3} cpm/1,000 cells. Experimental conditions were similar to those described for table 3. Astrocytes were exposed to γ-IFN for 72 hours.

Table 5. Astrocyte Proliferation in Response to Various Effectors

Experimental Conditions	DNA Synthesis % Activity	Protein Synthesis % Activity	G S * * % Activity
No additions	100	100	100
+ Soluble brain extract (200 µg protein)	145	153	160
+ Soluble brain extract (100 µg protein)	130	142	142
+ 1 /µm hydrocortisone	200	279	155
+ 10 mg/mL norepinephrine	300	400	221

Control neonatal brain astrocytes were exposed to either 9-day-old neonatal soluble brain extract, hydrocortisone, or norepinephrine for 72 hours and then labeled with ^{35}[S]-methionine and ^{3}[H]-thymidine for 4 hours. The 100 percent activities for DNA synthesis, protein synthesis, and glutamine synthetase were 5,200 ^{3}[H]- cpm/1,000 cells, 10,200 ^{35}[S]- cpm/1,000 cells, and 2.2 µmol/g G-glum formed/mg protein/h, respectively. GS = glutamine synthetase. G-glum formed/mg protein/h = γ-glutamate hydroxamate.

molecular weight of 102,000, was determined (Tewari et al. unpublished data). Occurring naturally in jack bean, Con A is known to increase RNA and DNA synthesis in astrocytes, possibly as a result of CNS inflammation (Fontana et al. 1981). A 300 x Con A supernatant fraction was prepared from lymphocytes derived from spleen by stimulating with 5 µg/mL Con A (Fontana et al. 1980). Table 8 demonstrates a large stimulation of both ^{3}[H]-thymidine (167 percent) and ^{35}[S]-methionine (203 percent) in astrocyte cultures exposed for 72 hours to Con A supernatant fraction. Under these experimental conditions, Con A had no effect when added directly to the incubation medium. Since Con A interaction with lymphocytes results in the production of γ-IFN (Fierz et al. 1985), the stimulatory effects were possibly mediated by γ-IFN in the present system. The experiments described in the previous section on γ-IFN–induced proliferation of astrocytes support this conclusion. Ethanol studies on Con A activation of astrocytes are now in progress.

Response to Endotoxin Exposure

The endotoxin LPS is an important effector that stimulates cells to release cytokines. Furthermore, LPS produces functional and morphological changes in the CNS during endotoxemia or following intracerebral injection (Fontana et al. 1981). Biologically, LPS, a B-cell mitogen (approximate molecular weight of 4,000), is of Gram-negative bacterial origin. Several clinical and experimental studies are available which have studied the interaction between LPS and alcohol and the secretion of cytokines. Cytokines are regulatory polypeptides secreted during the generation of an immune or inflammatory response by lymphocytes, cells of the monocyte/macrophage series, and a variety of other cell types.

Most investigations involving ethanol have been carried out either in vivo or in

Table 6. Response of Ethanol-Exposed Cells to Norephinephrine and Hydrocortisone

Experimental Conditions	DNA Synthesis %	Protein Synthesis %	GS Activity %
Untreated cultures	100	100	100
+ 48 h NE	164	175	170
+ 48 h hydrocortisone	132	125	75

Experimental conditions for NE or hydrocortisone exposure were similar to those described elsewhere (Tewari et al. 1993). Astrocyte cultures were prepared from neonatal brain exposed prenatally to ethanol. The 100 percent activities were (1) for thymidine uptake, 3.4 x 10^3 cpm/1,000 cells; (2) for methionine uptake, 1.6 x 10^3 cpm/1,000 cells; and (3) for GS activity, 1.8 µmol of γ=GLUMA formed/h/mg protein/h. Data represent an average of three experiments. Reprinted from Tewari, S.; Komanapalli, L.; Van, T.; and Carson, V. Astrocyte dysfunction and its implications in prenatally ethanol exposed rats. In: Watson, R.R., ed. *Advances in Biosciences. Vol. 86. Alcohol, Drugs of Abuse and Immuno-modulation*. Pergamon Press, 1993. pp. 483–491.

vitro using serum or spleen cells. Although data are lacking on ethanol-LPS interactions in the CNS, several clinical and experimental studies have examined the relationship between ethanol administration and the release of cytokines in non-CNS systems. Khoruts and colleagues (1991) studied the relationship between cytokines and metabolic consequences of alcoholic liver disease using an enzyme-linked immunosorbent assay to measure plasma TNF and IL-1 and a bioassay to measure serum IL-6 in three groups of alcoholic men. In this study, TNF-α and IL-1 concentrations were found to remain elevated for up to 6 months after the diagnosis of alcoholic hepatitis, but IL-6 normalized in parallel with clinical recovery.

Ethanol in the presence of LPS produced significant impairment of the synthesis and the release of TNF and GM-CSF and membrane expression of TNF receptors by human macrophages (Bermudez et al. 1991). These data show that ethanol exposure could induce significant reductions in the human macrophage's response to phorbol ester (phorbol-12-myristate-132-acetate) lasting for approximately 6 hours following the removal of ethanol. In addition, significant impairment of the expression of TNF receptors after γ-IFN stimulation was also observed. An ethanol-induced inhibition of macrophage functions was suggested as a potential mechanism for suppression of the host's immune response, resulting in increasing the susceptibility for infectious diseases. In an earlier study, Nelson and colleagues (1989) observed acute ethanol intoxication leading to a marked suppression of both serum and lung TNF elicited in response to intravenous LPS administration. The authors concluded that the anti-inflammatory effects of ethanol may be secondary to suppression of macrophage-derived TNF. A pronounced inhibition of serum TNF-α has been observed in rats following ethanol administration, confirming the clinical studies (D'Souza et al. 1989).

Chen and colleagues (1993) examined the relationship between ethanol and cytokine production in mice infected with retrovirus. Ethanol effects were tested both in vitro at concentrations of 0.1–1.0 percent vol/vol and in vivo where the C57BL/6 female mice were fed an ethanol-containing

Table 7. Requirement of Protein Synthesis for Norepinephrine-Induced Elevation of Glutamine Synthetase Activity

	GS Activity	
Experimental Conditions	**% Control Cells**	**% Ethanol Cells**
Astrocyte cultures	100	100
+ NE	200	131
+ NE + cycloheximide	95	102
+ NE + actinomycin D	95	110

Experimental conditions were similar to those described elsewhere (Tewari et al. 1993). Cycloheximide (10 µg) or actinomycin D (1 µg/mL) was added to the culture medium along with NE for 48 hours. Cultures were used on the 16th day. Values represent an average of two experiments. The 100 percent GS activity represents 2.6 ± 0.3 µmol γ-GLUM formed/h/mg protein. Reprinted from Tewari, S.; Komanapalli, L.; Van, T.; and Carson, V. Astrocyte dysfunction and its implications in prenatally ethanol exposed rats. In: Watson, R.R., ed. *Advances in Biosciences. Vol. 86. Alcohol, Drugs of Abuse and Immuno-modulation.* Pergamon Press, 1993. pp. 483–491.

Table 8. Astrocyte Activation by Glia Stimulatory Factors (GSF's)

Experimental Conditions	**^{3}[H]-Thymidine (cpm/1,000 cells)**	**^{35}[S]-Methionine (cpm/1,000 cells)**
No addition	7,200	10,800
+ Con A SN (GSF) fraction	12,000	21,900
% Activity	167	203
+ 5 µg Con A*	7,400	11,000
% Activity	103	101

The Con A SN (supernatant) fraction containing GSF was prepared by treating spleen cells with 5 µg Con A for 24 hours. Astrocytes were exposed to this GSF for 72 hours before labeling with precursors.
*Con A was added during the incubation period along with the labeled precursors.

liquid diet (27 percent of calories derived from ethanol) for 5 months. For this study, splenocytes from the ethanol-treated and control mice and purified macrophages from normal and retrovirus-infected mice were used. Culturing the cells with ethanol down-regulated the secretion of TNF and γ-IFN by LPS- or Con A–stimulated spleen cells. Although murine retrovirus infection per se increased TNF production, ethanol continued to down-regulate the LPS and Con A response in vitro. An increased HIV-1 expression, as measured by reverse transcriptase activity, was demonstrated by Vitkovic and colleagues (1990, 1991) in chronically HIV-1 infected human promonocytic cells, when exposed to media conditioned with LPS-stimulated astrocytes.

Very few studies on ethanol's effects on the LPS response of astrocytes in the brain exist. In a preliminary study, effects of prenatal ethanol exposure were determined on the astrocytic response to LPS exposure (Tewari et al. unpublished data). Based on the length of exposure, LPS was found to differentially affect the initial astrocytic proliferative response, as measured by DNA and protein synthesis. Following the exposure to 1.0 µg LPS/mL culture medium for 72 hours, neonatal brain astrocytes showed an increased ^{35}[S]-methionine incorporation into protein (200 percent control activity) with little effect on ^{3}[H]-thymidine uptake (table 9).

Similar to the Con A effects described in table 8, LPS added directly during the incubation had no effect on either DNA or protein synthesis. However, LPS differed from astrocytic responses to γ-IFN or the Con A–treated supernatant fraction in having no effect on ^{3}[H]-thymidine uptake but stimulating only ^{35}[S]-methionine incorporation. Ethanol exposure under these conditions significantly reduced the LPS response.

The observed increase in protein synthesis as shown in table 9 may reflect the increased RNA synthesis observed by Fontana and colleagues (1981) in mouse brain astrocytes exposed 72 hours to LPS. Since DNA synthesis was not inhibited under these conditions, data reflect LPS-induced changes at the posttranscriptional level. Astrocytes in response to LPS can secrete TNF-α and IL-6. Both of these cytokines have been demonstrated in the CNS during neurological diseases associated with inflammation (Chung and Benveniste 1990; Wesselingh et al. 1990). Whether ethanol exposure of astrocytes, similar to the serum, results in altered secretion of TNF-α under the present conditions needs to be determined. These results would provide significant information, because ethanol has been implicated in the onset of a variety of immune defects in vivo involving the cytokines TNF-α, IL-1, and IL-6. Alterations in the production, site of action, or metabolism of cytokines by exogenous factors such as ethanol could be expected to result in deleterious effects on the immune system as a whole.

Table 9. LPS-Induced Stimulation of Protein Synthesis: Effects of Ethanol

	Percent Control Activity	
Experimental Conditions	**Protein Synthesis**	**DNA Synthesis**
Control astrocytes	100*	100**
+1 h LPS exposure	100	95
+72 h LPS exposure	200	101
Ethanol astrocytes	68	65
+72 h LPS exposure	92	68

Control or in utero ethanol–treated astrocytes were exposed to 1.µg LPS/mL culture media for 1 hour or 72 hours. All other experimental conditions were similar to those described in table 3.
*100 percent ^{35}[S]-methionine activity for control cells = 3.68 x 10^{3} cpm/1,000 cells.
**100 percent ^{3}[H]-thymidine activity for control cells = 3.1 x 10^{3} cpm/1,000 cells.

VIRAL AND RETROVIRAL INFECTION OF ASTROCYTES: CONSEQUENCES OF ETHANOL EXPOSURE

It is very clear that besides affecting the immune-response properties, ethanol exposure can negatively affect the metabolism of astrocytes at the CNS level, which would render these cells vulnerable to viral infection. This notion is not far-fetched, since viral infections are responsible for several chronic disorders of the CNS (Koprowski et al. 1985) and can be inhibitory to the expression of MHC genes that are stimulated by interferons (Fellous et al. 1981; Schrier et al. 1983). The induction of class I antigen by the murine coronavirus JHM, which causes primary demyelination, is thought to occur through the release of a soluble factor or factors by the infected astrocytes (Suzumura et al. 1988). Although a significant amount of clinical and experimental research has shown viral infection of astrocytes and/or microglia, some of these studies are not conclusive. The evidence for astrocyte infection is reviewed here by examining (1) viruses in general and (2) retroviruses, specifically the AIDS virus and the Moloney murine leukemia virus (M-MuLV). The possibility of using M-MuLV exposure as an alternate animal model for AIDS is discussed.

Viral Infection

In an experimental model for multiple sclerosis, the release of TNF-α by astrocyte cultures infected in vitro with Theiler's murine encephalomyelitis virus (TMEV) strongly supports an active role for astrocytes as accessory immune cells (Sierra and Rubio 1993). Borrow and Nash (1992) demonstrated that the ability of astrocytes to present TMEV to virus-specific T cells in vitro was dependent upon prior induction of MHC class II antigens by γ-IFN treatment. This appears to be a key step in the amplification of immunopathological responses within the CNS during the development of demyelinating disease.

The involvement of astrocytes in CNS demyelination was also demonstrated in mice infected with herpes simplex virus type 1 (HSV-1) by Itoyama and colleagues (1991). In this experiment, the HSV-1 infection in mice provided an unique model of acute CNS demyelination preceded by an early loss of astrocytes. The reactions of astrocytes during HSV-induced brain inflammation in rabbits were examined by Nasyrov and colleagues (1992) using immunocytochemical procedures. The HSV infection produced changes in astrocytes by first forming glia complexes (astrocyte agglomerations) in the affected sites. Later, proliferating astrocytes replaced the injured spots of brain tissue. On the other hand, studies by Matsumoto and colleagues (1992) showed that in the CNS of rats with experimental autoimmune encephalomyelitis (EAE), the expression of Ia antigens occurred on the microglia but not on the astrocytes.

Lieberman and colleagues (1989) stimulated the production of TNF, lymphotoxin, IL-6, α-IFN, and β-IFN, and activated the IL-1 and IL-6 genes in rat brain astrocytes with LPS or a neurotropic virus (paramyxovirus, Newcastle disease virus). Significant roles for astrocytes were proposed along with the cytokines they produce in the pathogenesis of immunologically and/or virally mediated CNS disease, in CNS intercellular communication, and in the interactions between the nervous and immune systems.

Retroviral Infection

An extension of the studies on viral infection of the CNS to include retroviral infection was deemed necessary because of the increased CNS involvement in AIDS. Retroviruses are a diverse group of RNA viruses containing the enzyme reverse transcriptase, an RNA-dependent polymerase producing an intermediate DNA (provirus) which is integrated into the host genome and utilized in the infected cell for the transcription of virion RNA (McArthur 1987). The highest concentrations of the viral nucleic acid are found within the brain rather than in spleen, lymph nodes, liver, or other tissues. Retrovirally induced infections do not in general result in cell lysis but rather in a carrier state (Varmus and Swanstrom 1982).

The AIDS or M-MuLV retroviruses are slow viruses which are not cleared from the body and, therefore, undergo persistent replication and antigenic drift. Similar to most of the retroviruses which do not carry an oncogene, the M-MuLV genome consists of three genes: *gag*, *pol*, and *env* (Wong 1990). M-MuLV is composed of two identical RNA molecules joined at their 5' ends by dimer linkage structures (Roy et al. 1990). The integration of a DNA copy of the M-MuLV, or HIV viral RNA genome, into the chromosome or genome of the infected host cell is necessary for the normal replication of these viruses (Bushman and Craigie 1990) and is dependent upon myristylation of *gag* polyprotein precursors for assembly (Bryant et al. 1989; Vink et al. 1990).

Similar to HIV-1, the etiologic agent of AIDS, M-MuLV is lymphomagenic and neurovirulent in wild mice, inducing non-inflammatory lesions and spongiform encephalopathy (Lohler 1988) or inducing Kaposi's sarcoma–like lesions in 24- to 48-hour-old Balb/c mice injected intraperitoneally with supernatant from M-MuLV sarcoma virus 349 cells (Stoica et al. 1990). Originally isolated as a complex with a sarcoma virus by Moloney (1960), M-MuLV is a leukemia virus inducing T-cell lymphomas in 100 percent of the neonatally infected mice within 3 to 6 months postinoculation. When M-MuLV is injected intraperitoneally to rats within 24 hours after birth, it has a latency period ranging between 2 and 6 months (Bellacosa et al. 1989). M-MuLV causes infection of astrocytes, oligodendrocytes, and spinal ganglia in the CNS of postimplantation embryos (Sharpe et al. 1989).

Increased expression of H-2K, H-2D, and H-2L proteins occurs following the infection of mouse fibroblasts by M-MuLV (Wilson et al. 1987). Shikova and colleagues (1993) found that both M-MuLV-TB and ts1 (a highly neuropathogenic and lymphocytopathic mutant of M-MuLV-TB) replicated and induced differential cytopathic effects in astrocyte cultures, where the ts1 appeared to be more lethal than M-MuLV-TB. Neither ts1 nor M-MuLV-TB infections of endothelial or TB cells were cytopathic. Sharpe and colleagues (1987, 1988, 1989) observed that M-MuLV can be transmitted very efficiently from viremic mothers to offspring, and that the development of viremia in the offspring could be suppressed by azidothymidine administration.

Much work is now in progress using human or animal retroviruses that appear to identify astrocytes or microglia as target cells in the CNS. AIDS-related dementia reflects the infection of the CNS. The disorder is characterized by the presence of infected and giant microglial cells, astrocytosis, demyelination, and neuronal loss (Fauci 1988).

Epstein and Gendelman (1993) suggested that HIV-1 infected macrophages can initiate neurotoxicity, which is then amplified through cell-to-cell interactions with astrocytes. The secretion of TNF-α, IL-1β, and arachidonic metabolites through astrocyte activation could induce astroglial proliferation resulting in neuronal injury. Studies by Sharpless and colleagues (1992) on the HIV-1–infected CNS strongly suggest that neurotropism of HIV-1 is tightly restricted to the microglial cells. Swingler and colleagues (1992) observed that TNF-α augmented the HIV-1 long terminal repeat (LTR)–driven gene expression in human neuroblastoma and glioblastoma and in primary murine astrocyte cultures, while IL-6 could enhance HIV gene expression only in the primary astrocyte cultures.

In experimental studies, cultured feline astrocytes were found to be highly susceptible to infection and cell death by the feline immunodeficiency virus (FIV) infection in vitro, followed in susceptibility by microglial cells, which remained persistently and productively infected without obvious cytopathic effects (Dow et al. 1992). Because of relative resistance of brain endothelial cells to FIV infection, FIV entry into the CNS probably does not involve these cells. The findings from this study tend to support both astrocytes and microglia as primary target cells for FIV.

M-MuLV Infection

The growing recognition of alcohol as a cofactor in the transmission of viral infection, specifically as it relates to AIDS, provides a strong basis for examining the infection of neural cells using animal retroviruses like M-MuLV. Most people with AIDS have a history of alcohol or other drug use. Both of these populations are thus high-risk groups, and alcohol remains a serious cofactor. Beresford and colleagues (1986) demonstrated that AIDS and its complications mimicked psychiatric disorders related to alcoholism. In a study carried out with homosexual AIDS patients in San Francisco, it was found that an overwhelming majority (95 percent) used alcohol (Abrams 1987). In another study, 84 percent of AIDS cases had a history of intravenous drug abuse. However, no one in this group was homosexual.

According to Ostrow (1987), high-risk sexual activity probably carries about a 10 percent risk of infection per exposure. Data generated by Ostrow from the multicenter AIDS cohort study, when coupled with the San Francisco AIDS project, strongly point to an association between drug and alcohol abuse in a subpopulation of homosexual men and unsafe sexual practices known to transmit AIDS infection. Alcohol is used by the two high-risk AIDS-contracting populations, homosexual men and intravenous drug abusers (men and women). High-risk men are 1.5 times more likely to use alcohol, poppers, or marijuana during sexual activity than low-risk men (Stall 1988). Alcohol ranked highest among all drugs used during sexual activity. Stall developed AIDS risk scores dependent upon the use of drugs during sexual activity. In the no-risk group, 38.5 percent of the individuals used alcohol as opposed to 66.7 percent in the medium-risk group and 79.4 percent in the high-risk group (Stall 1988). On the other hand, Kaslow and colleagues (1989), in a multicenter AIDS cohort study of homosexual men, did not find strong evidence for a relationship between the use of psychoactive drugs or alcohol and progression of AIDS infection. However, no neuropsychiatry test-

ing was carried out by these investigators. DesJarlais and colleagues (1987) reported that intravenous drug abusers who reduced or stopped using drugs were less likely to show evidence of progressive HIV-1 infection when compared with individuals who continued to use drugs. Thus, it is clear that more work is needed to clarify the relationship between alcohol/drug use and the clinical progression of AIDS.

MacGregor (1988) has discussed at length the potential effects of alcohol and drug use on the AIDS epidemic. According to MacGregor, (1) the risk of primary infection is increased when an individual is first exposed to HIV and (2) further suppression of the immune system by alcohol could result in more rapid progression from asymptomatic to clinical infection in a person already infected with AIDS. Alcohol consumption was found to be a particular risk factor for both the development and adverse outcome of bacteria and pneumonia (50–53 percent mortality rate in heavy drinkers compared with 18.5 percent in abstainers and 22 percent in occasional drinkers). The pneumonia mortality rate is higher for alcoholic women than men as observed in a Canadian study reported by MacGregor. Alcoholism has also been found to be a predisposing factor in 30 percent of individuals with aspiration lung abscess. Therefore, it is logical to assume that factors and diseases that interfere with normal immune defenses would have an impact on the progression of HIV-1 infection.

The frequency of HIV transmission from an infected woman to her fetus or newborn infant can be 25–50 percent. Alcohol exposure was found to increase the frequency of HIV transmission from the mother to the fetus by more than 50 percent (MacGregor 1988).

Although the role of alcohol in producing most of the effects described in this section is now well recognized, and the neurochemical mechanisms have been investigated, very little is known or understood about what modifying effects alcohol might have on two clinical consequences: the transmission/rapid progression of AIDS and viral replication/infection in the brain. Because it is difficult to have access to clinical neural tissue, studies with animal models for HIV are urgently needed.

The infectibility of neural cells in an animal model following exposure to M-MuLV and the effects of ethanol exposure were determined by studies in our laboratory (Tewari et al. 1992*b*, unpublished manuscript). This report is the first to demonstrate the infection of astrocytes and C_6 glioma cell lines following M-MuLV exposure and describe the effects of prior ethanol exposure on this process. Primary cultures of astrocytes and C_6 glioma cells were infected with virus stocks obtained from NIH-3T3 mouse fibroblast cells (Tewari et al. 1992*a*). After 7 days, cells were irradiated with ultraviolet light and overlaid with 10^6 rat XC cells to form syncytia (evident as clusters of nuclei within a large region of cytoplasm) in the presence of M-MuLV-infected cells (Rowe et al. 1970). M-MuLV-infected cultures showed XC syncytia formation (after fixing the plates and staining with Giemsa blood stain), which is indicative of a productive M-MuLV infection. In contrast, the noninfected cultures did not show the formation of XC syncytia. A photograph of the stained culture infected with undiluted M-MuLV is presented in figure 3. At virus concentrations of 10^{-5} and 10^{-6} PFU/mL, the number of plaques were 6.6 x

10^6 XC PFU/mL and 3.5 x 10^6 XC PFU/mL, respectively.

The effects of ethanol exposure on astrocyte proliferation following infection with M-MuLV were examined in our laboratory (Tewari et al. unpublished manuscript). Prenatal ethanol–exposed neonatal brain astrocytes and C_6-glioma cells (exposed in vitro to 100 mM ethanol) were examined for infectibility with M-MuLV as described in the preceding paragraph. Control cells were infected with M-MuLV without ethanol treatment. Cells were then tested for syncytia formation (similar to that shown in figure 3) and ^{3}[H]-thymidine labeling.

Data in table 10 show a greater than 200 percent increase in the ^{3}[H]-thymidine uptake in cells that were preexposed to ethanol. Similarly, an increased thymidine labeling of 400 percent was observed when ethanol was included during the incubation period of the infected C_6 glioma cells. In contrast, under these conditions, ethanol was found to have an inhibitory effect on the ^{3}[H]-thymidine uptake in control noninfected cells. Results strongly suggest that M-MuLV expression in neural cells is facilitated by prior ethanol exposure.

SUMMARY

In this chapter we have carried out an in-depth review examining alcohol's role in regulatory mechanisms of the neuroimmune system. The evidence establishes unequivocally the damaging consequences of alcohol exposure in general and in particular during the prenatal phase. The following para-

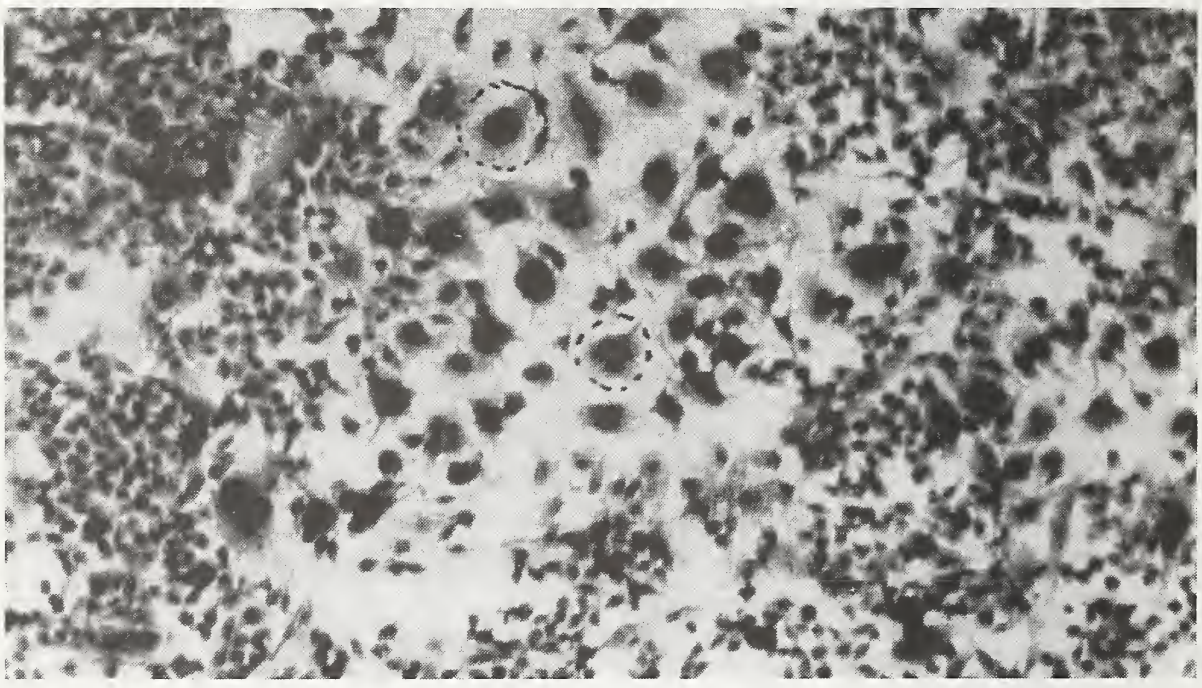

Figure 3. XC syncytia formation in astrocytes infected with M-MuLV. Astrocyte cultures were infected with M-MuLV, irradiated with ultraviolet light, and overlaid with 10^6 rat XC cells according to the procedures of Rowe et al. (1970). Plates were fixed and stained with Giemsa blood stain. XC syncytia formation is indicated as clusters of nuclei within the cytoplasm and is indicative of a productive M-MuLV infection. Virus concentrations were 10^{-5}/ml PFU/mL.

Table 10. Ethanol Effects on ^{3}[H]-Thymidine Uptake by Infected Astrocytes or C_6 Glioma Cells

Experimental Conditions	Control Noninfected Cells (%)	MMuLV Infected Cells (%)
Ethanol exposure in utero	68	275
Ethanol exposure in vitro	65	400

Astrocytes or C_6 glioma cells were exposed to MMuLV at concentrations of 10^{-5} PFU/mL. Labeling with ^{3}[H]-thymidine was carried out for 4 hours. Ethanol concentrations for in vitro exposure were 100 mM.

graphs provide a brief summary of the effects of alcohol on the CNS and the immune systems.

Both clinical and experimental studies demonstrated immunological dysfunctions following ethanol exposure. Ethanol was shown to alter host defense mechanisms and specifically impair cell-mediated immunity, an important defense mechanism against various types of infections. The inhibition of IL-2 synthesis and its interactions with IL-2 surface receptors, and suppression of TNF-α, were proposed as major mechanisms for the immunosuppressive effects of ethanol.

Clinical and experimental studies on ethanol/LPS-induced cytokine secretion found impaired synthesis and release of TNF levels in serum, lung, and spleen cells, including the expression of TNF receptors. In chronic alcoholic men with a diagnosis of chronic hepatitis, TNF-α, IL-1, and IL-6 were elevated; only IL-6 levels returned to normal in parallel with clinical recovery.

Prenatal ethanol exposure caused reduced translation of poly[A]+ mRNA in the developing and mature brain, with the hippocampal region sustaining the most injurious and irreversible effects. The learning problems and intellectual deficits suffered by the FAS children could be linked to ethanol-induced changes, at the posttranscriptional level, involving hippocampal mRNA.

Ethanol was found to interfere with the biological, metabolic, and physiological functions of astrocytes. These disturbances include delayed maturation of Bergmann glia, inhibition of the polypeptide chain initiation reaction, reduced proliferative response, and an inhibition of GS activity following exposure to NE, hydrocortisone, and soluble brain extracts containing specific differentiation factors. It was concluded that ethanol exposure is responsible for interfering with neuronal migration and cellular maturational processes.

In the CNS, the γ-IFN–induced Ia antigen expression was found to be regulated differentially in astrocytes versus microglia. A 72-hour γ-IFN exposure produced increased proliferation and growth activities in control astrocytes but only a subnormal γ-IFN response in the prenatal ethanol–exposed neonatal astrocytes. Ethanol effects could be related to interference with the expression of Ia antigens on astrocytes, although a differential regulation by microglia cannot be ruled out.

Neonatal brain astrocytes exposed to LPS for 72 hours demonstrated increased protein synthesis, but not DNA synthesis, indicating alterations at the posttranscriptional level.

The response level was significantly reduced by ethanol. These data suggest potential mechanisms for the suppression of host's immune response by ethanol, resulting in increased susceptibility to infectious diseases.

In EAE-infected rat CNS, the expression of Ia antigens occurred only on microglia and not on astrocytes. However, TNF, lymphotoxin, IL-6, α-IFN, and β-IFN were secreted by rat brain astrocytes when stimulated with LPS or neurotropic viruses. Under these conditions, IL-1 and IL-6 genes were also activated. Data support significant roles for astrocytes in virally mediated CNS disease.

Differences were present in the augmentation of HIV-1 LTR-driven gene expression, which was expressed by TNF-α in primary murine astrocyte cultures and in human neuroblastoma and glioblastoma, and by IL-6 in astrocyte cultures only. Microglia, on the other hand, were shown to be the targeted cells for neurotropism by HIV-1 in the CNS and were also infectible by FIV.

Astrocytes and C_6 glioma cells were both infectible when exposed to M-MuLV. Prior ethanol exposure resulted in increased syncytia formation and higher thymidine uptake in astrocyte or C_6 glioma cultures exposed to M-MuLV. Astrocytes were found to be more susceptible to retroviral infection when exposed to ethanol.

FUTURE DIRECTIONS FOR ALCOHOL RESEARCH

In reviewing the information, it is clear that there are significant gaps in the field of alcohol research and immune function. The gaps are even wider when one examines the alcohol field in relationship to the neuroimmune system. The potential areas for new alcohol research which have emerged from this review and deserve critical attention are as follows:

- Studies on MHC gene regulation and Ia antigen expression in neural cells, specifically astrocytes, are very much needed. Because there is much controversy in the field with respect to the types of neural cells involved, coupled with the fact that Ia antigen expression is regulated differentially in astrocytes and microglia, any investigation on effects of alcohol must design studies using both cell types.
- The viral infection of neural cells and the role of alcohol in the infectivity process is another area that requires immediate attention. Virtually no studies, either clinical or experimental, are available on this subject, and it is important because alcohol may affect the clinical progression of the disease.
- Investigations on astrocytic and microglial infection by human or animal retroviruses under alcohol exposure conditions are highly significant in understanding CNS infection and the progression of clinical symptoms in AIDS. In conjunction with clinical studies, investigations must include appropriate animal models for the AIDS retrovirus. Available data on M-MuLV and its various strains make the virus an ideal murine model system in the study of neurodegenerative diseases. Furthermore, because M-MuLV is transmitted maternally and the prenatal models of alcohol are also well established, an in vivo model combining the two systems would be extremely valuable for studying the effects of alcohol on maternally transmitted viral infection.

The recommendation of the M-MuLV system in the study of CNS infection under alcohol exposure conditions gains support from the recent National Institutes of Health consensus report (Wiley and Gardner 1993) generated from the workshop sponsored by the National Institute of Neurological Disorders and Stroke on the pathogenesis of murine retroviral infection of the CNS. The report also recognizes the controversy that exists in the field regarding astrocytes versus microglia, and the crucial need for experimental models in AIDS research. In dealing with effects of alcohol on the course of retroviral infection, the field is ready now and can take advantage of the new strains of the M-MuLV system.

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Chapter 9

The Stimulation of Compensatory Functional Changes and Repair Mechanisms in Chronic Alcoholism: Activated Astrocytes as a Source of Neurotrophins

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Neuronal degenerative disorders are frequently associated with the degeneration of specific populations of cells. In Alzheimer's disease, Parkinson's disease, and Wernicke-Korsakoff syndrome, the degeneration of neurons in the basal forebrain cholinergic system is an early and constant feature and is most probably linked to the progressive deterioration of cognitive function (Arendt et al. unpublished manuscript). The finding that neurotrophic factors act on these cholinergic neurons (see Ebendal 1989) has raised the possibility that trophic factor therapies could eventually prove effective in these disorders. A successful development of such strategies to enhance the survival of damaged neurons by increasing the concentration of growth factors available to them requires a detailed knowledge of the role of endogenous trophic factors in the mechanism of spontaneously occurring regeneration and repair.

Trophic actions relevant to repair and regeneration in both the central nervous system (CNS) and the peripheral nervous system can be divided into two broad categories. The first category contains neurotrophic molecules arising from glial cells and other sources. These molecules are soluble and act over limited distances on receptor-bearing neurons to influence their survival and the expression of genes that are important in neuronal maintenance and repair. The second category contains molecules that enhance growth or regeneration of axonal and dendritic processes. These molecules are mostly present at the cell surface or in the extracellular matrix, where their effects are either permissive or inhibitory.

This chapter focuses on the diffusible polypeptide growth factors that are liberated by glial cells and other cells and that are thought to be relevant to the survival and repair of injured neurons under various degenerative conditions, including those induced by chronic intake of ethanol.

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NERVE GROWTH FACTOR

The Nerve Growth Factor Gene Family and Their Receptors

Substantial progress has recently been made in research on nerve growth factor (NGF) and the other members of the gene family of neurotrophic factors collectively referred to as neurotrophins. Apart from NGF, the neurotrophins include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and neurotrophin-5 (NT-5) (Ebendal 1992). These factors are produced in limited amounts in the target tissue and mediate the cell interactions regulating neuronal survival during the period of naturally occurring neuronal death in development. In the adult nervous system, neurotrophic factors are important in neuronal maintenance and repair.

The best characterized neurotrophic factor is β-NGF, a basic 118 amino acid protein which in the peripheral nervous system acts on sympathetic neurons and the majority of neural crest–derived sensory nerve cells (Levi-Montalcini 1987). Nerve growth factor is synthesized by target organs innervated by sympathetic and sensory neurons and binds to specific receptors on the cell surface, forming a NGF/NGF receptor complex, which is internalized and transported retrogradely to the cell body where physiological actions are mediated (Thoenen and Barde 1980). In the CNS, NGF serves a trophic function in the development and maintenance of cholinergic neurons of the basal forebrain (Ebendal 1989). The target areas of these neurons, the hippocampus, the neocortex, and the olfactory bulb, contain the highest levels of NGF messenger RNA (mRNA) and protein in the brain (Goedert et al. 1986; Whittemore et al. 1986). As demonstrated by in situ hybridization, NGF is synthesized in dentate gyrus granule cells and hippocampal pyramidal neurons (Ayer-LeLievre et al. 1988; Rennert and Heinrich 1986; Whittemore et al. 1988). The observation that [^{125}I]-labeled NGF infused into the hippocampus results in retrograde transport of NGF to the cholinergic neurons in the medial septal/diagonal band, but not to other hippocampal afferents, suggests that NGF is selectively taken up by cholinergic nerve terminals in hippocampus and neocortex and transported to the cell bodies of cholinergic neurons in the basal forebrain (Schwab et al. 1979).

The neurotrophins exert effects on the responsive neurons by binding to cell surface receptors. Two classes of neurotrophin receptors have been identified. Initially, molecular cloning studies characterized a 75- to 80-kd NGF receptor protein ($p75^{NGFR}$) (Johnson et al. 1986; Radeke et al. 1987) that binds several neurotrophins with equivalent low affinity, thus being designated the low-affinity NGF receptor (Rodriguez-Tebar et al. 1990; Squinto et al. 1991). However, this protein by itself is not capable of mediating cellular responses to neurotrophins. A second class of neurotrophin receptors is encoded by the trk proto-oncogene family (trkA, trkB, and trkC). The trk genes encode receptor tyrosine kinases, which, in the absence of $p75^{NGFR}$ expression, are capable of mediating cellular responses to neurotrophins (Cordon-Cardo et al. 1991; Glass et al. 1991; Lamballe et al. 1991). Expression of $p75^{NGFR}$, however, appears to enhance the sensitivity to neurotrophins of cells expressing trk receptors, perhaps in part by increasing the binding affinity of the trk receptors (Hempstead et al. 1991; Kaplan et al. 1991). Since

neurotrophin-responsive neurons generally express $p75^{NGFR}$ as well as one of the trk genes, it appears most likely that $p75^{NGFR}$ is a functional component of neurotrophin receptors in neurotrophin-responsive neurons.

Autoradiographic studies have demonstrated that neurons which bind radiolabeled NGF codistribute with cholinergic neurons of the basal forebrain in the rat (Raivich and Kreutzberg 1987; Richardson et al. 1986). These early findings have been confirmed more recently by means of immunohistochemical techniques, demonstrating a very high degree of colocalization between $p75^{NGFR}$ and the specific cholinergic marker choline acetyltransferase in rat (Batchelor et al. 1989), monkey (Kordower et al. 1988), and human brain (Hefti et al. 1986; Mufson et al. 1989). The expression of detectable levels of trk mRNA, colocalized with $p75^{NGFR}$ mRNA, has been found to be restricted to cholinergic basal forebrain nuclei (Buck et al. 1987; Vazquez and Ebendal 1991).

A second protein with neurotrophic activities reminiscent of but not identical to NGF was isolated from the pig brain and termed "brain-derived neurotrophic factor" (Barde et al. 1982). A genomic clone isolation for the porcine BDNF led to the finding that the mature BDNF and NGF proteins show striking amino acid similarities (Leibrock et al. 1989). During development BDNF is expressed in the brain at initially low levels but later increases to become the most widespread neurotrophin in different areas of the brain, with the hippocampus containing the highest amounts, followed by cerebral cortex, pons, and cerebellum (Ernfors et al. 1990*c*; Hofer et al. 1990; Phillips et al. 1990; Wetmore et al. 1990). Similarly to NGF, BDNF also supports the survival of neuronal crest–derived embryonic sensory neurons (Barde et al. 1982; Leibrock et al. 1989) and of septal cholinergic neurons (Alderson et al. 1990; Knüsel et al. 1991) in vitro. Furthermore, BDNF has been reported to influence dopaminergic neurons of the mesencephalon (Knüsel et al. 1991) and spinal motoneurons (Oppenheim et al. 1992; Sendtner et al. 1992; Yan et al. 1992) which do not respond to NGF. Unlike NGF, BDNF and its tyrosine receptor kinase B (trkB) mRNA are expressed within the same cells in the hippocampal formation (Klein et al. 1990, 1991; Soppet et al. 1991; Squinto et al. 1991). This finding, as well as the higher levels of BDNF mRNA (greater than tenfold) compared with NGF mRNA (Hofer et al. 1990) in brain together with the broader distribution of BDNF mRNA expression, suggests that BDNF has a broader and perhaps different kind of influence on CNS neurons than NGF.

Based on cloning strategies utilizing the partial sequence similarities between NGF and BDNF, a third member of the NGF family, NT-3, was isolated (Ernfors et al. 1990*a*; Kaisho et al. 1990; Maisonpierre et al. 1990; Rosenthal et al. 1990). In the adult brain, its expression is largely confined to the hippocampus. Like other members of the neurotrophin family, it can support survival of sensory and sympathetic neurons. Although the members of the NGF family share considerable sequence similarities, NGF, BDNF, and NT-3 each have unique biological activities (Ernfors et al. 1990*b*; Ibanez et al. 1991) and probably cooperate to support the development and maintenance of the vertebrate nervous system.

Actions of NGF on Survival and Growth of Cholinergic Neurons

Application of exogenous NGF to rat cholinergic neurons stimulates the expression of choline acetyltransferase (Gnahn et al. 1983;

Higgins et al. 1989; Mobley et al. 1986; Williams and Rylett 1990) and promotes the survival and fiber growth of forebrain cholinergic neurons both in vitro (Gähwiler et al. 1990; Hartikka and Hefti 1988) and in vivo (Hefti 1986). High-affinity choline uptake and acetylcholine release in the forebrain are also enhanced by chronic treatment with NGF (Williams and Rylett 1990). After acute brain injury, a number of degenerative changes can be counteracted in NGF-responsive neurons by administration of exogenous NGF. Degeneration of the cholinergic afferentation of the hippocampus and neocortex, which would normally occur after axotomy by fimbria-fornix transection and after excitotoxic basal forebrain lesion, respectively, can be almost completely alleviated by intraventricular administration of exogenous NGF in both rat and nonhuman primate (Gage et al. 1988*a*; Hagg et al. 1988; Koliatsos et al. 1990; Santucci et al. 1993; Tuszynski et al. 1990), as can the behavioral impairments associated with these lesions (Mandel et al. 1989; Ogawa et al. 1993). Chronic intraventricular infusion of NGF, furthermore, has an attenuating effect on the age-related atrophy of cholinergic basal forebrain neurons and its accompanying impairment in memory function (Fischer et al. 1987).

The recent finding that several neuronal populations and their target tissues express neurotrophins simultaneously (Lauterborn et al. 1991; Schecterson and Bothwell 1992) has raised serious questions about the neurotrophic hypothesis predicting that neuronal maintenance is critically dependent on target-derived neurotrophic support. This concept, furthermore, has been contradicted by several studies demonstrating the persistence of cholinergic basal forebrain neurons despite the removal of their respective target neurons in the neocortex and the hippocampus (Kordower et al. 1992; Minger and Davies 1992; Sofroniew et al. 1990). It has, therefore, been proposed that some neurons, including those of cholinergic basal forebrain nuclei, may provide their own trophic support by autostimulation in an autocrine fashion (Lauterborn et al. 1991; Lu et al. 1989; Schecterson and Bothwell 1992).

Changes in NGF Content Under Neurodegenerative Conditions

Many types of experimental brain lesions induce an increase in the content of polypeptide growth factors such as glia maturation factor (Nieto-Sampedro et al. 1988), ciliary neurotrophic factor (Nieto-Sampedro et al. 1983), basic fibroblast growth factor (Finkelstein et al. 1988; Kiyota et al. 1991), acidic fibroblast growth factor (Nieto-Sampedro et al. 1988), interleukin-1 (Nieto-Sampedro and Berman 1987), NGF (table 1), and BDNF (table 2). Furthermore, an increase in the expression of trophic factors and their receptors has been observed in Alzheimer's disease, a disorder that, like Wernicke-Korsakoff syndrome, shows an early and constant degeneration of cholinergic basal forebrain neurons (Arendt 1993; Arendt et al. 1983) (table 3). Mimicking this cholinergic deafferentation of the hippocampus and the neocortex observed in Alzheimer's disease and Wernicke-Korsakoff syndrome in rat brain by transecting the fimbria-fornix or by placing a lesion to basal forebrain neurons, an increase of NGF is found both at the site of neuronal death and in loci deafferented by the lesion (Gasser et al. 1986; Korsching et al. 1986; Lorez et al. 1988; Otten et al. 1989*a*; Weskamp et al. 1986*a*, 1986*b*). Moreover, an increase in NGF has been described in the septum and in the cere-

bral cortex of aged rats that show a severe impairment of learning and memory, indicating that NGF levels were maintained at supranormal levels under conditions where the nervous system is undergoing a progressive degeneration, a process that might be of importance for the stimulation of compensatory functional changes and repair mechanisms (Hellweg et al. 1990; Otten et al. 1989*c*).

Regulation of NGF and BDNF Synthesis

Increased levels of both NGF mRNA and BDNF mRNA not only occur as a result of brain injury but also have been found after seizures induced by a variety of treatments such as focal electrolytic lesions of the dentate gyrus hilus (Gall and Isackson 1989; Isackson et al. 1991; Rocamora et al. 1992), intracerebroventricular injection of kainic acid (Gall et al. 1991*b*), and systemic administration of kainic acid (Dugich-Djordjevic et al. 1992; Zafra et al. 1990). The observation, moreover, that even brief bursts of epileptiform activity are sufficient to induce large changes in the expression of NGF and BDNF in target regions of the adult basal forebrain neurons has led to the suggestion that the activity-dependent regulation of synthesis might be an inherent property of this class of neurotrophic factors and might be related to their role in maintaining neuronal viability (Isackson et al. 1991). Both the glutamatergic system, being intrinsic to the cortical mantle, and the cholinergic afferentation of neocortex and hippocampus arising in the basal forebrain have been shown to be involved in this activity-related stimulation of NGF and BDNF expression (Lapchak et al. 1993; Lindefors et al. 1992; Zafra et al. 1990, 1991). Therefore, the existence of a feedback loop can be proposed whereby the activity of the

Table 1. Increase in NGF Content in Rat Brain After Experimental Brain Lesion and in Aged Animals

Type of Lesion	Site of NGF Increase	Study
Hypoxic injury (carotid artery ligation)	Neocortex, hippocampus, striatum	Lorez et al. 1989
Excitotoxic injury (kainic acid) of neocortex, striatum	Neocortex, striatum	Otten et al. 1989*b*
Cortical ablation (suction lesion)	Striatum, basal forebrain	Lorez et al. 1988
Basal forebrain lesion (electrolytic lesion)	Neocortex, hippocampus	Weskamp et al. 1986*b* Otten et al. 1989*a*
Fimbria-fornix transection	Hippocampus, septum	Gasser et al. 1986 Korsching et al. 1986 Weskamp et al. 1986*a* Whittemore et al. 1987
Aging	Neocortex, basal forebrain	Otten et al. 1989*c* Hellweg et al. 1990

Table 2. Increase in BDNF mRNA in the Adult Rat Brain After Experimental Manipulation

Type of Manipulation	Site of BDNF mRNA Increase	Study
Forebrain ischemia	Hippocampus	Lindvall et al. 1992
Insulin-induced hypoglycemic coma	Hippocampus	Lindvall et al. 1992
Mechanical damage	Hippocampus	Ballarin et al. 1991
Excitotoxic injury of septum or hippocampus	Hippocampus	Ballarin et al. 1991 Lindefors et al. 1992
Kainic acid/electrically induced seizure activity	Hippocampus, cerebral cortex, amygdaloid nuclei	Dugich-Djordjevic et al. 1992 Isackson et al. 1991 Rocamora et al. 1992 Zafra et al. 1990

afferent cholinergic innervation of the cortical mantle may influence their own structural stability.

Activated Astrocytes as a Source of Endogenous NGF

Possible therapeutic strategies aimed at an increase of the available concentrations of NGF in order to facilitate mechanisms of repair require a detailed knowledge of the cellular origin and regulation of the endogenous NGF. The cellular source of endogenous NGF in the CNS, however, is still somewhat controversial. In the adult CNS not affected by injury, NGF and BDNF mRNA is predominantly localized in neurons as demonstrated by in situ hybridization (Ayer-LeLievre et al. 1988; Bandtlow et al. 1990; Ernfors et al. 1990*c*; Hofer et al. 1990; Rennert and Heinrich 1986; Senut et al. 1990; Wetmore et al. 1990; Whittemore et al. 1988). No expression of neurotrophins has been detected so far in astrocytes under in vivo conditions in the normal adult brain. Astrocytes, however, can synthesize and secrete NGF in culture (Assouline et al. 1987; Furukawa et al. 1986, 1987; Houlgatte et al. 1989; Rudge et al. 1992; Yoshida and Gage 1991). During certain developmental stages of the CNS and under conditions of brain injury, astrocytes may synthesize and secrete NGF and BDNF as well. For example, in the developing optic nerve, which contains only glial cells, NGF mRNA can be detected using a sensitive nuclease protection assay (Lu et al. 1991). However, in the adult optic nerve, NGF mRNA is barely detectable unless the nerve is transected, resulting in reactive gliosis and de novo synthesis of NGF mRNA.

The expression of NGF in neurons and astrocytes appears to be regulated differentially. The activity-dependent increase in synthesis of NGF and BDNF in hippocampal neurons, for example, is mediated by non-NMDA glutamate receptors (Zafra et al. 1990). In glioma cells, on the contrary, non-NMDA agonists induce a decrease in NGF mRNA, while NMDA causes an up-regulation (Amano et al. 1992). Glucocorticoid hormones, which are impor-

Table 3. Increase in the Content of Neurotrophic Factors and Their Receptors in Alzheimer's Disease

Factor/Receptor	Method of Detection/Quantification	Study
Factor unspecified	Brain extract/tissue culture	Atterwill and Bowen 1986 Uchida et al. 1988
	Transplantation techniques	Arendt et al. 1991
NGF	ELISA	Allen et al. 1991 Crutcher et al. 1993
NGF-receptor	In situ hybridization Immunocytochemistry	Ernfors et al. 1990*b* Mufson and Kordower 1992
bFGF	Immunocytochemistry	Gomez-Pinilla et al. 1990 Stopa et al. 1990
bFGF-receptor	Immunocytochemistry	Kato et al. 1991
EGF	Immunocytochemistry	Birecree et al. 1988
EGF-receptor	Immunocytochemistry	Styren et al. 1990
APP/β-A4 amyloid	Tissue culture	Schubert et al. 1989 Alvarez et al. 1992 Milward et al. 1992

APP = amyloid precursor protein; bFGF = basic fibroblast growth factor; EGF = epidermal growth factor; ELISA = enzyme-linked immunoadsorbent assay; NGF = nerve growth factor.

tant mediators during development, aging, and stress, increase the levels of NGF mRNA in cultured neurons, whereas they down-regulate the NGF mRNA levels in astrocytes (Lindholm et al. 1992). This might be one factor that contributes to the low levels of NGF mRNA present in astrocytes in vivo.

The expression of NGF mRNA in astrocytes under in vitro conditions is regulated by various cytokines and growth factors, including acidic and basic fibroblast growth factor, interleukin-1 (Carman-Krzan et al. 1991; Ono et al. 1991; Spranger et al. 1990; Yoshida and Gage 1991), and transforming growth factor β (Lindholm et al. 1990). These growth factors are also effective in increasing the NGF mRNA levels in vivo in the hippocampus of neonatal rats (Lindholm et al. 1990; Spranger et al. 1990). Furthermore, immunohistochemical techniques have provided evidence that brain astrocytes activated in vivo by brain lesions (Gage et al. 1988*b*; Lu et al. 1991; Oderfeld-Nowak et al. 1992; Sofroniew et al. 1990; Weskamp et al. 1986*a*) or by pharmacological stimulation by interleukin-1β (Fagan and Gage 1990; Oderfeld- Nowak et al. 1992; Otten et al. 1989*a*; Spranger et al. 1990) may behave just like the astroglia in vivo—that is, they synthesize and secrete NGF. Subconvulsive electrical stimulation resulting in kindling causes a dramatic increase in NGF and BDNF mRNA in the dentate gyrus, parietal cortex, and piriform cortex after 4 hours (Ernfors et al. 1991; Gall et al. 1991*a*). It has not been addressed yet whether this increase in neurotrophin synthesis might also be related to

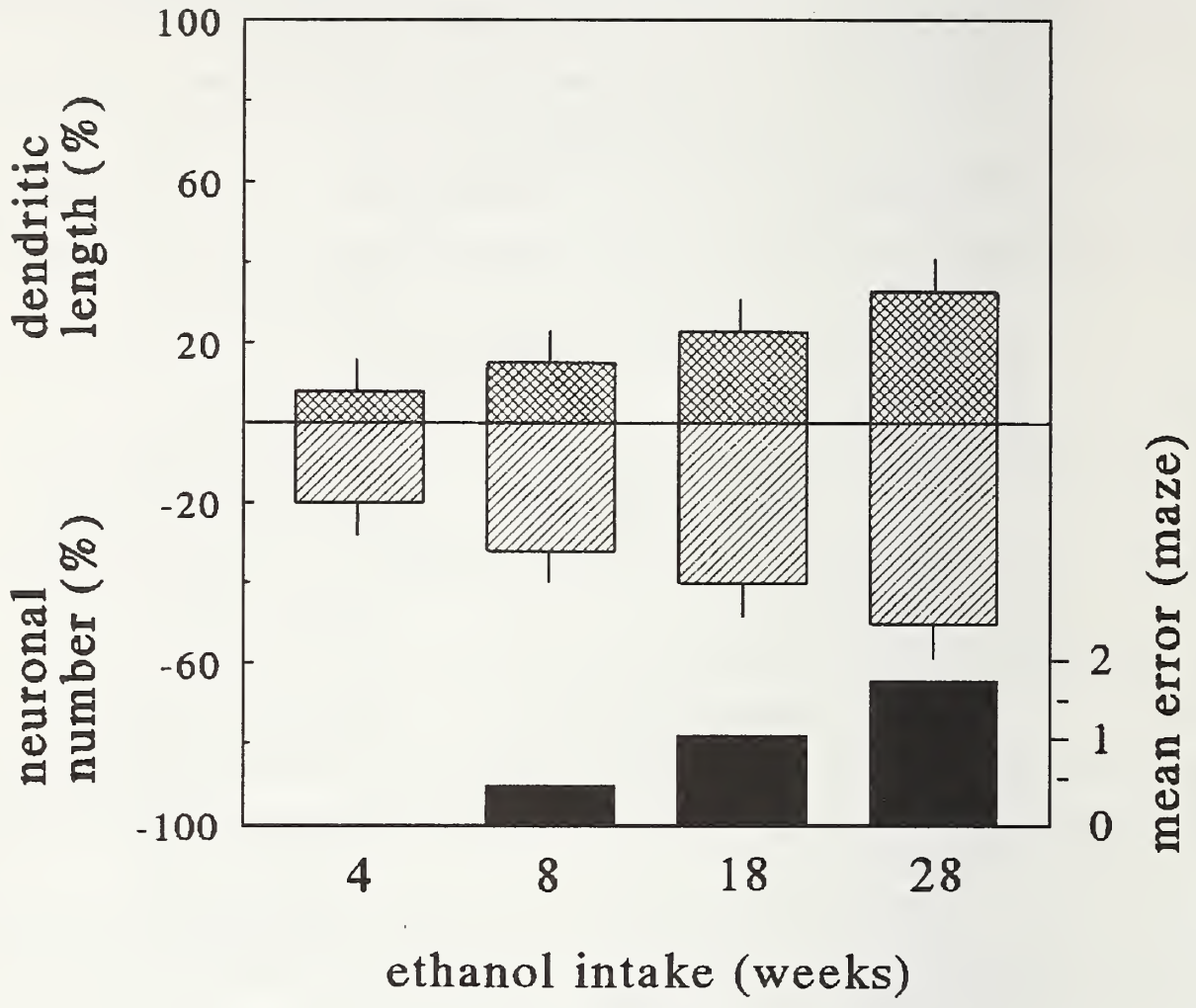

Figure 1. Degeneration in the cholinergic basal forebrain projection system, plastic dendritic response, and memory impairment as a function of the duration of ethanol intake in rat. Animals treated with ethanol (20 percent solution in the drinking water) showed a continuously increasing impairment in maze performance (solid bars, right scale). The number of cholinergic basal forebrain neurons declined over the duration of treatment (hatched bars, left scale; histochemical reaction of acetylcholinesterase). Simultaneously, an increase in dendritic length could be observed on remaining neurons (crosshatched bars, left scale; Golgi impregnation). Changes are expressed as a percentage of control values (+ SD) obtained on non–ethanol-treated animals (each group n = 12 animals).

an activation of astrocytes, which was demonstrated in a similar experimental paradigm (Steward et al. 1991).

Astrocytes have been shown to be capable of synthesizing basic fibroblast growth factor (Ferrara et al. 1988; Fukumoto et al. 1991), which stimulates their own proliferation (Barotte et al. 1989) and NGF synthesis. Furthermore, it has been demonstrated that astrocytes in culture might be able to express $p75^{NGFR}$, trkA, and trkB mRNA (Hutton et al. 1992). On astrocytes (Hutton et al. 1992) as well as on cholinergic basal forebrain neurons (Fusco et al. 1991; Higgins et al. 1989; Kojima et al. 1992), the expression of $p75^{NGFR}$ can be up-regulated in response to NGF. Interleukin-1, which under the condition of injury is synthesized and secreted by microglia, might be one factor mediating the stimulation of NGF synthesis in astrocytes, which in turn would increase the expression of $p75^{NGFR}$ on both astrocytes and cholinergic neurons

(Gadient et al. 1990; Giulian and Baker 1986; Heumann et al. 1987; Lindholm et al. 1987). Taken together, these results suggest that some neurotrophins act on astrocytes as part of autocrine cascades associated with neuronal-glial interactions, which are of importance for neuronal maintenance and repair.

DEGENERATION, COMPENSATION, AND REPAIR IN THE CHOLINERGIC BASAL FOREBRAIN SYSTEM AFTER CHRONIC INTAKE OF ETHANOL

Chronic intake of ethanol both in humans (Arendt et al. 1983, 1989) and in rodents (Arendt et al. 1988, 1989; Beracochea et al. 1987) results in a degeneration of the cholinergic basal forebrain projection system. This degeneration, which most probably is causally linked to the impairment in memory function, is revealed by a loss of cholinergic basal forebrain neurons and a concomitant decrease of choline acetyltransferase activity in the target areas such as the neocortex and the hippocampus (see Arendt 1993).

The loss of neurons in the cholinergic basal forebrain nuclei induced by chronic ethanol consumption is associated with a plastic response—that is, a remodeling of the dendritic organization of remaining neurons. A quantitative study performed in our laboratory on the topology and size of the dendritic arborization demonstrated an increase in the degree of dendritic branching associated with an increase in the total length of dendrites in both human and rodent brain (figures 1 and 2).

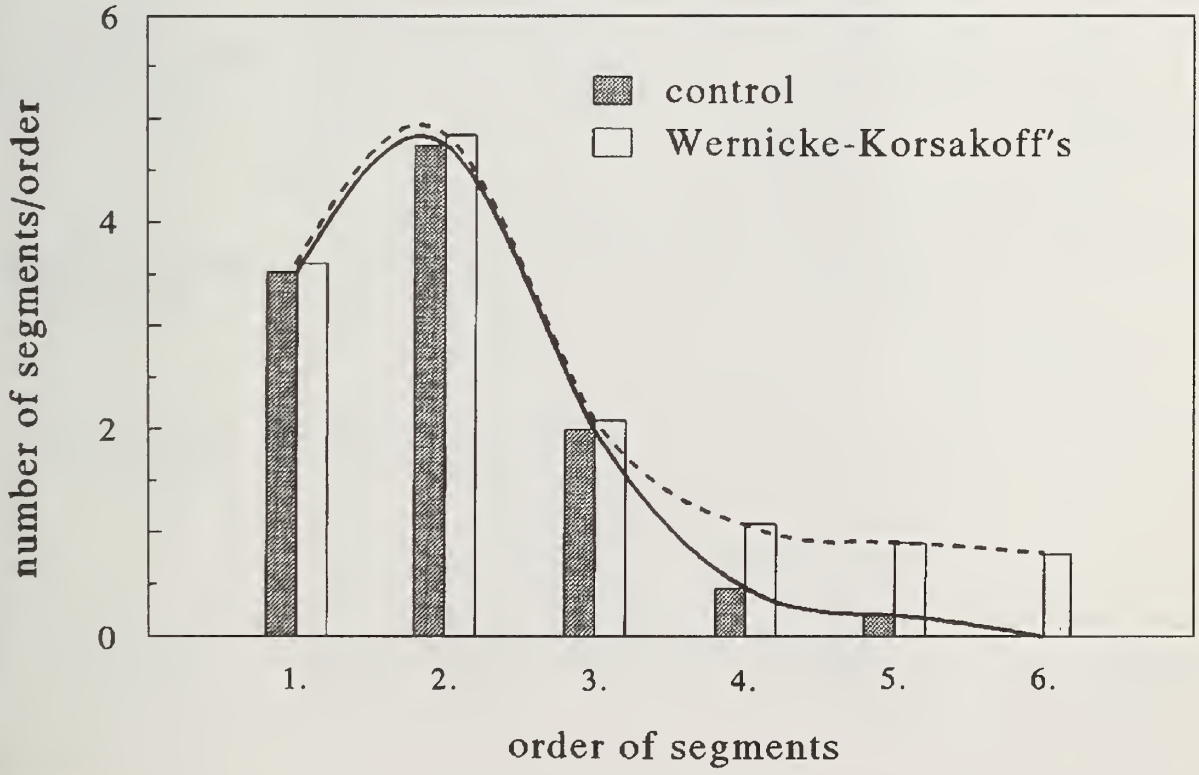

Figure 2. Changes in the frequency distribution of segments of different orders (centrifugal ordering system) obtained on basal forebrain neurons in Wernicke-Korsakoff syndrome compared with control (Golgi impregnation; data are mean of $n = 6$ cases in each group).

Table 4. Plastic Changes of Dendritic Organization in Rodent Brain Induced by Chronic Intake of Ethanol and Subsequent Abstinence

Neuronal Type	Dendritic Parameter		Species	Paradigm of Ethanol Treatment	Study
Hippocampal CA1 pyramidal neurons	Branching of basilar dendrites	↓ ↑	Rat	5 months, 15 g/kg/d (liqu.d.) 5 months + 2 months abstinence	McMullen et al. 1984
	Spine density on apical and basal dendrites	↑	Rat	2 months, 6.8 g/kg/d (tub.)	Kunz et al. 1976
		↓	Rat	20 weeks, 13.3 g/kg/d (liqu.d.)	King et al. 1988
		↑	Rat	20 weeks + 20 weeks abstinence	
	Spine density on apical dendrites	↓ ↑	Mouse	9.5 months, 15% aqueous solution 9.5 months + 2 months abstinence	Lescaudron et al. 1989
Prefrontal pyramidal neurons	Branching of basal dendrites	↑ ↓	Rat	6/18 months, 20% acqueous solution 12 months + 6 months abstinence	Cadete-Leite et al. 1990
Dentate gyrus granule cells	Spine density	↑ ↓	Rat	20 weeks, 13.3 g/kg/d (liqu.d.) 20 weeks + 20 weeks abstinence	King et al. 1988
	Number and length of segments	↑ ↑	Rat	6 months, 20% aqueous solution 12 months + 6 months abstinence	Cadete-Leite et al. 1988*b*, 1989
	Dendritic length Distal dendritic branching Proximal dendritic branching	↑ ↓ ↑	Rat	5 months, 10g/kg/d (liqu.d.)	Durand et al. 1989
Cerebellar Purkinje cells	Length of terminal segments	↑	Rat	48 weeks, 10g/kg/d (liqu.d.)	Pentney et al. 1989 Pentney 1991 Tavares et al. 1985
	Number of terminal segments	↑	Rat	48 weeks liquid diet + abstinence	Pentney and Quackenbush 1990

NOTE: ↓ decreased; ↑ increased.

The pattern of degeneration as well as of the plastic dendritic response showed a gradient over the rostrocaudal extension of the cholinergic basal forebrain nuclei, with the septum/diagonal band nuclei—giving rise to the cholinergic afferentation of the hippocampus—being most severely affected. Plastic changes found on cholinergic neurons were largely restricted to the distal parts of the dendritic tree, indicating a regrowth of terminal and preterminal dendritic segments (see figure 2). Alterations in number and length of dendrites, particularly the terminal branches, are consistent with current theories on dendritic plasticity. Neuronal dendritic arbors are continually changing and adapting by retraction and expansion in response to changes in function and neuronal environment throughout the entire lifespan (Purves and Hadley 1985). Both during development and under the conditions of aging and degeneration, plastic dendritic changes are largely confined to terminal and preterminal dendritic branches (Fritschy and Gareg 1986; Pentney 1993; Scheibel 1983).

Plastic changes of the dendritic organization during aging and a variety of other degenerative conditions primarily affect the

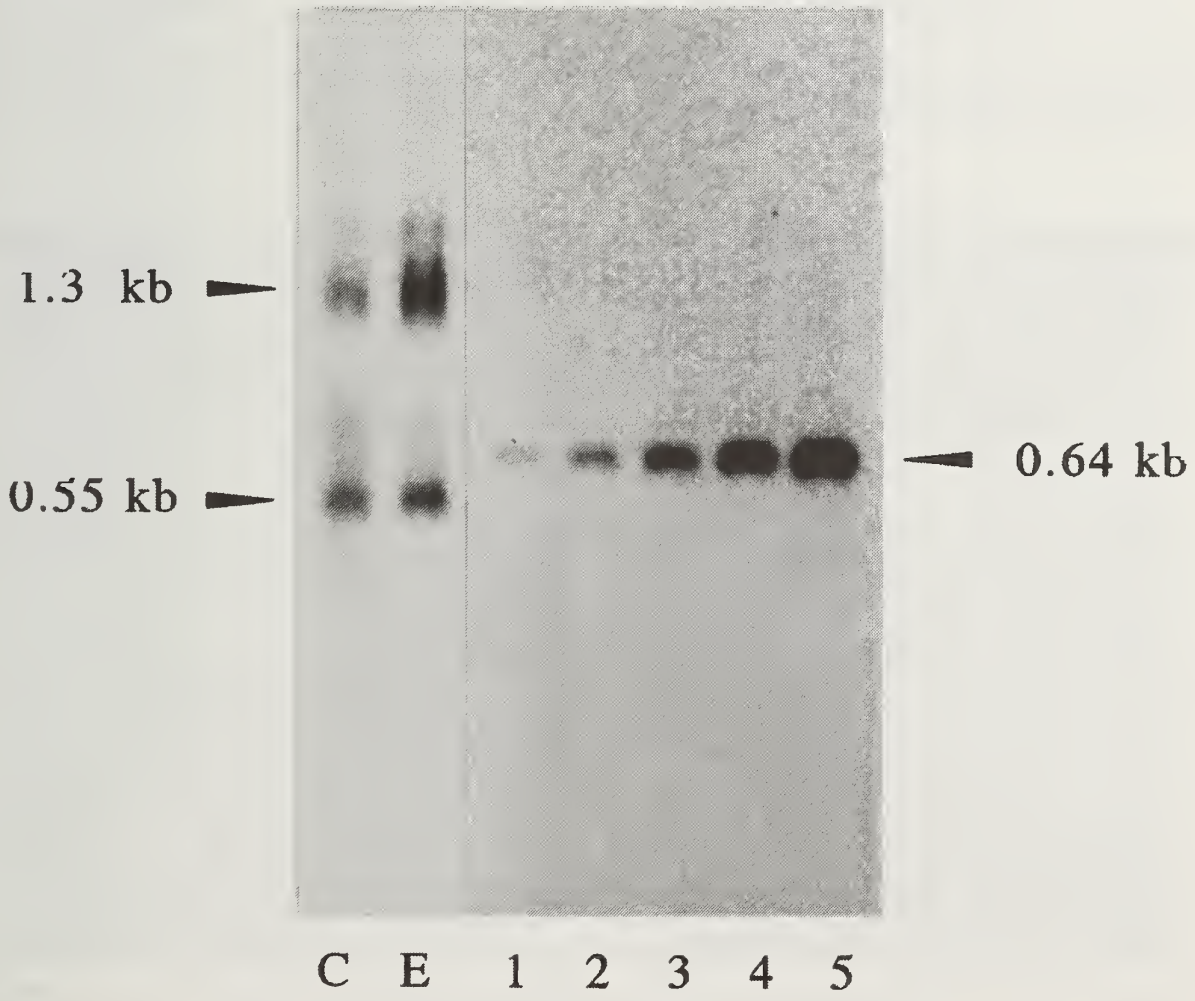

Figure 3. Northern blot analysis of NGF mRNA in the hippocampus of rat chronically treated with ethanol compared with control. Lanes: C = control animal; E = ethanol-treated animal; 1–5 = external standards, 1.25 pg, 2.5 pg, 5 pg, 10 pg, 20 pg mouse NGF mRNA.

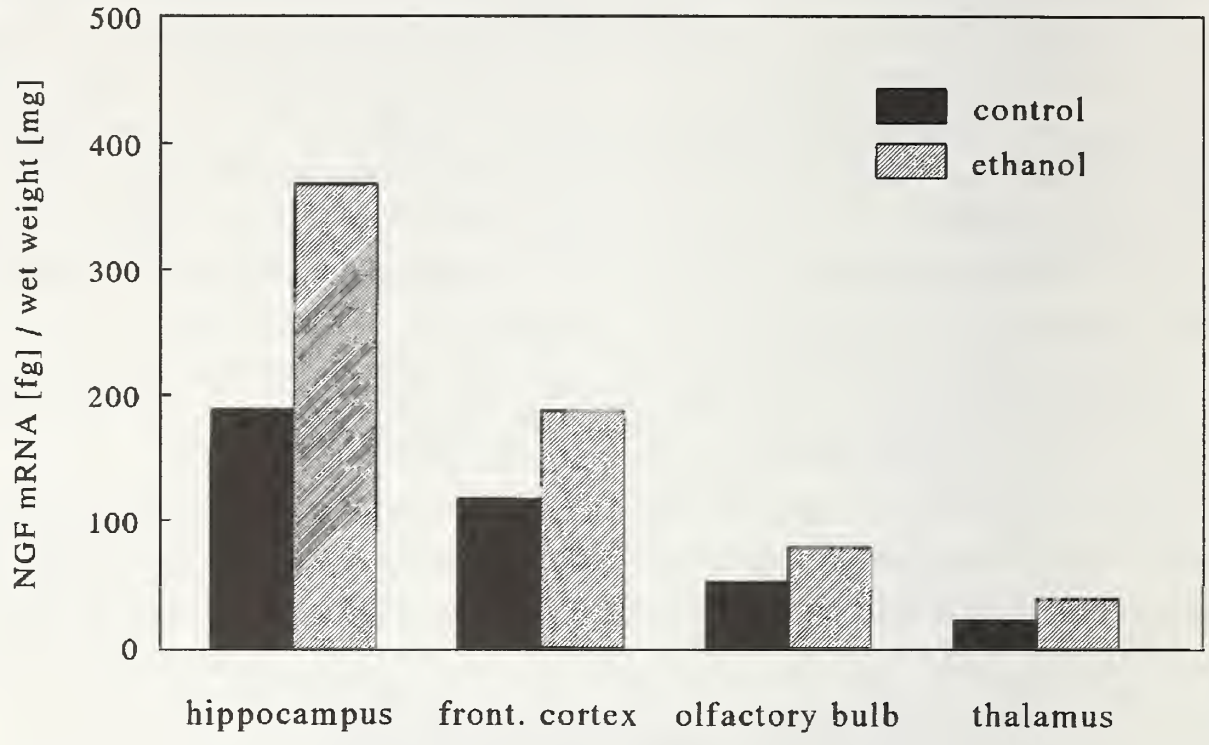

Figure 4. Quantitative changes in the NGF gene expression after chronic intake of ethanol as determined by quantitative NGF mRNA Northern blot technique.

same neuronal population that appears to be particularly vulnerable to degeneration and cell death, indicating that certain types of neurons exert a high degree of structural plasticity under these degenerative conditions. Our observations of both degeneration and dendritic remodeling on cholinergic basal forebrain neurons after chronic intake of ethanol are in agreement with a number of studies reporting on similar changes for other neuronal types (table 4). Thus, chronic treatment with ethanol has been reported to induce dendritic growth on cortical and hippocampal pyramidal neurons (Cadete-Leite et al. 1990; Kunz et al. 1976; McMullen et al. 1984), dentate gyrus granule cells (Durand et al. 1989; King et al. 1988; Paula-Barbosa et al. 1993), and cerebellar Purkinje cells (Pentney 1993), neurons that at the same time have been reported to be particularly susceptible to ethanol-induced degeneration (Cadete-Leite et al. 1988*a*; Pentney 1993; Walker et al. 1980).

Both regressive and plastic adaptive changes of dendritic parameters follow a rather complex pattern during ethanol treatment and subsequent withdrawal depending on the neuronal type subjected to investigation and the experimental paradigm used. A compensatory dendritic hypertrophy—that is, an increase in spatial extension and branching of the dendritic tree—eventually occurs after a longer period of abstinence or can already be observed after a few weeks of treatment. These compensatory changes in the size and geometry of the receptive surface area of surviving neurons, which usually follow a period of regression, are accompanied by the formation of new synapses (Tavares et al. 1987).

Dendritic growth under the conditions of ethanol-induced neurodegeneration either rep-

resents structural equivalents of repair processes or might be a direct effect of ethanol, which has been shown to enhance NGF-induced neurite outgrowth in vitro (Messing et al. 1991). Little is known about the mechanisms by which ethanol alters the formation of neural processes. Studies on the interference of ethanol with neurotrophic factor activity and responsiveness using different culture systems and NGF bioassays have produced conflicting results (Aloe and Tirassa 1992; Dow and Riopelle 1985; Heaton et al. 1992; Messing et al. 1991). The question has not been addressed yet, however, by means of the more conclusive direct quantification of NGF and BDNF expression. Therefore, in order to investigate whether the progressive plastic changes induced by chronic ethanol intake are related to trophic signals derived from efferent neuronal connections and neuroglia, we studied the regional expression of NGF and BDNF throughout different brain areas by quantitative Northern blot analysis and in situ hybridization (Arendt et al. unpublished manuscript).

INCREASE IN THE NEUROTROPHIN EXPRESSION IN NEURONS AND GLIAL CELLS AFTER CHRONIC ETHANOL INTAKE

Northern Blot Analysis of NGF mRNA

Wistar rats were treated with a 20 percent (vol/vol) ethanol solution in the drinking water for 28 weeks resulting in blood ethanol levels between 40 mg/100 mL (daytime) and

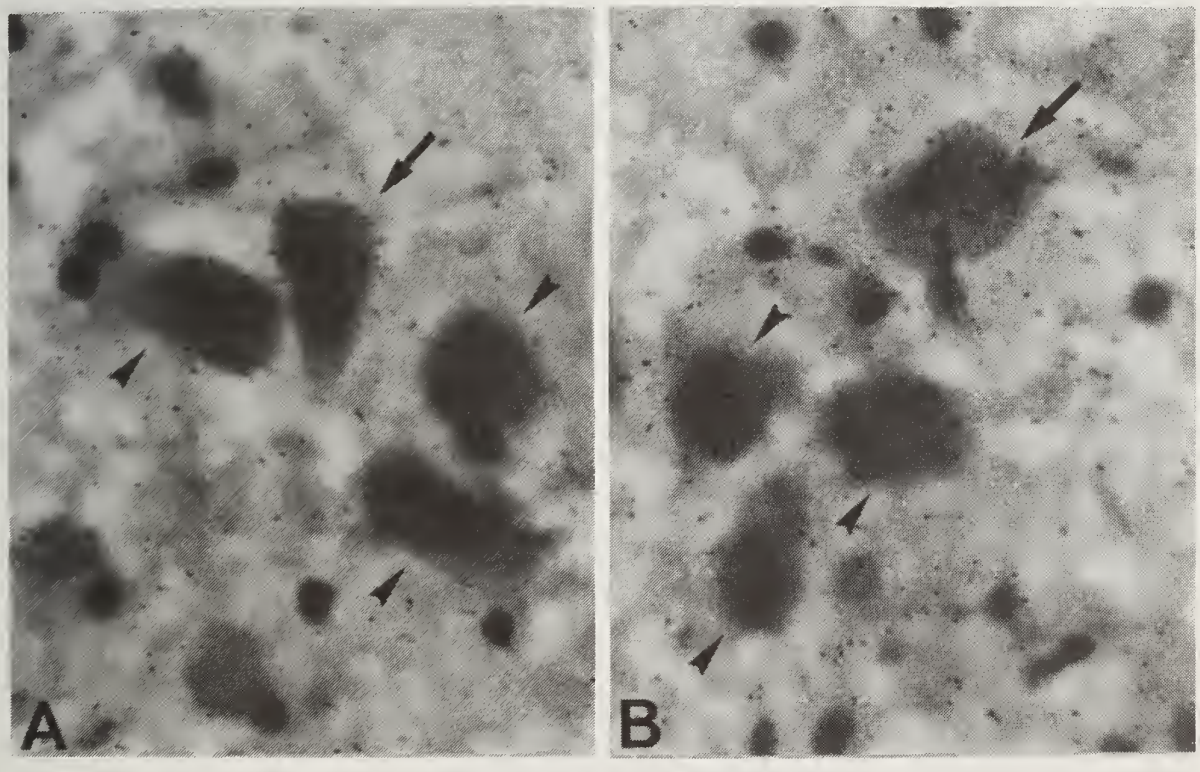

Figure 5. Expression of NGF mRNA and BDNF mRNA in hilar neurons of the hippocampus of control animal. The hybridization signal of NGF (A) and BDNF (B) antisense oligonucleotides on hilar neurons is confined to a subset of neurons (arrows), while adjacent neurons show no specific signal over background (arrowheads).

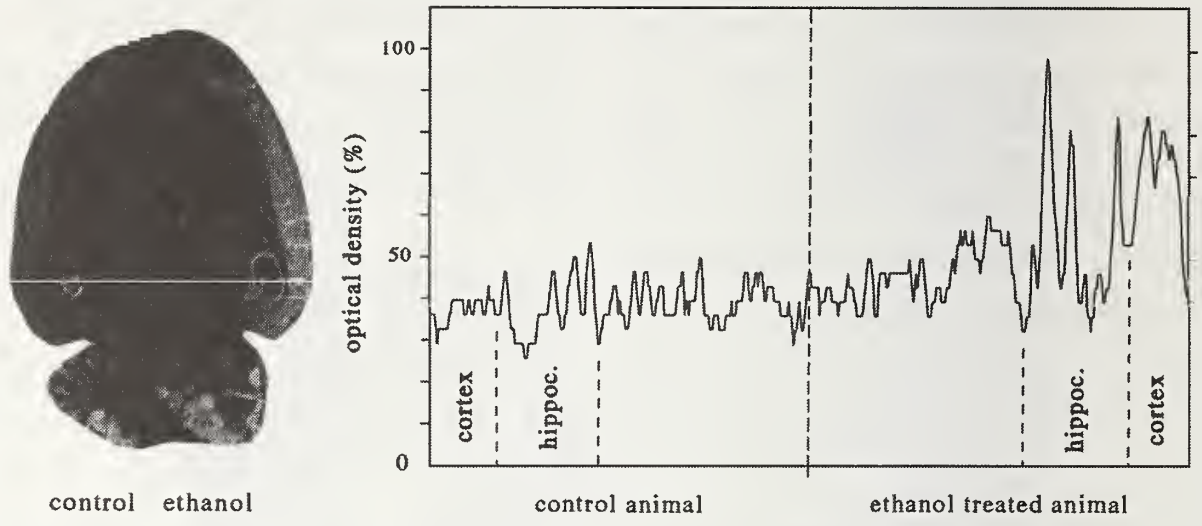

Figure 6. Signal of hybridization to BDNF mRNA in ethanol-treated animal compared with control. (Left) The left hemisphere of an untreated control and the right hemisphere of an ethanol-treated animal were mounted together to ensure identical processing. Dark field view of an autoradiogram of a section hybridized with a BDNF antisense probe and exposed for 7 days. Note the higher expression in cortical areas of the ethanol-treated animal. (Right) Cross-sectional profile of optical density obtained on the autoradiogram along the line indicated. The hybridization signal in the hippocampus and the cortex of the ethanol-treated animal was about doubled compared with control.

120 mg/mL (darkness). Daily intake was 9–10 g/kg body weight. Poly(A)-RNA was isolated from various brain regions and subjected to quantitative Northern blot analysis (Arendt et al. unpublished manuscript) (figure 3). In ethanol-treated animals, NGF mRNA was increased in a number of brain areas (figure 4). The changes were most pronounced in the target areas of cholinergic basal forebrain neurons such as the hippocampus and the neocortex, where they increased by 92 percent and 133 percent, respectively.

In Situ Hybridization

In order to relate these changes in NGF expression obtained on Northern blot to defined neuronal and glial cell populations, we used mRNA in situ hybridization of NGF and BDNF in combination with immunocytochemical methods. Rat brain horizontal sections were hybridized with antisense ^{35}S-oligonucleotide probes and with digoxigenin-labeled RNA probes specific for NGF and BDNF, respectively, and quantitatively examined by image analysis (Arendt et al. unpublished manuscript).

In both control and ethanol-treated animals, expression of NGF and BDNF was observed throughout the brain gray matter, with the highest level of expression seen in the hippocampus and the neocortex and in particular within the dentate gyrus and CA2–CA4 areas. Cerebellar cortex, thalamic, and hypothalamic nuclei were also prominently labeled. The strong signals observed over the large hippocampal pyramidal cells and the granule cells of the dentate gyrus allowed the localization of NGF mRNA and BDNF mRNA in neurons (figure 5). Neurons that were strongly labeled were found adjacent to neurons that were not labeled over background. In the ethanol-treated animals, the

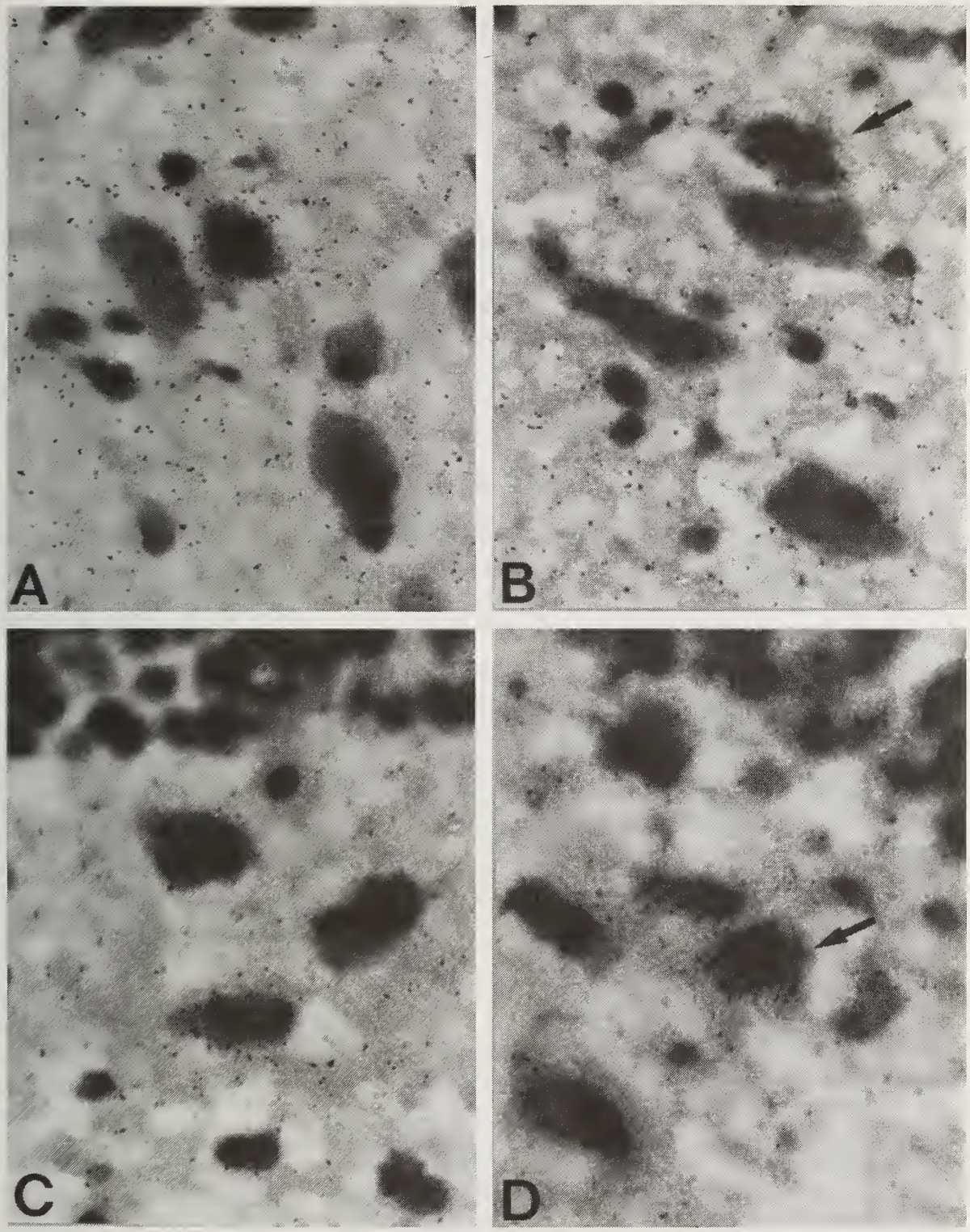

Figure 7. In situ hybridization of NGF mRNA (A, B) and BDNF mRNA (C, D) in hippocampal hilar neurons of control (A, C) and ethanol-treated (B, D) animals. Note the higher grain density over labeled neuron for both NGF and BDNF after ethanol treatment (arrows).

expression of both NGF and BDNF was increased throughout many brain areas, with changes being most pronounced in the neocortex and hippocampus (figure 6). The hybridization signal of both NGF and BDNF, which was found to be increased in ethanol-treated animals, was localized over neurons (figure 7) as well as over glial cells.

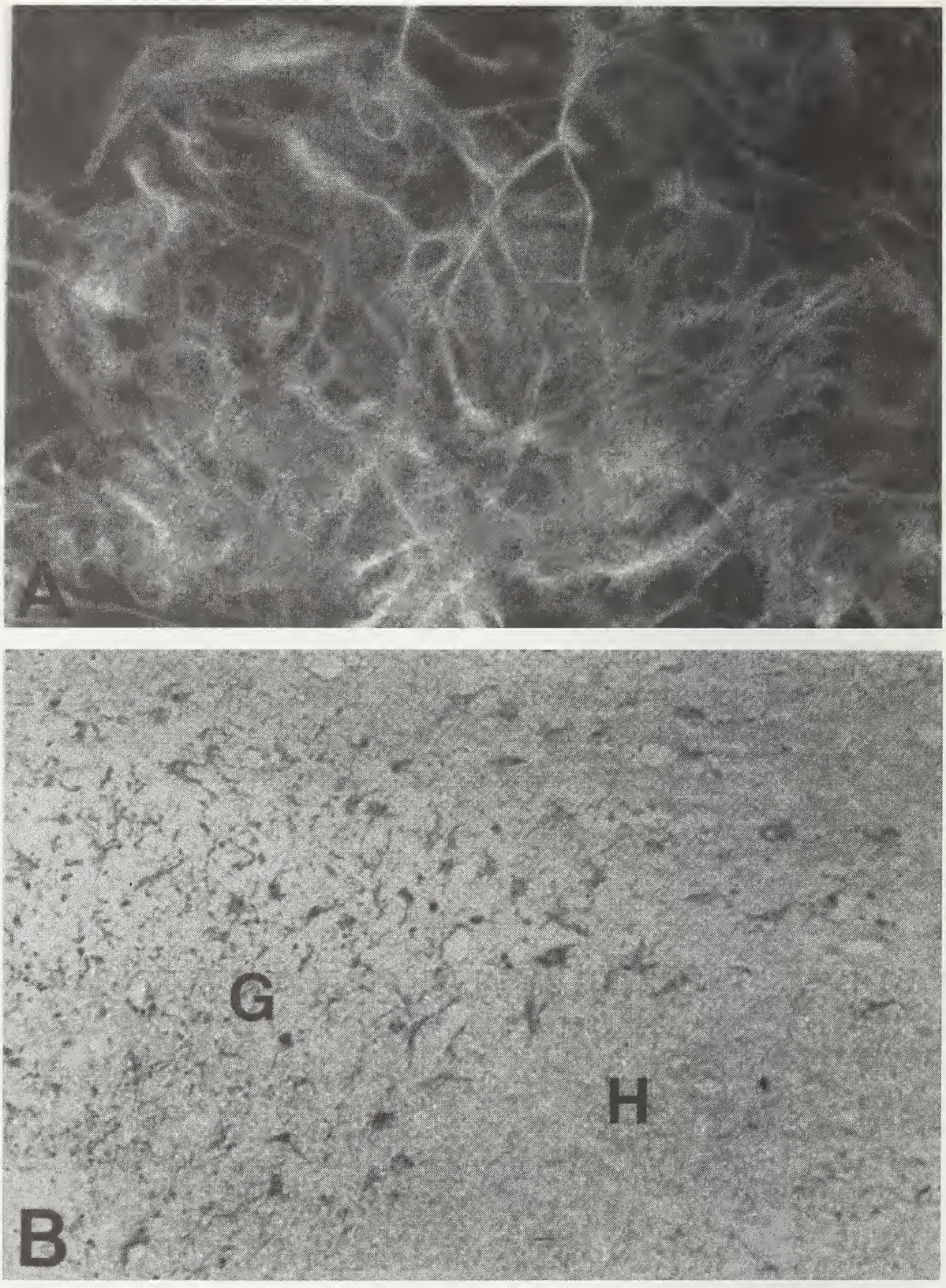

Figure 8. Transplantation of purified astrocytes into rat neocortex. (A) Astrocytes in culture. (B) Host-graft interface, 2 days after transplantation (G = graft, H = host). Labeling of glial fibrillary acidic protein specific to astrocytes with a monoclonal antibody (Boehringer, Mannheim; 1:5). Immunoreaction was visualized using a fluorescein isothiocyanate conjugated secondary antibody (A) or biotinylated secondary antibody, the Extravidin-peroxidase complex (Sigma) and diaminobenzidine/H_2O_2 as chromogen (B).

TRANSPLANTATION OF NEUROTROPHIN-LIBERATING ASTROCYTES: A THERAPEUTIC STRATEGY TO AMELIORATE ETHANOL-INDUCED BEHAVIORAL IMPAIRMENT

The data presented above provide direct evidence for the ability of activated astrocytes to express NGF in vivo. These findings offer a new insight into the potential role of glial NGF in rescuing the ethanol-damaged brain. Transplantation of activated astrocytes might, therefore, be one possible therapeutic strategy to increase the endogenous concentration of NGF available to degenerating neurons.

In a set of experiments, we investigated the effects of intracortical and intrahippocampal grafting of purified astrocytes on the amelioration of the impaired memory function that is a sequel of the partial cholinergic deafferentation of the cortical mantle induced by chronic intake of ethanol (Brückner and Arendt 1991, 1992) (figure 8). Grafting of purified activated astrocytes into the neocortex and/or hippocampus substantially improved cognitive performance (figure 9). Transplantation was equally effective in restoring working and reference memory. The time course of behavioral recovery, which could be detected as early as 2 weeks after operation, was matched by the substantial recovery of synthesis, content, and release of acetylcholine both in the basal forebrain and in target areas such as neocortex and hippocampus (figure 10, table 5). This concordance between the cognitive improvement and the recovery of cortical cholinergic function as well as the alleviation of the ethanol-induced atrophy of cholinergic neurons (figure 11) makes it most likely that the graft-induced behavioral recovery in the present paradigm is due to glia-derived neurotrophic stimulation of remaining cholinergic basal forebrain neurons.

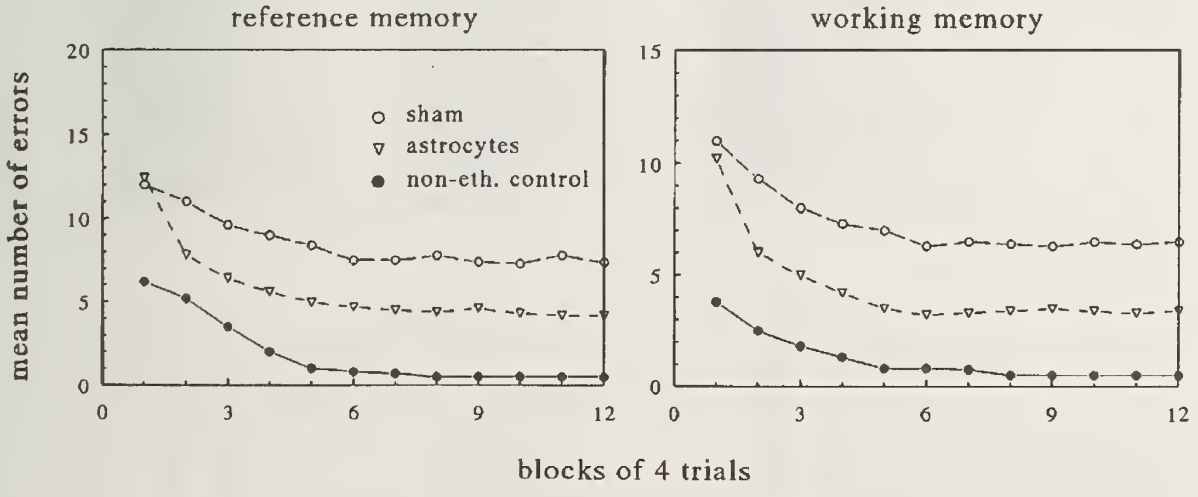

Figure 9. Effects of purified astrocytes grafted to the neocortex and the hippocampus on the impaired memory function induced by chronic intake of ethanol. Mean number of errors cumulated over blocks of four trials in the eight-arm radial maze. Errors made by animals treated with ethanol (20 percent solution in the drinking water) for 28 weeks were analyzed separately for reference memory and working memory after receiving grafts of purified astrocytes simultaneously at four sites in the neocortex and the hippocampus or being sham operated (group size: $n = 8$ animals).

CONCLUSIONS

The results summarized in this chapter indicate that neuronal degeneration of cholinergic basal forebrain neurons induced by chronic intake of ethanol is accompanied by a reactive increase in the neurotrophin expression of both neurons and glial cells. It might, therefore, be suggested that this local increase in trophic activity provides the microenvironment for a stimulation of the process of dendritic remodeling which accompanies the process of chronic neuronal degeneration. Grafting of neurotrophin-liberating

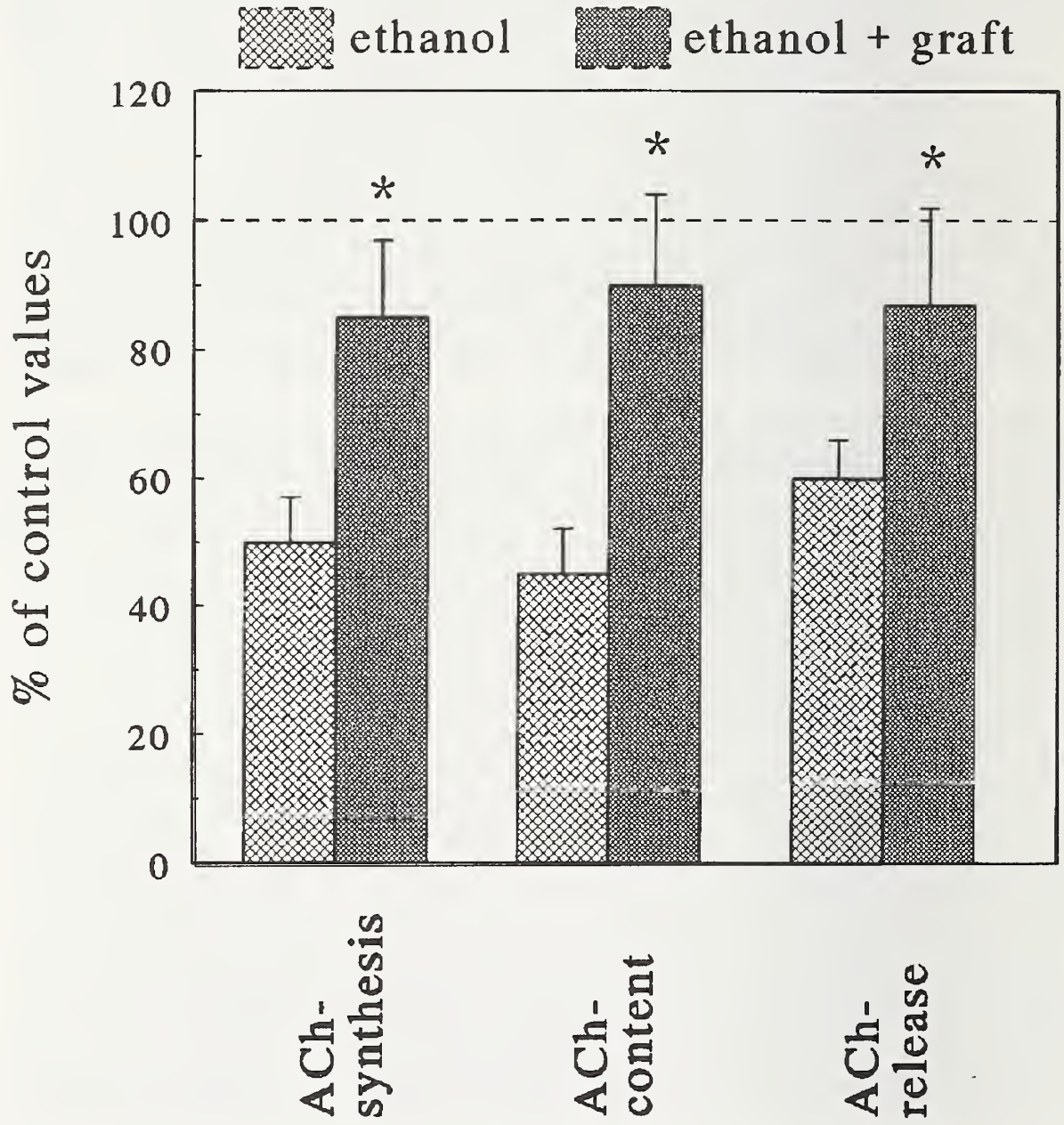

Figure 10. Effects of grafts of purified astrocytes placed to the neocortex and the hippocampus on the synthesis, content, and release (K^+-evoked) of acetylcholine (ACh) in the basal forebrain previously reduced by chronic intake of ethanol (20 percent in the drinking water, 28 weeks). Data are mean (+ SD), obtained 13 weeks after surgery (n = 12 animals). *Effects are significant for $p < 0.01$ (t test).

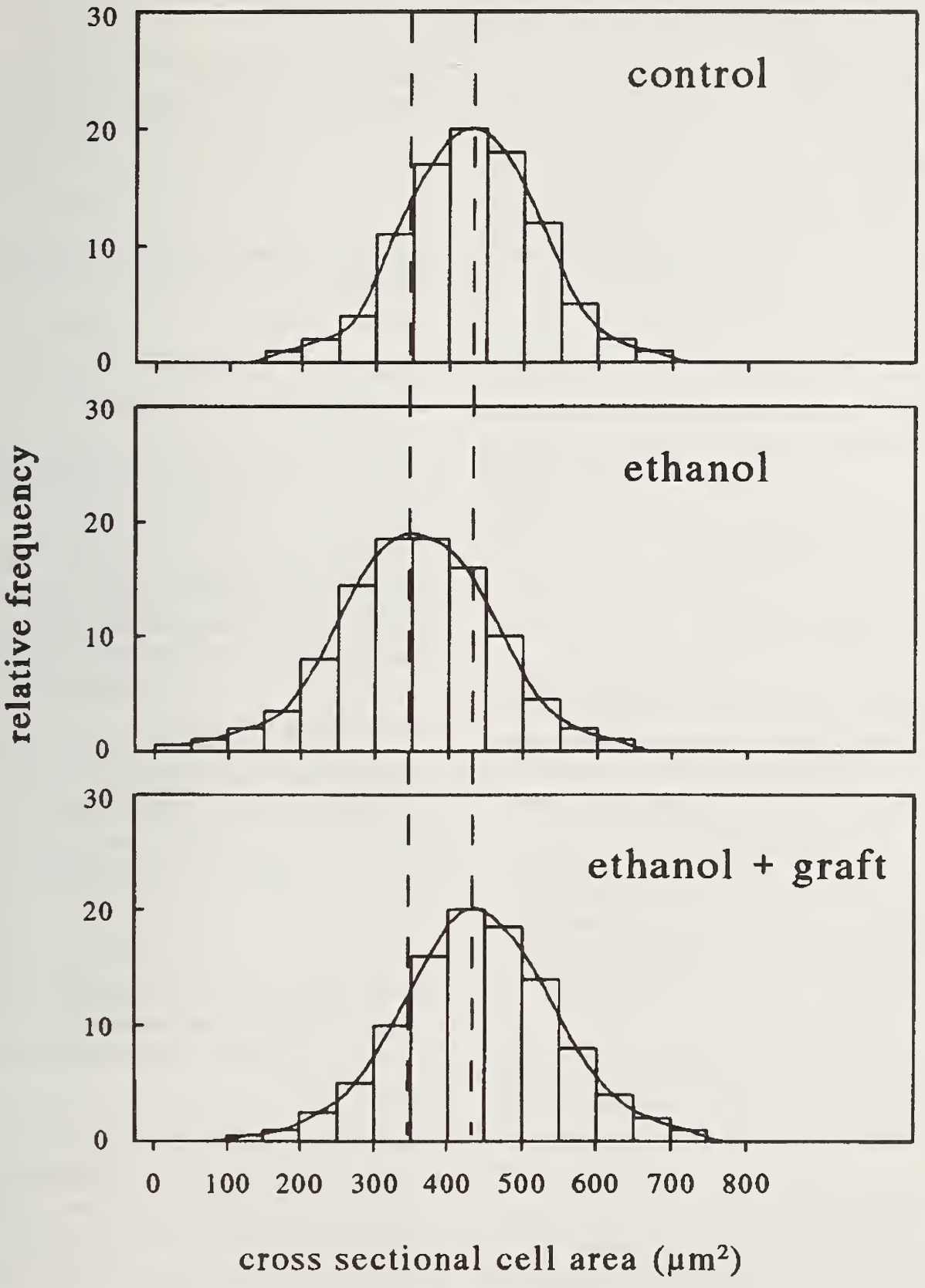

Figure 11. Effects of grafts of purified astrocytes placed to the neocortex and the hippocampus on the atrophy of cholinergic basal forebrain neurons induced by chronic intake of ethanol in rat. Neuronal shrinkage induced by ethanol treatment (20 percent) for 28 weeks was completely reversed by grafting (histochemical reaction of acetylcholinesterase; each group n = 350 neurons).

Table 5. Correlations Between the Content of Neurotransmitters in the Neocortex and the Pre-/Post-treatment Differences in Maze Performance

	Acetylcholine	Noradrenaline	Serotonin	Dopamine
Astrocytes	0.92*	0.18	0.16	0.08
Sham	0.13	0.21	0.09	0.11

Note: The rank correlation coefficient was calculated from the difference between the asymptotic maze performance prior to transplantation and 13 weeks after receiving either grafts of purified astrocytes to the neocortex or being sham operated ($n = 8$ animals. $*p < 0.01$; Mann-Whitney *U Test*).

astrocytes onto the rat brain affected by ethanol-induced brain damage might be an experimental model suitable for developing strategies of therapeutic benefit by increasing the concentration of neurotrophins available to degenerating neurons.

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Chapter 10

Induction of Nitric Oxide Synthase in Brain Glial Cells: Possible Interaction With Ethanol in Reactive Neuronal Injury

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Much of the current interest in neuroimmunopathology stems from the recent understanding that the immune system and central nervous system (CNS) undergo bidirectional communication and that inflammatory processes in the brain involve cooperation between cells of the immune system (macrophages, T and B lymphocytes) and glial cells (astrocytes, microglia, and oligodendrocytes). Some types of communication help maintain homeostasis, whereas some immune responses may be pathological in nature. Although the precise role inflammatory and immune processes play in brain pathology is not clear, most, if not all, types of neurological conditions appear to have an inflammatory component. In addition, accumulating evidence has implicated immune and inflammatory responses in the pathogenesis of neurological diseases, including alcohol-induced brain damage to both gray and white matter. Crosstalk between the immune and nervous system occurs through the release of growth factors, neuropeptides, and, most importantly, cytokines, which play a fundamental role in inflammatory and immune responses. Injury, infection, trauma, alcohol exposure, and other pathological events can result in breakdown of the blood-brain barrier and infiltration of cytokines and cytokine-releasing cells from the peripheral immune system. It has recently been demonstrated that glia not only respond to cytokines, but also secrete cytokines and neurotrophic and neurotoxic substances; they also express class I and class II major histocompatibility complex (MHC) antigens (for a review, see Benveniste 1992). Thus, glial cells provide cellular responses to immunological challenge and injury which may contribute to the neuropathological outcome.

We are grateful to Dr. Soloman Snyder (Johns Hopkins University) for the brain cNOS-cDNA probe and to Drs. Qiao-wen Xie and Carl Nathan (Cornell University) for the mouse macrophage iNOS-cDNA probe and rabbit anti-iNOS antibody. This work was supported by National Institute on Alcohol Abuse and Alcoholism grants AA00127 and AA06069, Public Health Service grant NS–19441, and American Heart Association (Florida Affiliate) grant 92GIA/846.

Another area of investigation which has further linked immune function and neuropathology is the finding that both the nervous system and immune system produce nitric oxide (NO) in response to bacterial endotoxin (lipopolysaccharide [LPS]) or cytokine exposure. This is an intriguing observation from a pathological standpoint since bactericidal and tumoricidal actions of macrophages are mediated in part by NO. This chapter presents evidence that cytokines and endotoxin stimulate production of NO in astroglia and microglia cells. It is hypothesized that NO produced by either infiltrating immunocompetent cells or resident astroglia and microglia may act as a neurotoxic agent following immunological challenge, injury, or brain trauma and may play a role in the pathogenesis of alcohol-induced brain damage.

REGULATION OF NITRIC OXIDE FORMATION

Nitric oxide is a short-lived and highly reactive gas that participates in diverse physiological responses. It was first identified as a messenger molecule in vascular endothelium and has since been shown to be synthesized in a number of tissues and cell types, including macrophages and neutrophils, macula densa and mesangial cells of the kidney, Kupffer cells and hepatocytes, nonadrenergic/noncholinergic neurons of the peripheral nervous system, and neurons and glial cells in the brain (for a review, see Stuehr and Griffith 1992).

Our understanding of the biochemistry, physiology, and tissue distribution of NO formation was greatly advanced by isolation and molecular cloning of nitric oxide synthase (NOS), the enzyme responsible for NO formation. A number of isoforms of NOS have been identified; they can be divided into two general types. Endothelial cells and neurons possess constitutively expressed forms of NOS that have been isolated and cloned (figure 1, panel A) (Bredt and Snyder 1990; Bredt et al. 1991*b*; Sessa et al. 1992). A second type of NOS, which is not normally present in the cell but is synthesized de novo in response to inducing agents, has also been cloned from macrophage cells (Lyons et al. 1992; Xie et al. 1992).

A distinguishing characteristic between the constitutive forms of NOS (cNOS) and inducible forms of NOS (iNOS) is that cNOS is activated by rapid and transient increases in intracellular calcium, while iNOS activity is independent of Ca^{2+} and is regulated at the transcriptional level by various stimulating agents. Neuronal cNOS, endothelial cNOS, and macrophage iNOS have common cofactor requirements that include flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), heme, and tetrahydrobiopterin. All known forms of NOS have consensus sequences for binding of calmodulin and NADPH in addition to the flavins and catalyze the formation of NO from the guanidino nitrogen of L-arginine using NADPH and O_2 as cosubstrates in a stoichiometric reaction that also produces L-citrulline as a coproduct with NO (figure 1, panel B). Neuronal cNOS and endothelial cNOS have absolute requirements for both calcium and calmodulin. It is thought that increased calcium activates cNOS by stimulating the binding of calmodulin to its consensus sequence on the enzyme. Although it is not yet clear what role phosphorylation plays in regulating its activity, cNOS has been reported to undergo phosphorylation

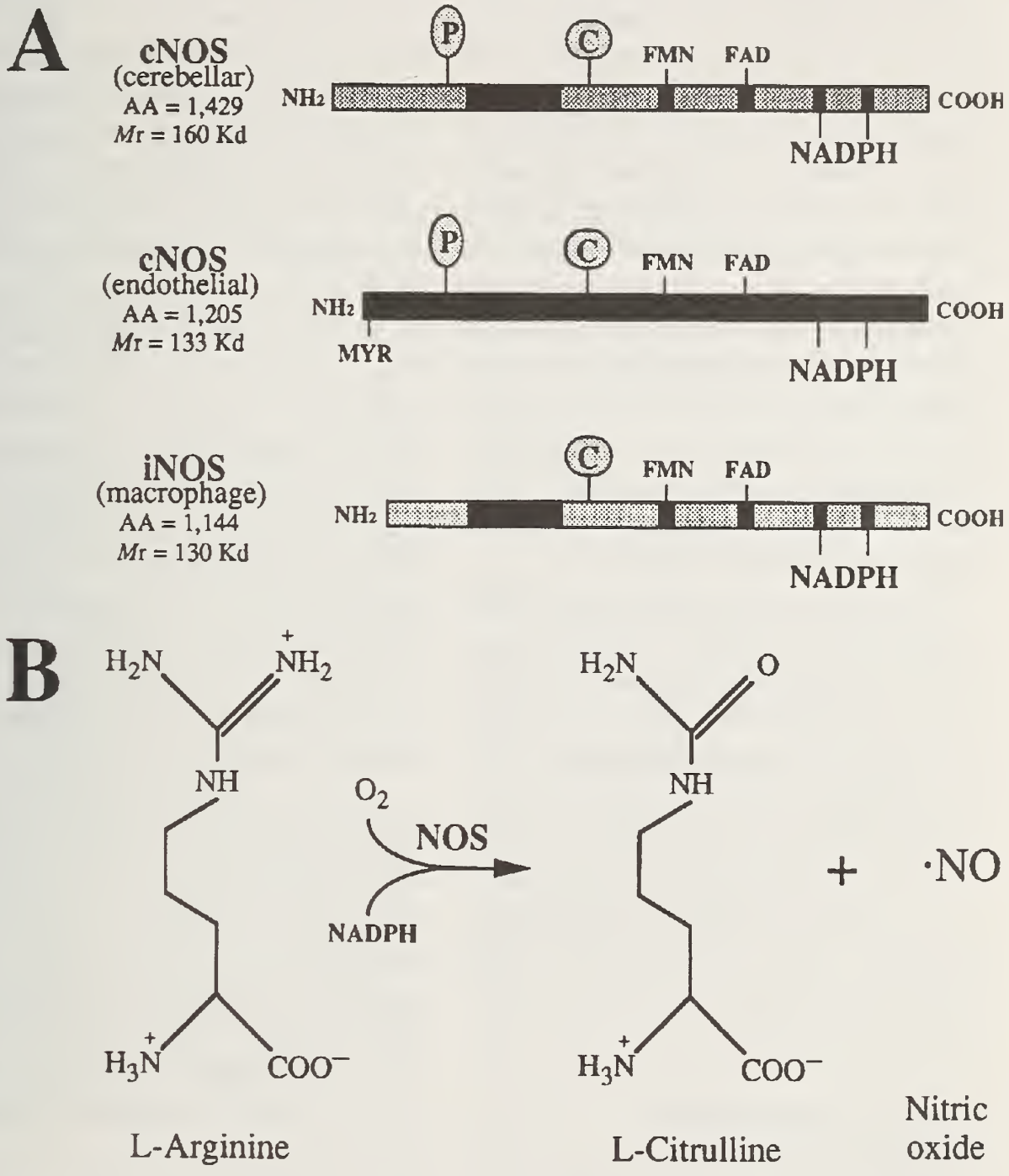

Figure 1. (A) Schematic representation of NOS isozymes showing relationships of consensus binding sequences for various cofactors. Constitutive forms of NOS (cNOS) have been cloned from cerebellum and endothelium and an inducible form (iNOS) from macrophages. All three isozymes possess predicted cofactor binding sequences for flavins, NADPH, and calmodulin. Endothelial cNOS also possesses a consensus sequence for *N*-myristylation that may relate to coupling to different calcium signaling pathways (e.g., phospholipase C) by anchorage of the enzyme to the plasma membrane. (B) Proposed model for formation of NO which involves hydroxylation of the guanidino group of L-arginine giving rise to hydroxyarginine intermediate (not shown) that is further oxidized to L-citrulline and NO with incorporation of molecular oxygen into both NO and L-citrulline. AA = amino acids; C = calcium/calmodulin binding sequence; FAD = flavin adenine dinucleotide; FMN = flavin mononucleotide; *Mr* = molecular weight; MYR = myristylation site; P = phosphorylation sites.

by protein kinase A, protein kinase C (PKC), and calcium/calmodulin-dependent kinase (Bredt et al. 1992; Brune and Lapetina 1991). Inducible NOS is not normally present in the cell but must be synthesized de novo in response to cytokines and endotoxin. In contrast to cNOS, calmodulin binds so tightly to iNOS that it is always activated and functions essentially independently of both calcium and calmodulin (Cho et al. 1992). Thus, once formed, iNOS continuously produces NO. Processes that may be involved in long-term posttranslational maintenance or regulation of iNOS activity are not known but may involve availability of substrate and synthesis of cofactors.

The differences in regulation of cNOS versus iNOS activity have important consequences in terms of the amounts of NO produced and thus the cellular processes in which they are involved. Production of NO by cNOS is immediate and frequently transient and tends to be involved in servomechanical regulatory processes (e.g., vascular tone), whereas that produced by iNOS is delayed and sustained and tends to be involved in reactive processes (e.g., inflammatory/immune responses).

PHYSIOLOGICAL RESPONSES AND MOLECULAR TARGETS OF NITRIC OXIDE

Nitric oxide can easily pass through cell membranes and rapidly diffuse to nearby target tissues, and many of the cellular effects of NO in target tissues are mediated by cyclic GMP (cGMP) via activation of soluble guanylyl cyclase. The best known response mediated by the NO-cGMP signaling pathway is vasodilation caused by relaxation of vascular smooth muscle. Although the precise role NO plays in brain function is not yet clear, it appears to represent a new type of neurotransmitter. Constitutive NOS is found in highest concentrations in cerebellar granule and basket cells and the olfactory and accessory olfactory bulb and is also present in discrete populations of neurons in the cortex, hippocampus, striatum, hypothalamus, posterior pituitary, and midbrain (Bredt et al. 1990, 1991*a*). Activation of neuronal cNOS is coupled primarily to glutamate receptor stimulation and, although controversial, evidence suggests that NO may play an important role in learning and memory formation by acting as a retrograde messenger to carry information from the postsynaptic to the presynaptic neuron (Bohme et al. 1991; O'Dell et al. 1991; Schuman and Madison 1991; Zhuo et al. 1993).

In addition to its role in normal cellular signaling, NO can be cytotoxic. The large and continuous production of NO by macrophages following immunological challenge may contribute to host defense against invading organisms (Moncada et al. 1991; Nathan 1992). Proteins and enzymes containing heme Fe and nonheme Fe_4-S_4 clusters are particularly sensitive to NO (Drapier et al. 1991; Lancaster and Hibbs 1990; Reif and Simmons 1990). Nitric oxide can nitrosylate Fe-S complexes of aconitase (tricarboxylic acid cycle enzymes) and complex I and II enzymes of the electron transport chain, and inhibition of mitochondrial function by NO may contribute to the bacteriostatic and bactericidal activity of macrophages (Hibbs et al. 1990; Nathan and Hibbs 1991). The tumoricidal activity of macrophages may relate to inhibition of tumor cell DNA

synthesis by nitrosylation of the Fe_4-S_4 cluster enzyme ribonucleotide reductase that is associated with DNA replication (Kwon et al. 1991; Lepoivre et al. 1990).

It has also been reported that NO generation enhances auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which appears to inhibit its activity in brain (Zhang and Snyder 1992). Thus, disruption of glycolysis may contribute to the neurotoxic actions of NO.

Free radicals are known to cause cellular damage and NO is itself a free radical. NO can also form other reactive nitrogen intermediates which may also contribute to NO cytotoxicity. For example, NO can react with superoxide ions to form reactive peroxynitrate anions ($ONOO^-$) that can also further break down to hydroxyl radicals (OH-), both of which have been shown in vitro to damage cells through membrane lipid peroxidation (Beckman et al. 1990).

The preceding discussion suggests that NO formation can be a double-edged sword. When produced in small amounts by the transient activation of cNOS, it participates in normal physiological responses. When production is sustained and continuous, such as following inappropriate stimulation of cNOS or synthesis of iNOS, it can be toxic.

GLUTAMATE AND EXCITOTOXICITY

Excitotoxicity refers to neuronal damage or toxicity due to excessive excitation. This process has been strongly linked to glutamate receptors and their excitatory input to neurons. Seizures, including those that occur during ethanol withdrawal, hypoxia/ischemia, hypoglycemia, trauma, and other insults to the brain, can lead to an excessive release of glutamate, neuronal depolarization, and increased extracellular K^+, which blocks glial uptake of glutamate, thus further increasing synaptic glutamate concentrations (figure 2).

Glutamate stimulation of the NMDA receptor and the resulting large Ca^{2+} conductance appear to play an important role in excitotoxicity since this process occurs only in neurons that possess NMDA receptors and not in glia, which have kainate/α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and do not appear to have NMDA receptors (Cull-Candy et al. 1991). Glutamate-mediated excitotoxicity has been divided into two components: a rapid component associated with osmotic swelling and immediate neuronal death and a delayed component that occurs as a progressive degenerative process over a period of hours to days. Both processes are likely due at least in part to the excessive neuronal accumulation of intracellular calcium (Clark 1989; Meldrum and Garthwaite 1990; Olney 1990).

In addition to Ca^{2+}, the early process likely involves Na^+ flux through AMPA/kainate and other activated membrane channels, an increase in intracellular volumes, and a depletion of intracellular energy stores. The slow progressive neuronal degeneration (that is, delayed neuronal cell death) appears to involve the following events: glutamate depolarizes neurons through NMDA, AMPA, and kainate receptors, leading to increased $[Ca^{2+}]i$ through NMDA receptor ion channels and voltage-sensitive calcium channels (Choi et al. 1988; Clark 1989). Both glutamate and the increase in $[Ca^{2+}]i$ activate phospholipases generating inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which then releases (1) intracellular calcium stores; (2) diacylglycerol,

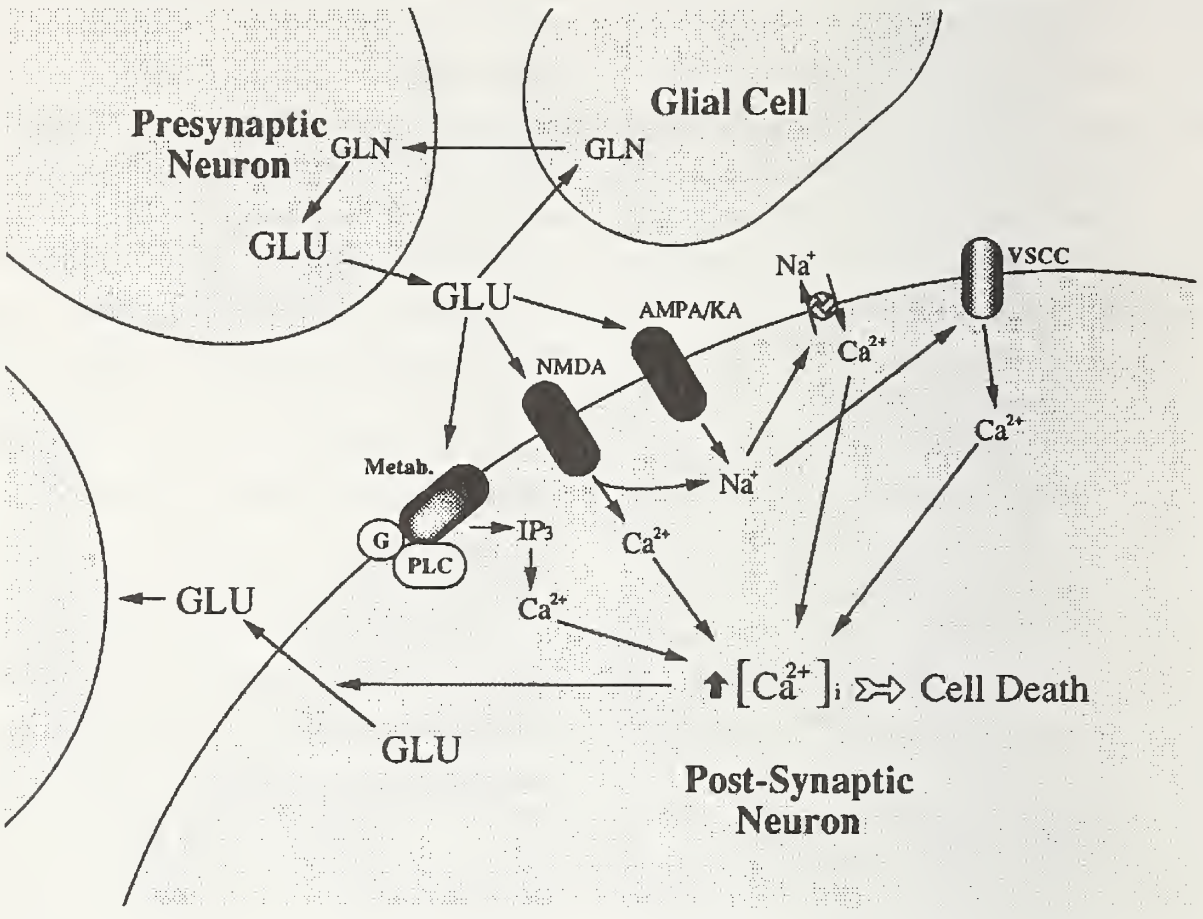

Figure 2. Schematic representation of excitatory amino acid–mediated neurotoxicity. Overstimulation of glutamate receptors initiates a series of events that lead to delayed neuronal cell death. Glial cells also play a role in this process by controlling extracellular glutamate concentrations. AMPA/KA = α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate; G = G-protein; GLN = glutamine; GLU = glutamate; PLC = phospholipase C; VSCC = voltage-sensitive calcium channels.

which activates PKC; and (3) arachidonic acid, which is metabolized by prostaglandin synthetases generating free radicals and prostaglandins. Ultimately, additional proteases and endonucleases are activated, leading to cell death (Orrenius et al. 1989).

Although a brief exposure to glutamate initially increases $[Ca^{2+}]i$ even after removal of glutamate, $[Ca^{2+}]i$ remains increased throughout the progression to delayed neuronal death. This sustained increase in $[Ca^{2+}]i$ appears to involve activation of PKC, since PKC inhibitors or down-regulation of PKC have been reported to prevent the sustained increase in $[Ca^{2+}]i$ and block delayed neuronal cell death (Favaron et al. 1990; Manev et al. 1989). The sustained increase in intracellular Ca^{2+} would likely cause sustained and inappropriate increases in cNOS activity and, in fact, recent evidence suggests that NO may be an important mediator of NMDA receptor–stimulated excitotoxicity. Inhibition of NO synthase activity in vitro has been reported to protect against NMDA receptor neurotoxicity in primary neuronal cultures (Dawson et al. 1991). Inhibition of NO synthase in vivo has been shown to dramatically reduce the volume of cortical

infarcts (a measure of neuronal cell death which is strongly linked to excitotoxicity) following irreversible focal ischemia in the mouse (Nowicki et al. 1991). Thus, an important factor in excitotoxicity is likely to be the formation of NO subsequent to NMDA receptor activation.

ETHANOL AND EXCITOTOXICITY

Exposure of cerebral cortical neurons in culture to NMDA for a little as 5 minutes results in a progressive neuronal death over the next 20 hours. When ethanol is added during NMDA treatment it reduces the neurotoxic effects of NMDA (Chandler et al. 1993*b*; Greenberg et al. 1992). At maximal concentrations of NMDA, ethanol can block excitotoxicity in a dose-dependent manner with approximately 40 percent inhibition of excitotoxicity at 25 mM ethanol and complete inhibition at 200 mM ethanol (Chandler et al. 1992). Ethanol appears to act as a noncompetitive antagonist of excitotoxicity by reducing excessive and inappropriate increases in Ca^{2+} influx through NMDA receptors. Thus, acute in vitro ethanol can actually protect neurons from excitotoxicity.

Tolerance to ethanol is remarkably rapid, and ethanol withdrawal represents a hyperexcitable state, which can include seizures. Seizures have been associated with glutamate neurotransmission and excitotoxic neuronal lesions at the site of the seizure focus. During ethanol withdrawal, neuronal hyperexcitability is likely due in part to increased glutamate neurotransmission. NMDA and voltage-sensitive calcium channel antagonists reduce ethanol withdrawal symptoms (Grant et al. 1990; Little et al. 1986). These data suggest that ethanol withdrawal hyperexcitability involves increased neuronal calcium flux, which could sensitize neurons to excitotoxicity. Studies in cell culture have found that chronic ethanol exposure can sensitize neurons to NMDA neurotoxicity (Chandler et al. 1993*a*). The findings that alcoholics suffer a loss of cortical pyramidal neurons (Harper and Kril 1990) and decreased cognitive function (Oscar-Berman and Ellis 1987) are consistent with chronic ethanol sensitizing cortical neurons to excitotoxicity. Thus, while acute ethanol may provide protection against excitotoxic damage (at least in vitro), chronic ethanol may increase sensitivity to excitotoxicity, which could underlie the neuropathology of chronic ethanol abuse.

The mechanism by which chronic ethanol increases excitotoxicity may be due to adaptive changes in neuronal ion channels that regulate excitability and Ca^{2+} flux during chronic ethanol abuse. Studies have found that chronic ethanol increases the density of [^{3}H]L-glutamate binding in brains of rat and humans and the density of [^{3}H]MK-801 binding (a ligand used to identify the NMDA ion channel itself) in the rat hippocampus (Grant et al. 1990). Other studies in cultured cerebellar neurons have found that chronic ethanol can significantly increase NMDA-stimulated calcium influx (Iorio et al. 1992). Increases in NMDA receptor ion channels and NMDA-mediated calcium flux are not the only changes that would sensitize neurons to excitotoxicity. Increases in voltage-dependent calcium influx, decreased GABA-stimulated Cl^- flux, and reduced Na^+/Ca^{2+} exchange have been reported following chronic ethanol treatment (see Crews and Chandler 1993).

Thus, a variety of data suggest that chronic ethanol treatment may disrupt calcium

homeostatic mechanisms and that this disruption enhances excitotoxicity, increases intracellular Ca^{2+}, and likely increases cNOS production of NO. In addition, recent studies with cultured endothelial cells have indicated that chronic ethanol may increase cNOS production of NO (Davda et al. 1993), and we have observed increased NMDA-stimulated NO formation in primary cortical cultures exposed chronically to ethanol (unpublished observations). Thus, increased Ca^{2+} during ethanol withdrawal may increase cNOS activity and, although ethanol does not change the sensitivity of neurons to NO toxicity (Greenberg et al. 1992), increased NO production would be expected to increase neuronal damage.

INDUCTION OF NITRIC OXIDE SYNTHASE IN GLIAL CELLS

Astroglia, oligodendroglia, and microglia make up approximately one-half of the total volume of the brain. Microglia are resident macrophages derived from precursor mononuclear bone marrow cells that populate the CNS at some point during fetal development (see chapter 3). They have a number of properties in common with peripheral macrophages but also possess several unique features. In normal tissue, microglia exist as inactive "down-regulated" mononuclear phagocytes called ramified ("resting") microglia that do not resemble macrophages in either morphology or function. Although the signaling mechanisms involved are complex and poorly understood, microglia differentiate, migrate, and proliferate at sites of inflammation and injury. Ramified microglia undergo transformation to different morphological states that correspond to different functional states. Following insult, injury, or immunological challenge, ramified microglia convert to ameboid ("activated") microglia that exhibit macrophage-like properties (including MHC and cytokine expression) but, unlike macrophages, are mitotic and nonphagocytic. Upon cellular degeneration, activated microglia are transformed into "reactive" phagocytic microglia that may function as "traditional" macrophages.

Astroglia, which outnumber neurons 10 to 1 in the CNS, also become reactive in response to injury leading to proliferation (astrogliosis) and the eventual formation of astroglial scars. Reactive astrocytes have many features in common with reactive microglia, including phagocytic properties and MHC and cytokine expression. Thus, astroglia are not simply support cells for neurons but may participate along with microglia in cellular responses to injury and immunological challenge.

Reactive microglia are associated with almost all types of CNS disorders, including multiple sclerosis (MS), Alzheimer's disease, Parkinson's disease, acquired immunodeficiency syndrome (AIDS)–related dementia, trauma, stroke, and the progressive loss of motor neurons and motor skills after ischemic injury to the spinal cord (Benveniste 1992; Giulian 1992; Giulian et al. 1989; Lipton 1992; Sherman et al. 1992; Thomas 1992). Cytokines, which play a vital role in coordination of immune and inflammatory responses, have been increasingly linked to neurological diseases and brain pathology. This association has focused attention on the interaction of excitotoxic processes with cytokines and neurotoxins in neuronal injury and recovery. Responses of astroglia and microglia to neuronal insult are com-

plex and are only now being elucidated. Glial cells and cells of the peripheral immune system have in common the ability to both secrete and respond to immunoregulatory cytokines and to present antigens to infiltrating T cells (Frei et al. 1987; Giulian and Baker 1986). Further, microglia have been shown to release superoxide radicals and a number of other neurotoxic compounds, including glutamate, that may stimulate excitotoxic processes (Colton and Gilbert 1987; Giulian et al. 1993; Piani et al. 1991, 1992). These observations have led to the suggestion that microglia and astroglia may be active participants in neuropathogenic processes and may control survival of neurons after damage to the CNS. Although glial cell responses may be beneficial in the short term via host defense and tissue repair, they may also be destructive in the long term and may contribute to neuropathology in certain disease states.

Several lines of evidence in our laboratory and others have indicated that microglia possess an inducible form of NOS (Boje and Arora 1992; Chandler et al. 1992; Chao et al. 1992; Zielasek et al. 1992). Using a cDNA probe of mouse macrophage iNOS, Northern blot analysis of total RNA isolated from cultured rat microglia treated with a combination of LPS and γ-interferon (IFN-γ) for 0 hour, 1 hour, 3 hours, and 6 hours caused a time-dependent increase in a 4.4-kb messenger RNA (mRNA) species (figure 3). This mRNA species was identical in size to an mRNA species found in RNA extracts from mouse RAW 264.7 macrophage cells that were also exposed to LPS and IFN-γ. Note that no mRNA bands were detected in either the control RAW or microglia time-zero extracts. In addition, immunocytochemical studies using an antibody to iNOS showed a large increase in microglial cell immunoreactivity after 24-hour exposure to a combination of LPS and IFN-γ, indicating synthesis of the iNOS protein after induction of the iNOS gene (figure 4).

Nitric oxide formation and NOS activity can be indirectly determined by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline (a relatively stable coproduct produced along with NO). Exposure of microglial cultures to LPS, IFN-γ or a combination of LPS and IFN-γ for 24 hours produced a large increase in the accumulation of [^{3}H]citrulline during a 30-minute incubation with [^{3}H]arginine (figure 5). The increase in NOS activity was delayed and time dependent and could be blocked by coincubation of LPS with dexamethasone (a known inhibitor of transcription of the iNOS gene in peripheral macrophages). Accumulation of [^{3}H]citrulline over the 30-minute time period was not altered by removal of extracellular calcium and was completely blocked by N^{ω}-nitro-L-arginine (NARG), a competitive inhibitor of NOS. Thus, the identification of an appropriate-sized mRNA transcript of iNOS, immunocytochemical detection of the iNOS protein, and functional expression of iNOS activity following exposure to LPS and IFN strongly indicate that microglial cells possess an inducible form of NOS that is similar to the peripheral macrophage iNOS.

In contrast to positive identification of iNOS in microglia, there have been conflicting reports of iNOS in astroglia. Chao and colleagues (1992) reported that treatment of purified astroglial cultures (secondary cultures obtained by subculturing of primary astroglial cultures) with LPS and IFN failed to stimulate

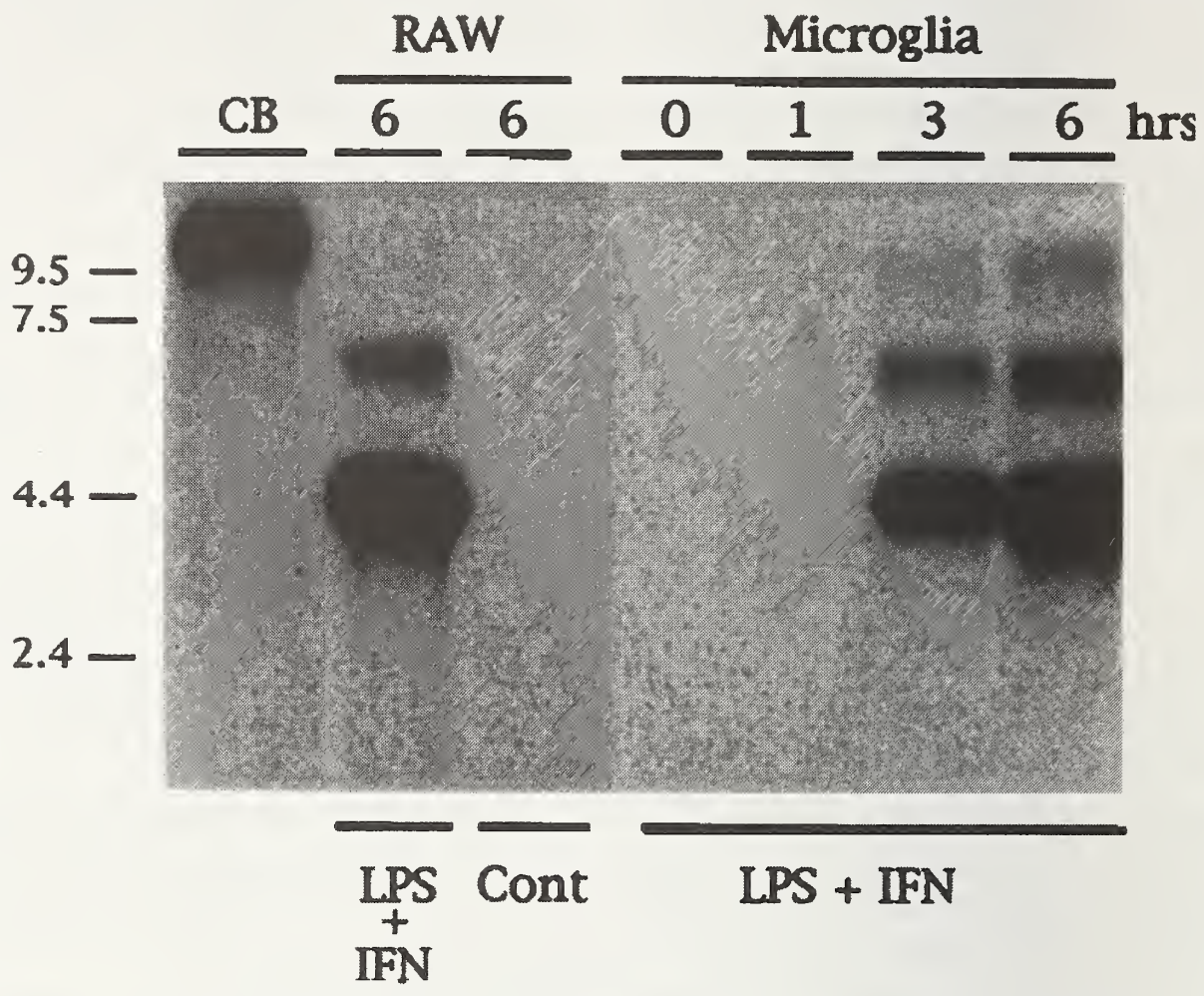

Figure 3. Transcriptional induction of iNOS. Northern blot analysis of total RNA (20 µg/lane) isolated from rat cerebellum (CB), control and LPS/IFN-γ–treated (6 hours) RAW 264.7 mouse macrophage cells, and LPS/IFN-γ–treated rat microglia. Cerebellar RNA was probed with a random prime ^{32}P-labeled cNOS-cDNA; RAW and microglia RNA were probed with a random prime ^{32}P-labeled iNOS-cDNA. Cerebellar RNA contained a cNOS message > 9.5 kb. RAW and microglia RNA contained an iNOS message approximately 4.4 kb in size that was only present after treatment with LPS/IFN-γ.

NO formation as determined by NO_2^- accumulation, whereas Simmons and Murphy (1992) observed induction of iNOS activity in secondary cultures as determined by stimulation of cGMP. Galea and colleagues (1992) also observed increased NO_2^- accumulation in primary astroglial cultures but not in purified astroglial cultures. Using the [^{3}H]citrulline formation assay, we have also observed induction of NOS activity in secondary astroglial cultures following LPS exposure. However, the difficulty of obtaining astroglial cultures that are not contaminated with microglia and variations in the levels of contamination between laboratories make interpretations of these studies difficult.

Observations by Galea and colleagues (1992) may help clarify whether astroglia possess an inducible form of NOS. Using an antibody to mouse macrophage iNOS, they found that approximately 15–20 percent of the cells in primary astroglial cultures that were identified by glial fibrillary acidic protein (GFAP) staining as astroglia showed intense

iNOS immunoreactivity following LPS treatment; the remaining astroglia showed only weak staining. Another finding was that treatment of C6 glioma cells produced no increase in nitrite accumulation in response to either LPS or tumor necrosis factor α (TNFα), but nitrite accumulation did increase when exposed to a combination of LPS and TNFα. We made a similar observation (figure 6) and noted that a combination of LPS and IFN-γ also failed to increase nitrite accumulation in C6 cells. These observations suggest that induction of NOS in astroglia may occur only in a subpopulation of cells and is dependent upon the culture conditions and the appropriate combinations of inducing agents.

ETHANOL AND NEUROPATHOLOGY: INTERACTION WITH NITRIC OXIDE, CYTOKINES, AND EXCITOTOXICITY

Immunoneuropathology likely involves a complex interplay of cytokine production and release, infiltration of immune cells, astroglia and microglia, and excitotoxic processes that may also involve NO production. The interactions of ethanol with these processes is likely to be complex as well. The demonstration that microglia and astroglia possess an inducible form of NOS and the association of

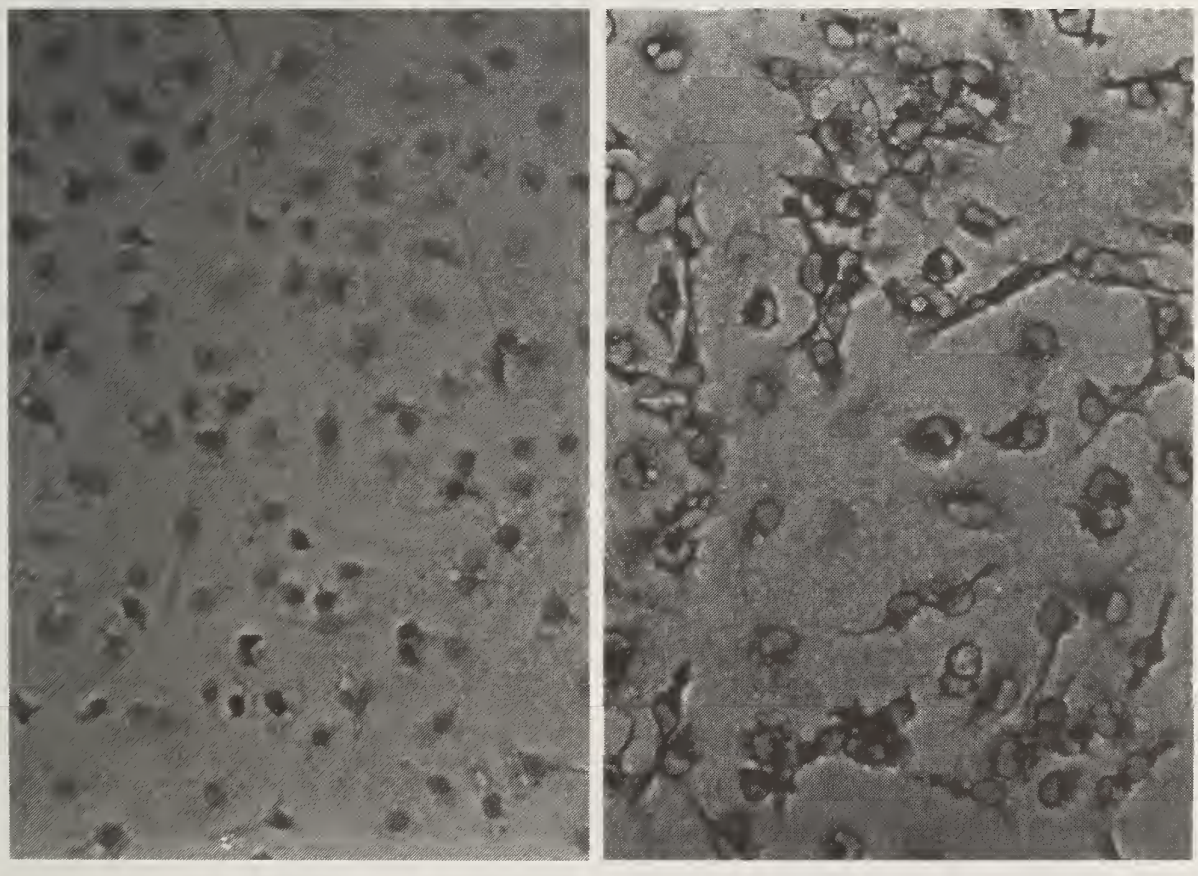

Figure 4. Immunocytochemical identification of iNOS in microglial cultures. Microglia were isolated by mechanical shaking from mixed astroglial cultures, allowed to attach to glass slides, exposed to LPS/IFN-γ for 24 hours, and processed for immunostaining using a rabbit anti-iNOS antibody. Antigenicity was visualized by peroxidase staining. Left panel shows immunostaining in control microglia not exposed to LPS/IFN-γ; right panel shows microglia that were exposed to LPS/IFN-γ.

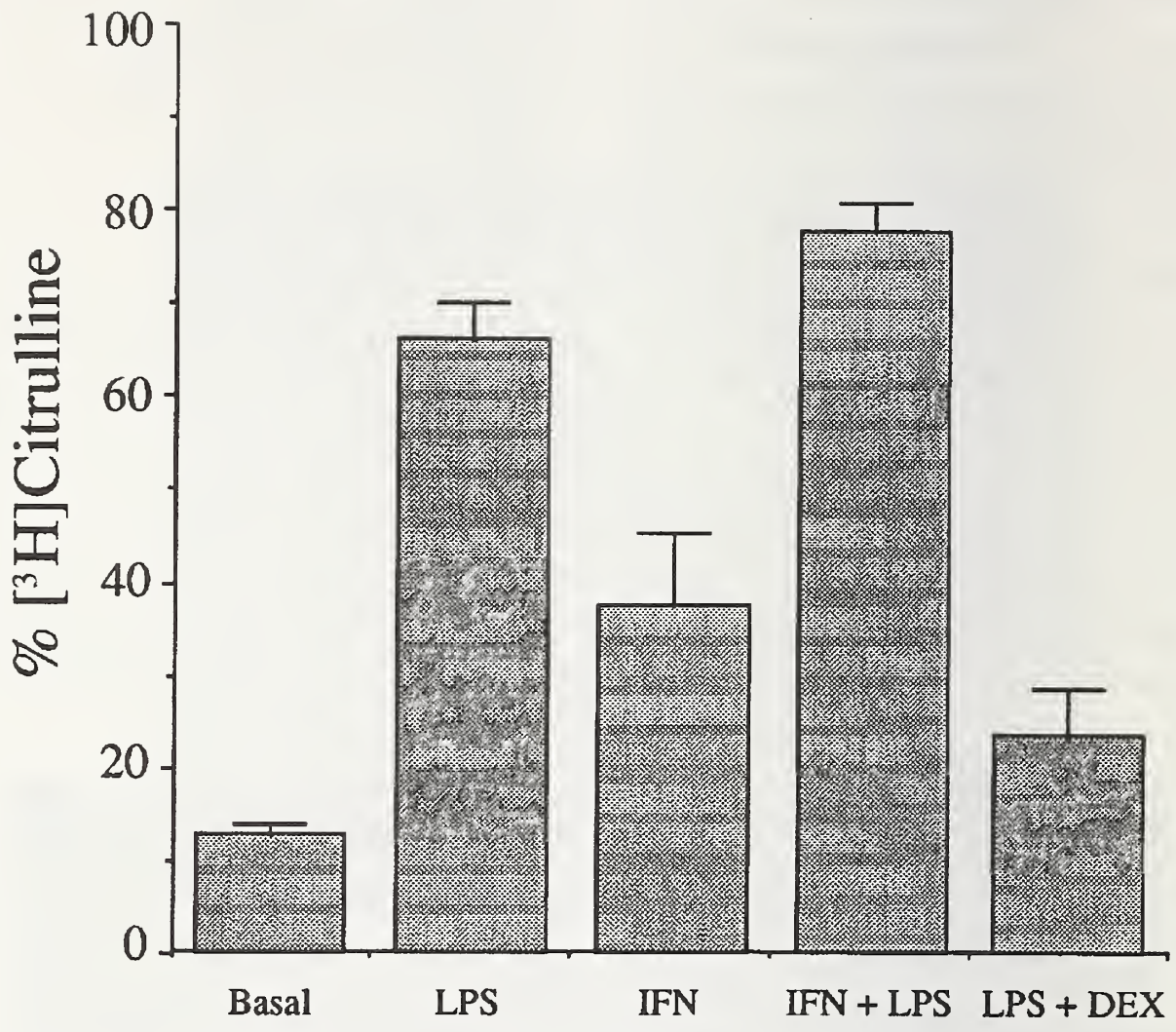

Figure 5. Induction of NOS activity in microglia cultures by LPS (1 µg/mL) and IFN-γ (100 U/mL) and its inhibition by dexamethasone (DEX) (1 µM). After 24-hour exposure, NOS activity was assayed by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline over a 30-minute time period. Formation of [^{3}H]citrulline is expressed as a percent total radioactivity taken up into the cell.

cytokines with glial cell responses support the suggestion that the neurotoxic effects of cytokines are due in part to production of NO and reactive nitrogen intermediates. To test this hypothesis, we examined the effects of NOS induction on cell survival in neuronal/microglial cocultures prepared by growing rat primary cortical neuronal cultures on a feeder layer of microglia isolated from mixed astroglial cultures. Ten days after plating, cocultures were exposed to LPS, LPS plus IFN-γ, or LPS plus the NOS inhibitor NARG. The culture media was assayed for lactate dehydrogenase (LDH) activity to quantitate cell death at 0 hour, 36 hours, 48 hours, and 68 hours after induction of NOS.

As shown in figure 7, LPS caused a time-dependent increase in the release of LDH into the culture media that was potentiated by IFN-γ. Morphologically, neurons showed a progressive degeneration and by 68 hours had completely disintegrated; microglia showed few signs of damage, although some appeared to have detached from the dish after 68 hours. The addition of NARG almost completely blocked LPS-induced neurotoxicity, suggesting

that neuronal death was dependent on NO formation. These observations are in close agreement with similar studies by Boje and Arora (1992) and Chao and colleagues (1992), who also demonstrated that immunostimulation-induced neurotoxicity in neuronal/microglial cocultures was not a glutamate receptor–mediated event (i.e., microglial release of glutamate producing excitotoxic damage). Although these in vitro studies show that microglia can kill neurons via production of NO, they await in vivo confirmation.

The BBB controls the passage of substances between the peripheral circulation and the brain and consists anatomically of capillary endothelial cells that are tightly connected ("tight junctions"), leaving little or no space for substances to pass between. Astrocytic processes (astrocytic end feet) also surround the capillaries and provide additional protection from infiltration of peripheral substances. Since the BBB limits communication between the brain and immune cells and inflammatory factors in the peripheral circulation, alterations in the integrity of the BBB may be a critical factor in initiating or promoting neuropathogenic processes in brain. For example, infiltrating T

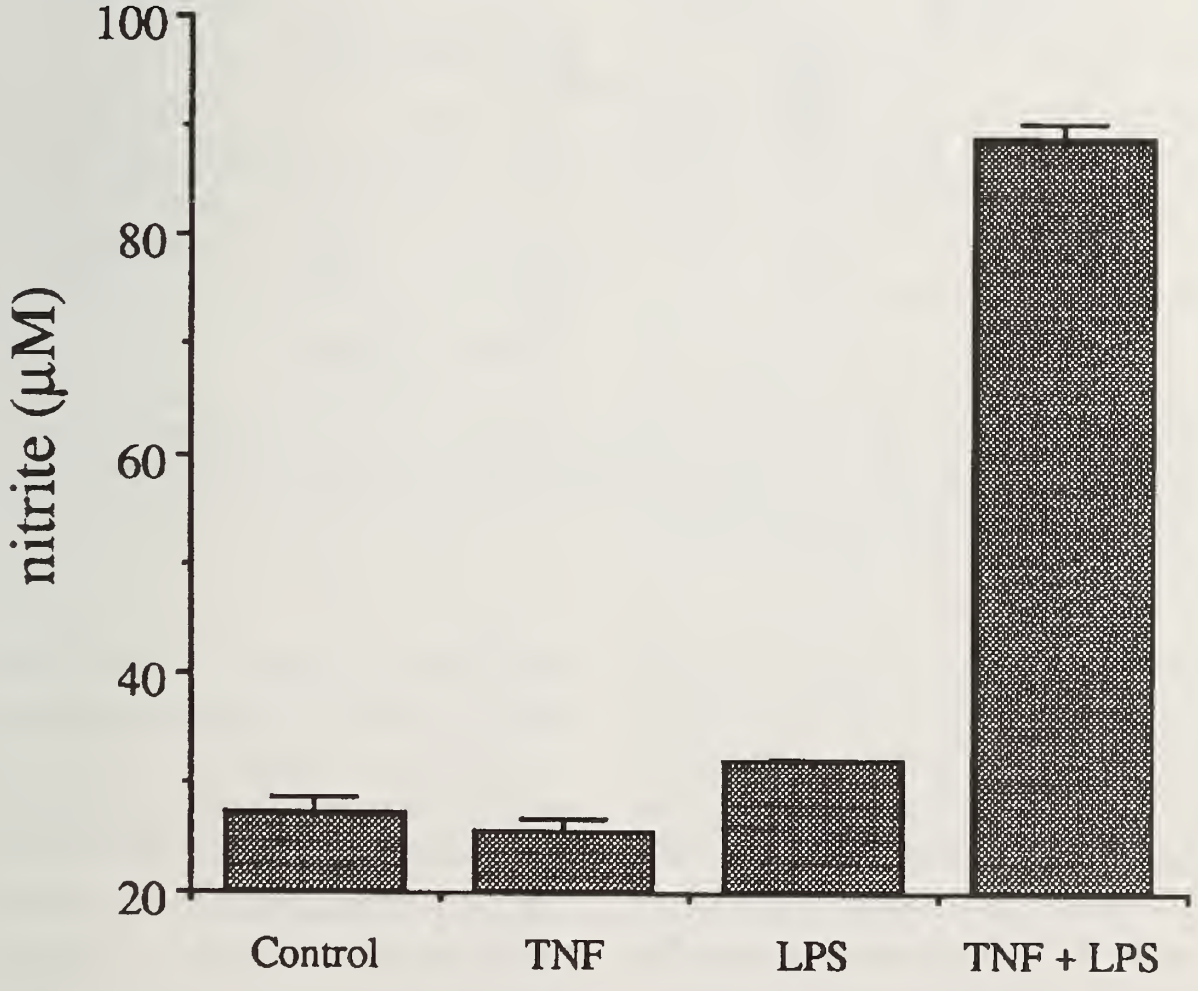

Figure 6. Effect of TNFα (100 ng/mL) and LPS (1 μg/mL) on nitrite accumulation over a 48-hour period in C6 glioma cultures. Nitrite in the media was assayed spectrophotometrically by the Griess reaction. Significant increases in nitrite accumulation occurred only when cultures were exposed to a combination of LPS and TNFα.

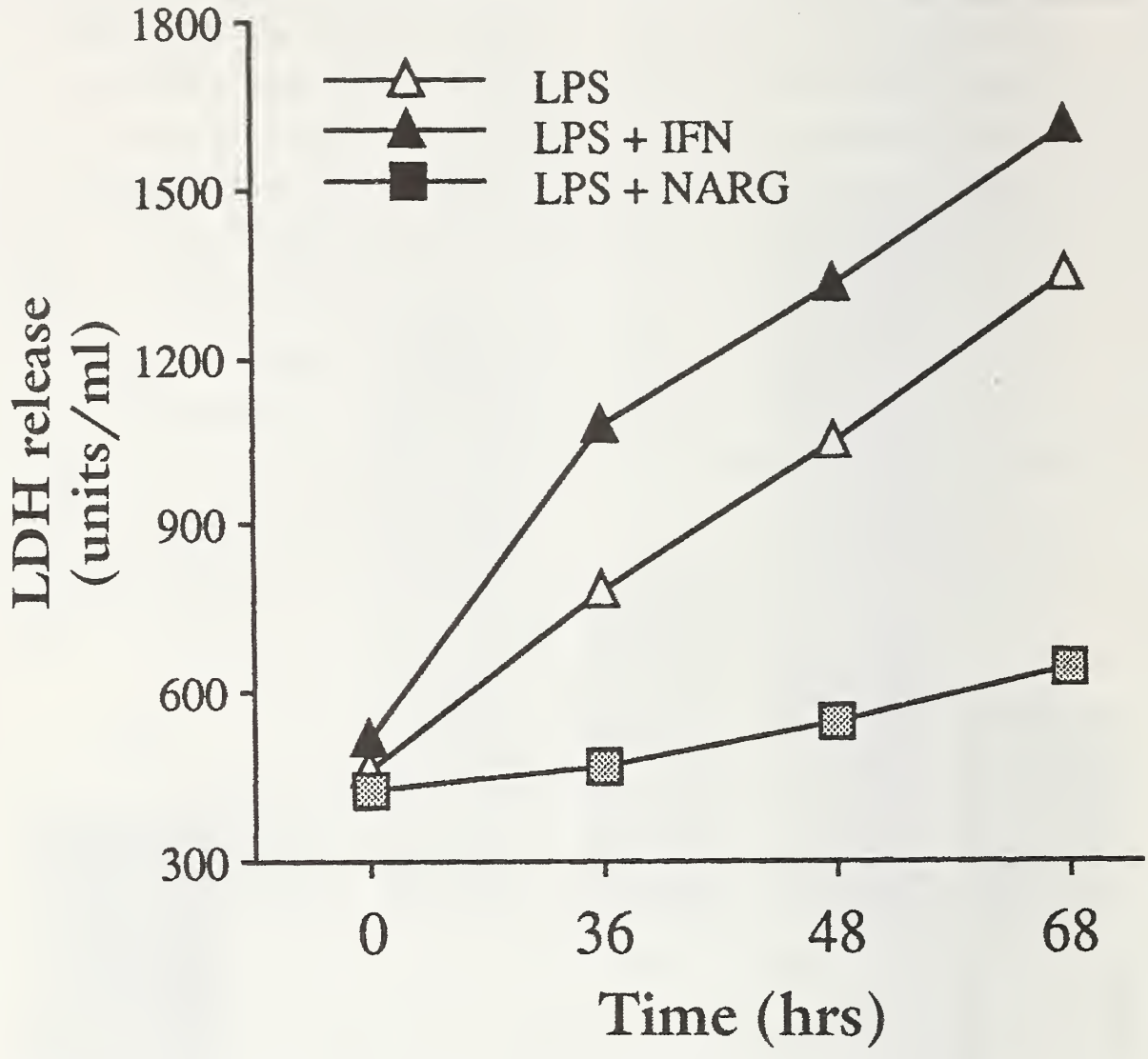

Figure 7. Neurotoxic effects of induction of NOS in primary cortical neuronal/microglia cocultures and its protection by NARG, a competitive inhibitor of NOS. Cocultures were exposed to LPS (1 μg/mL), LPS plus IFN-γ (100 U/mL), or LPS plus NARG (2 mM), and the cultured media were assayed for LDH release at the indicated times.

cells release IFN-γ, which can initiate synthesis and release of other cytokines, up-regulate MHC I and II expression in astroglia and microglia, and promote the inflammatory response leading to further breakdown of the BBB. Upon passage through the endothelial cells of the BBB, blood-borne cytokines could also act on surrounding astrocytes to promote release of neuroactive substances that either promote recovery following injury or infection or may contribute to further injury. The BBB is essentially composed of cell membranes, and ethanol is well known to disrupt membrane structure and function. It is therefore not surprising that acute and chronic ethanol exposure can increase the permeability of the BBB to infiltrates (Banks and Kastin 1993). Ethanol may not cause overt disruption of the BBB but instead may simply increase its residual leakiness or leave it more susceptible to disruption by other agents.

Certain cytokines (i.e., IFN-γ, TNFα, and interleukin-1 [IL-1]) play a pivotal role in inflammatory processes and have been

increasingly linked to neurodegeneration (Morganti-Kossmann et al. 1992). Multiple sclerosis is a progressive autoimmune CNS disease that manifests as destruction of myelin (demyelination of white matter) and myelin-producing cells (oligodendroglia) and is of particular interest in terms of cytokines, NO, and ethanol-related brain damage. Dexamethasone is commonly used to accelerate clinical recovery in MS patients experiencing relapse episodes, and transforming growth factor β (TGF-β) can ameliorate symptoms of experimental allergic encephalomyelitis (EAE), an animal model of MS (Sherman et al. 1992). Conversely, IFN-γ worsens the symptoms while TNFα also appears to play a pathogenic role in MS. Interestingly, both dexamethasone and TGF-β can inhibit induction of NOS, whereas IFN-γ and TNFα can promote its induction. These observations have led to the proposal that cytokine-mediated induction of NOS in microglia and infiltrating macrophages with production of NO contribute to killing of oligodendroglia in MS (Sherman et al. 1992). Interleukin-1 and TNFα are pleotropic cytokines commonly associated with neuronal damage, are elevated in cerebrospinal fluid of MS patients, and are known to induce NOS under certain conditions. Since TNFα is toxic to oligodendrocytes, TNFα production by IFN-γ–primed macrophages, astrocytes, and microglia may be a mechanism for destruction of white matter in MS.

The neuropathology of chronic ethanol exposure is also characterized by diffuse damage to white matter throughout the brain that is manifested as decreased myelin and myelin pallor, plaque formation, and ventricular dilation. It has been suggested that ethanol-induced damage to white matter may occur through an autoimmune reactive process akin to that of MS, but on a less severe scale (Lancaster 1993; Schubert 1990). In this model, ethanol damage to oligodendrocytes and/or myelin causes enhanced presentation of self-antigens and MHC class I and II antigens. This leads to activation of infiltrating cytotoxic T cells and initiation of an autoimmune reaction with further destruction of myelin and oligodendrocytes that may also involve NO. Chronic ethanol-induced damage to gray matter may also involve inflammatory/immune processes and NO formation via both cNOS and iNOS.

Figure 8 provides a schematic summary of the complex interactions between excitotoxicity, reactive gliosis, and inflammatory responses in brain pathology during chronic ethanol exposure. Chronic ethanol can kill neurons by sensitizing or potentiating the excitotoxic process or by nonexcitotoxic processes. Ethanol may also increase the permeability of the BBB, allowing infiltration of circulating cytokines and inflammatory cells. Cellular damage may initiate a cascade of events that either promote repair or limit further damage on one hand but that may become pathogenic on the other. The infiltrating cells and substances may directly mediate the inflammatory response, causing release of cytokines and excitotoxins (glutamate and glutamate-like compounds from microglia), and promote reactive gliosis and induction of NOS in microglia and astroglia. Released cytokines (e.g., TNFα) and NO and other reactive nitrogen intermediates may kill neurons and glial cells directly or, along with excitotoxins released from microglia, may kill cells indirectly via excitotoxic processes.

Ethanol consumption is known to impair normal immune responses that protect the body from disease, and alcoholics appear to be more susceptible to cancer and bacterial infections. In terms of ethanol and brain

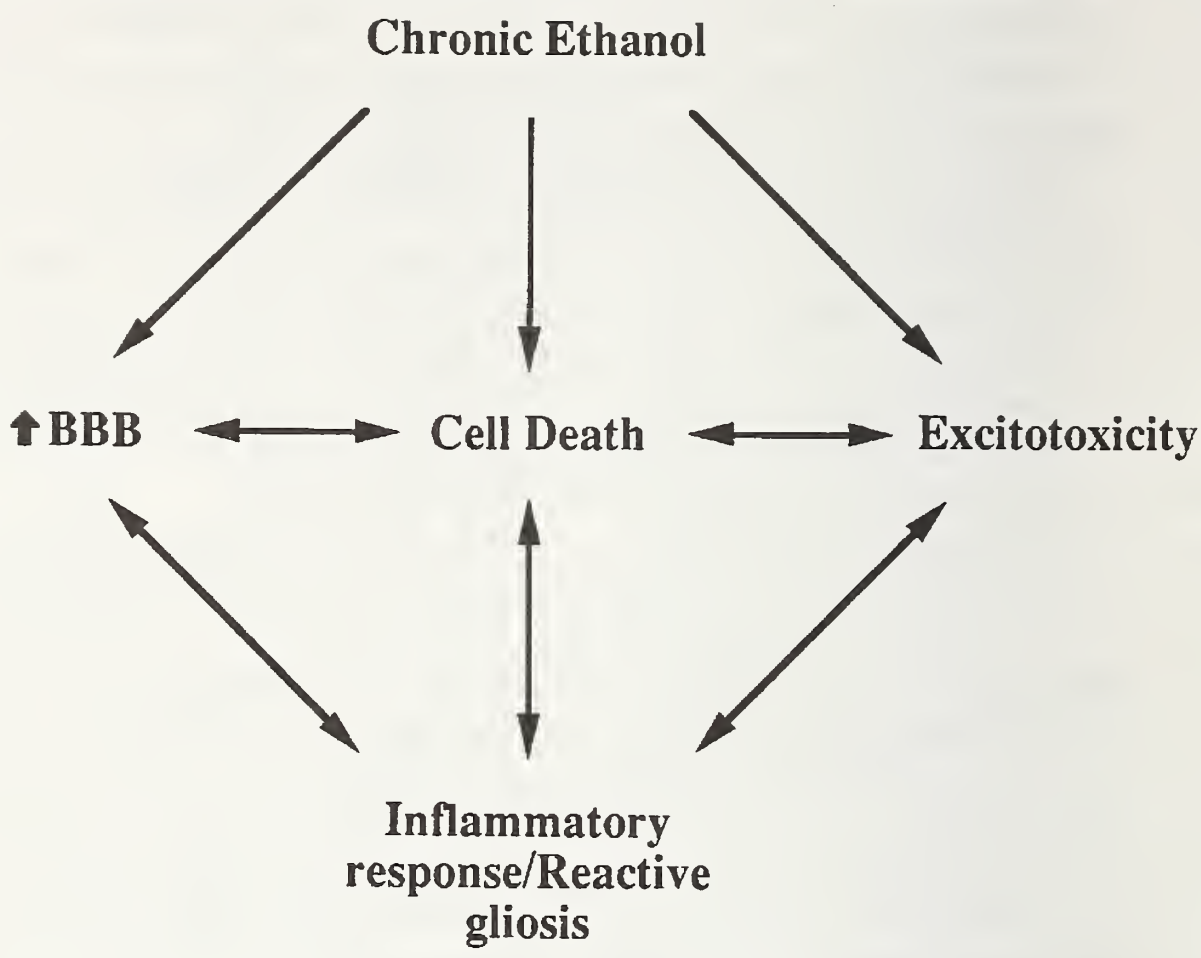

Figure 8. Model depicting interactions between chronic ethanol exposure and reactive neuronal injury. It is hypothesized that chronic ethanol exposure can lead to neuronal cell death through three interactive pathways: (1) increased permeability of the BBB to cytokines and inflammatory cells that are neurotoxic, (2) direct damage to the neurons themselves, or (3) sensitization of neurons to excitotoxicity (e.g., up-regulation of NMDA receptors). These three events can promote inflammatory responses that include reactive gliosis. Cytokine induction of NOS in infiltrating macrophages, astroglia, or microglia may also occur, with NO having either neurotropic or neurotoxic actions.

pathology, reduced immune function could interfere with normal immediate response mechanisms designed to limit or prevent further injury. Thus, what was previously a minor or nonpathological insult may become a pathological condition.

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Chapter 11

The Astrocyte in Alcoholic Liver Disease

Roger F. Butterworth, Ph.D.

Neurological symptoms associated with alcoholic liver disease have been recognized for centuries. In Shakespeare's play *Twelfth Night*, the alcoholic nobleman, Sir Andrew Aguecheek, could have been referring to the precipitation of neuropsychiatric symptoms by a high protein load in alcoholic liver disease when he remarked, "I am a great eater of beef, and I believe that does harm to my wit." Alcoholic liver disease is currently one of the most serious medical consequences of chronic alcohol use. Moreover, chronic excessive alcohol use is the single most important cause of illness and death from liver disease in the United States (National Institute on Alcohol Abuse and Alcoholism [NIAAA] 1993).

There are three alcohol-related liver conditions: fatty liver, alcoholic hepatitis, and cirrhosis. A patient may have only one of these three complications or any combination of them. Although generally considered to be sequentially related, progressing from fatty liver via alcoholic hepatitis to cirrhosis, some studies have shown that alcoholics may progress to cirrhosis without passing through a stage resembling hepatitis; that is, alcoholic cirrhosis can appear insidiously (NIAAA 1993).

Complications of advanced alcoholic liver disease include bleeding from esophageal varices, ascites, and hepatic encephalopathy. Indicators of liver malfunction may appear early in the development of alcoholism; a study of adolescent alcoholics undergoing treatment revealed at least one biochemical indicator of liver injury in up to 24 percent of those evaluated (Arria et al. 1991). Alcoholic liver disease develops more readily in women than in men, and women are more likely to develop multiple organ damage (Morgan and Sherlock 1977).

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PORTAL-SYSTEMIC ENCEPHALOPATHY: A MAJOR NEURO-PSYCHIATRIC COMPLICATION OF ALCOHOLIC LIVER DISEASE

The most common form of hepatic encephalopathy encountered in alcoholic liver disease is portal-systemic encephalopathy (PSE), which results, as its name suggests, from shunting of portal blood into the systemic circulation. Shunting may be spontaneous (resulting from the development of portal-systemic collaterals) or may result from surgically constructed portacaval anastomoses created to relieve portal hypertension and variceal hemorrhages in these patients. A new surgical shunt procedure, the transjugular intrahepatic portal-systemic shunt (TIPS), is rapidly gaining in popularity and promises to have a major impact in the treatment of portal hypertension and ascites; the procedure can also improve nutrition in cirrhotic patients and has the potential to "buy time" for patients needing liver transplantation (Conn 1993). However, patients with TIPS are susceptible to the development of PSE. Results of a study of the treatment of 108 consecutive TIPS patients, 50 percent of whom were alcoholic cirrhotics, revealed episodes of new or worsened PSE in 24 percent of patients (Somberg et al. 1992); 11 patients required hospitalization for hepatic encephalopathy and 3 died in hepatic coma. In a study by Sellinger and colleagues (1992), the risk of hepatic encephalopathy after TIPS was shown to be particularly high (in excess of 30 percent) in older patients.

In addition to overt PSE, cirrhotic patients manifest a high incidence of so-called latent or subclinical PSE. In one study, a group of 15 patients with alcoholic cirrhosis and 15 patients with nonalcoholic cirrhosis were studied using an extensive psychometric test scheme used, in part, for the assessment of fitness to drive an automobile; 100 percent of alcoholic cirrhotics were found to be unfit to drive (Schomerus et al. 1981). Patients with alcoholic cirrhosis scored consistently lower than did patients with nonalcoholic cirrhosis, but this was found to relate mainly to differences in liver function rather than to alcohol consumption. In a subsequent study, 22 alcoholic cirrhotics, 20 nonalcoholic cirrhotics, and 42 control subjects matched for age and educational background as well as for Pugh score (degree of liver impairment) were subjected to a battery of psychometric tests; 76 percent failed at least one test, and the degree of neurological impairment was comparable in alcoholic and nonalcoholic cirrhotics (Pomier Layrargues et al. 1991). These findings suggest that cirrhosis and its sequelae play a major role in cognitive disturbances in chronic alcoholics.

The spectrum of neuropsychiatric signs and symptoms typical of PSE are shown in table 1.

NEUROPATHOLOGY OF PSE: THE ALZHEIMER TYPE II ASTROCYTE

Neuropathological studies have consistently demonstrated astrocytic changes in the brains of patients with chronic liver disease and PSE. Protoplasmic (gray matter) astrocytes in particular are affected, being increased in size and in number; they take on a characteristic appearance first described by von Hösslin and Alzheimer in 1912 and now generally referred to as Alzheimer type II astrocytosis. In the most comprehensive assessment reported to date, Adams and Foley (1953) described neuropathological

Table 1. Progression of Neurological and Psychiatric Symptoms as a Function of Grade of Portal-Systemic Encephalopathy (PSE)

PSE Grade	Neurological/Neuromuscular Symptoms	Psychiatric Symptoms
0	None	None
1	Muscular incoordination Tremor	Inversion of sleep patterns Shortened attention span Euphoria Depression
2	Asterixis (flapping tremor)	Anxiety Apathy, lethargy
3	Ataxia Nystagmus Rigidity Opisthotonos	Somnolence Amnesia Paranoia, anger
4	Coma	Stupor Coma

changes in 50 cases of chronic liver disease of both alcoholic and nonalcoholic etiology and varying degrees of PSE at the time of death. The following changes were noted: (a) enlargement and increased numbers of protoplasmic astrocytes and (b) large pale nuclei, prominent nucleoli, and margination of chromatin.

Astrocytic changes were particularly prominent in brain regions such as cerebral cortex, putamen, globus pallidus, caudate nucleus, thalamic nuclei, inferior olivary nuclei, and the Purkinje layer of the cerebellum. Hypothalamic, brain stem, and spinal cord nuclei were relatively free from such changes. Fibrous (white matter) astrocytes retained their natural appearance. The number of protoplasmic astrocytes in the brains of liver disease patients who died in hepatic coma was twice that encountered in noncoma patients or controls, and astrocytic size was increased by 35 percent in the hepatic coma group (Adams and Foley 1953). Few, if any, neuronal changes were observed in the brains of these patients.

Studies undertaken in the years since the pioneering work of Adams and Foley have led to further characterization of the Alzheimer type II astrocyte and have strengthened the link between chronic liver disease, ammonia neurotoxicity, the presence of hepatic encephalopathy, and Alzheimer type II astrocytosis. For example, studies by Mossakowski and colleagues (1970) demonstrated that the exposure of cultured astrocytes to serum from patients in hepatic coma resulted in the formation of Alzheimer type II changes. An immunohistochemical study on human hepatic encephalopathy associated with chronic liver disease demonstrated that the Alzheimer type II change was associated with a loss of immunohistochemically detectable glial fibrillary

acidic protein (GFAP) in gray matter astrocytes (Sobel et al. 1981). More recently, a magnetic resonance imaging (MRI) evaluation of patients with chronic liver disease revealed pallidal hyperintensity (Kulisevsky et al. 1992). Subsequent neuropathological assessment of the brains of these patients showed the presence of Alzheimer type II astrocytosis and a positive correlation between the MRI and astrocytic changes.

EVIDENCE FOR A PATHOGENETIC LINK BETWEEN AMMONIA NEUROTOXICITY AND ALZHEIMER TYPE II ASTROCYTOSIS IN PSE

Around the time that Adams and Foley were conducting their studies of clinical-neuropathological correlation in chronic liver disease, Gabuzda and colleagues were investigating the diuretic properties of ammonium cation-exchange resins for the treatment of ascites in cirrhotic patients (Gabuzda et al. 1952). Oral administration of these resins (which bind sodium and liberate ammonium ions) did, as expected, result in diuresis in these patients but consistently resulted in neurological complications detected by electroencephalography (EEG) and other methods that were indistinguishable from PSE. Gabuzda and colleagues subsequently demonstrated that PSE could be induced in cirrhotic patients by the administration of ammonium salts, urea, or dietary protein and that withdrawal of these agents or restriction of protein led to remission of the neurological signs and normalization of EEG patterns.

Since that time, the results of numerous studies in experimental animals as well as with cultured astrocytes have reinforced the notion of a pathogenetic link between ammonia and Alzheimer type II changes. This evidence may be summarized as follows:

- Neuropathological studies in experimental PSE resulting from portacaval anastomosis and varying degrees of hyperammonemia demonstrated Alzheimer type II astrocytosis (Norenberg and Lapham 1974). As in the human studies, Alzheimer type II changes were significantly correlated with the severity of encephalopathy. The concomitant ultrastructural changes consisted of increased numbers of cytoplasmic organelles in Alzheimer type II astrocytes, mainly mitochondria and rough endoplasmic reticulum. This was accompanied by a broadening of astrocytic processes and enlargement of perikaryal cytoplasm. Figure 1 is an electron micrograph of an astrocyte process showing increased numbers of mitochondria in early PSE resulting from ammonia feeding following portacaval anastomosis in the rat.
- Alzheimer type II astrocytosis was encountered in the brains of children with hyperammonemic syndromes resulting from congenital deficiencies of urea cycle enzymes (Shih 1978), as well as in the brains of experimental animals made chronically hyperammonemic by the administration of urease (Gibson et al. 1974).
- Exposure of primary astrocyte cultures to ammonia for up to 10 days resulted in increased basophilia, prominent cytoplasmic processes, and increased cytoplasmic granularity and vacuolization. Nuclear size was increased, as were nucleolar/nuclear size ratios (Gregorios et al. 1985*a*). Changes were proportional to the concentration

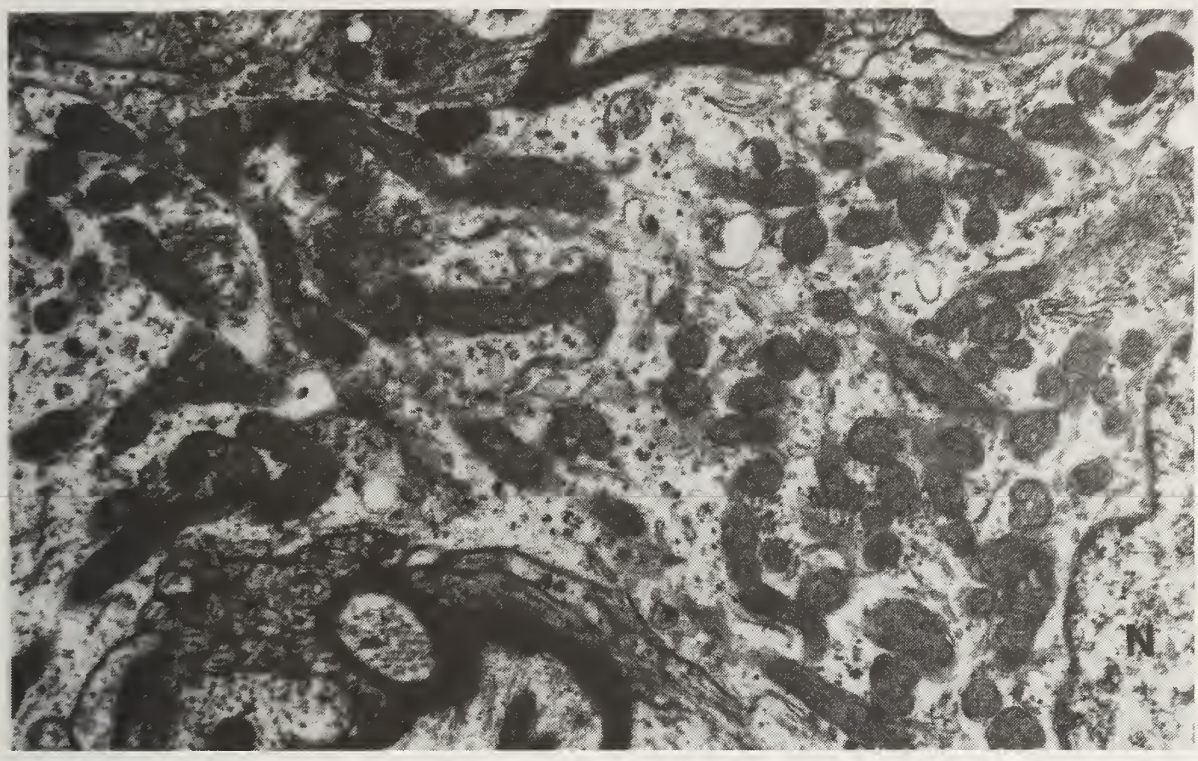

Figure 1. Electron micrograph of an astrocyte process showing increased numbers of mitochondria. From an in vivo model of PSE. N = nucleus; bar = 1 µm. Reprinted with permission from Norenberg, M.D. The role of astrocytes in hepatic encephalopathy. *Neurochem Pathol* 6:13–33, 1987.

and duration of ammonia exposure. Alzheimer type II cells were observed. Ultrastructural studies showed that the initial response consisted of mitochondrial proliferation and swelling (Gregorios et al. 1985*b*), similar to that observed in experimental PSE.

Taken together, these findings constitute a substantial body of evidence suggesting that ammonia neurotoxicity is a key factor in the pathogenesis of Alzheimer type II astrocytosis and of hepatic encephalopathy in chronic liver disease (for a complete review of the evidence, see Butterworth et al. 1987).

Brain relies almost exclusively on glutamine synthesis for the effective removal of excess ammonia because, unlike liver, brain is devoid of an effective urea cycle. The enzyme responsible for glutamine synthesis in brain, glutamine synthetase (GS) (figure 2), is localized almost exclusively in astrocytes; thus it is the astrocyte and not the neuron that "bears the brunt" of ammonia removal in brain.

Brain GS appears to operate at near-maximal capacity under normal physiological conditions (Cooper and Plum 1987). Furthermore, chronic hyperammonemic conditions, including chronic liver dysfunction, do not result in GS induction in brain (Butterworth et al. 1988; Cooper and Plum 1987), suggesting a limited capacity for brain to remove additional blood-borne ammonia. Consistent with this suggestion, two independent studies of the effects of portacaval shunting in experimental animals were unable to demonstrate any increase in glutamin formation in brain (Cremer et al. 1975; Ukida et al. 1988). To make matters worse, GS activities were significantly decreased in certain brain regions of cirrhotic patients who died in

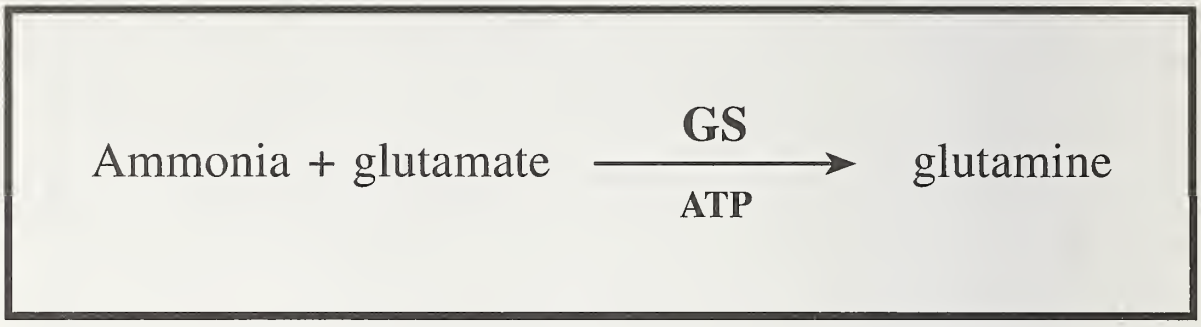

Figure 2. Removal of ammonia by brain: glutamine synthesis. Glutamine synthetase (GS) is a predominantly astrocytic enzyme.

hepatic coma (Lavoie et al. 1987) as well as in the brains of portacaval shunted rats (Butterworth et al. 1988; Girard et al. 1993). The limitation of the capacity of GS coupled with the findings of reductions of GS activity in brain could explain the precipitous increases in brain ammonia and ensuing neuropsychiatric symptoms observed in alcoholic cirrhotic patients following ingestion of an ammoniagenic high-protein diet.

ASTROCYTIC "PERIPHERAL-TYPE" BENZODIAZEPINE RECEPTORS IN PSE

"Peripheral-type" benzodiazepine receptors (PTBR's), so called as a result of their initial discovery in peripheral tissues, are also found in brain where they are highly concentrated on astrocytic outer mitochondrial membranes (Anholt 1986). In brain, these receptors are not allosterically coupled to GABA receptors or chloride channels. There is evidence to suggest that PTBR's as well as their endogenous ligands may mediate the response of astrocytes to ammonia in chronic liver disease and thereby contribute to the pathogenesis of PSE.

Increased densities of binding sites for the PTBR ligand [^{3}H]-PK11195 were first described in PSE by Lavoie and colleagues (1990) in autopsied brain tissue (frontal cortex and caudate nuclei) of alcoholic cirrhotic patients who died in hepatic coma. Histopathological studies revealed the presence of Alzheimer type II astrocytosis in these brains. That the increase of PTBR's was the result of hyperammonemia associated with chronic liver impairment rather than chronic alcohol exposure per se is borne out by the subsequent reports of (a) generalized increases of [^{3}H]-PK11195 binding sites in rat brain following portacaval anastomosis (Giguère et al. 1992) and (b) increased densities of [^{3}H]-PK11195 binding sites in the brains of mice with chronic hyperammonemia resulting from a congenital defect of the urea cycle enzyme ornithine transcarbamylase (Raghavendra Rao et al. 1993*b*).

Furthermore, PTBR changes are not limited to brain in hyperammonemic syndromes. [^{3}H]-PK11195 binding sites are increased in kidney following portacaval anastomosis in the rat (Raghavendra Rao et al. in press) and in kidney, heart, and testis of congenital hyperammonemic mice (Raghavendra Rao et al. 1993*b*).

Putative endogenous ligands for PTBR's include the neuropeptide diazepam binding inhibitor (DBI) and its active metabolite octadecaneuropeptide (ODN). Both DBI and ODN have been shown to displace [^{3}H]-benzodiazepines from PTBR's on astrocytes (Bender and Hertz 1987). Using an immunocytochemical technique and an antibody of high specific activity to synthetic ODN, we studied the effects of portacaval anastomosis on the distribution of ODN immunoreactivity in rat brain (Butterworth et al. 1991). Four weeks after shunt surgery, ODN immunolabeling was increased two- to fivefold in cerebral cortex, hippocampus, hypothalamus, and thalamic structures. As expected, increased ODN immunolabeling was confined mainly to astrocytes. Since PTBR's are highly localized on the outer mitochondrial membrane (Anholt 1986) and since exposure of cultured glioma cells to PTBR ligands results in proliferation and swelling of mitochondria (Shiraishi et al. 1990), it was suggested that PTBR's and their endogenous ligands may be involved in the astrocytic response (swelling and proliferation of mitochondria) characteristic of PSE (Giguère et al. 1992).

OTHER ASTROCYTIC ABNORMALITIES IN PSE

In addition to decreased expression of GFAP and increased densities of PTBR's in astrocytes, several other alterations of astrocytic integrity have been described in both human and experimental PSE. Monoamine oxidase type B (MAO_B) in human brain is a predominantly astrocytic enzyme. MAO_B activities were significantly increased in frontal cortex and caudate nucleus obtained at autopsy from cirrhotic patients, most of whom were alcoholics who died in hepatic coma (Raghavendra Rao et al. 1993*a*). This finding could relate to that of increased PTBR's in frontal cortex and caudate nucleus since treatment of mitochondrial preparations with digitonin resulted in the simultaneous release of MAO and PTBR's (Anholt 1986), suggesting that these two entities are localized in close proximity on the outer mitochondrial membrane. Reinforcing this notion are the findings of increased MAO_B activities and concomitantly increased densities of PTBR's in frontal cortex and caudate nuclei of cirrhotic patients who died in hepatic coma (Lavoie et al. 1990; Raghavendra Rao et al. 1993*a*).

To summarize, astrocytic alterations encountered in brain in human and experimental PSE are as follows:

- Increased number and size of mitochondria
- Decreased GFAP immunostaining
- Decreased GS activities
- Limited capacity to remove additional blood-borne ammonia
- Increased densities of PTBR's
- Increased ODN immunostaining
- Increased activities of MAO_B
- Decreased capacity to uptake glutamate and, ultimately, Alzheimer type II astrocytosis

EVIDENCE FOR DISRUPTION OF NEURON-ASTROCYTE METABOLIC INTERACTIONS IN PSE

Perineuronal astrocytes are essential participants in the supply and trafficking of substrates in the brain and, in particular, in synaptic regulation. In addition to taking part in the active removal of ions such as K^+, astrocytes take up released

neurotransmitter, express enzymes involved in neurotransmitter metabolism (e.g., MAO_B), and express receptors for both monoamine and amino acid neurotransmitters (Hansson 1988). Thus, modifications of astrocytic integrity in PSE would be expected to result in a loss of effective neuron-astrocyte metabolic interactions and, ultimately, to neuronal dysfunction.

Glutamatergic synaptic regulation, in particular, relies on the functional integrity of the perineuronal astrocyte. Glutamate released into the synaptic cleft is reuptaken by a high-affinity system mainly into the astrocyte, where it is transformed by GS into glutamine (figure 3). Glutamine is then returned to the presynaptic nerve terminal as the immediate precursor of releasable (neurotransmitter) glutamate.

Studies in cultured astrocytes from various brain regions demonstrate a positive correlation between GS activity and glutamate uptake capacity of these cells (Hansson et al. 1988). Administration of methionine sulfoximine to adult rats resulted in inhibition of GS and in parallel reductions in the size of the releasable (presynaptic) pool of glutamate (Rothstein and Tabakoff 1984). These findings suggest that the amount of releasable glutamate in the nerve terminal (figure 3) depends directly on the integrity of the perineuronal astrocyte and, in particular, on GS. As already mentioned, GS activities were significantly reduced in autopsied brain tissue from alcoholic cirrhotic patients who died in hepatic coma (Lavoie et al. 1987) as well as in the

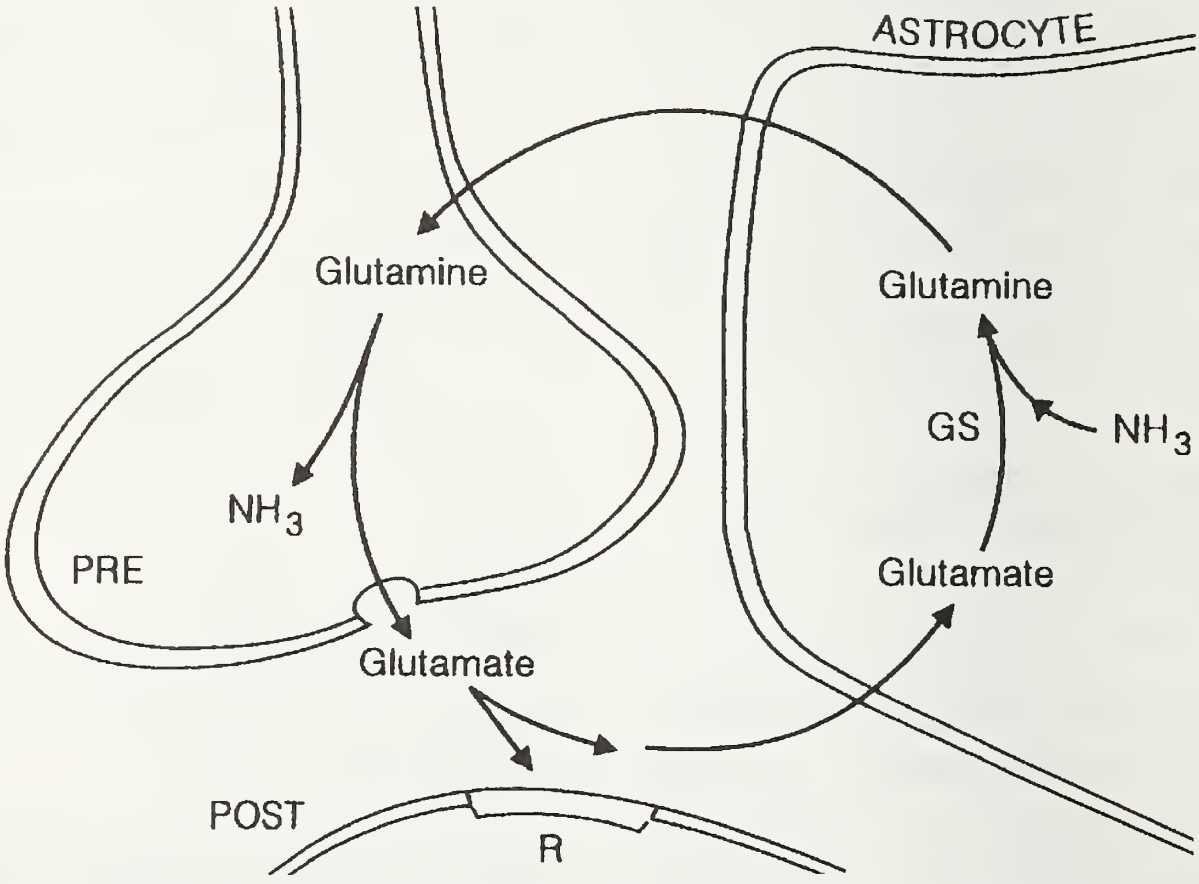

Figure 3. Simplified schematic representation of steps involved in glutamatergic synaptic regulation. Note key role of the perineuronal astrocyte. GS = glutamine synthetase; POST = postsynaptic neuron; PRE = presynaptic nerve terminal; R = receptor.

brains of rats following portacaval anastomosis (Butterworth et al. 1988; Girard et al. 1993).

Evidence from both in vitro and in vivo studies suggests that glutamate reuptake in brain is impaired in PSE, resulting in glutamatergic synaptic dysregulation. Evidence includes the following:

- There was a dose-dependent inhibition of D-aspartate uptake into rat hippocampal slices by blood extracts from patients with varying severity of PSE (Schmidt et al. 1990). (D-aspartate is a nonmetabolizable analog with high affinity for the l-glutamate uptake system). The relative inhibition of D-aspartate uptake was correlated with the ammonia content of the blood extracts from PSE patients.
- Exposure of cultured astrocytes to 2 mM ammonia for 4 days resulted in a 35-percent decrease in capacity for glutamate uptake (Norenberg et al. 1985).
- High-affinity uptake of glutamate into rat brain synaptosomal preparations was significantly decreased following exposure to 5 mM ammonia (Mena and Cotman 1985). Note that brain ammonia concentrations at coma stages of PSE in experimental animals are in the 2–5 mM range (Butterworth 1991; Butterworth et al. 1988).
- Electrically stimulated, Ca^{2+}-dependent release of glutamate from superfused hippocampal slices from portacaval shunted rats was significantly increased (Butterworth et al. 1991), consistent with decreased glutamate reuptake into perineuronal astrocytes and nerve terminals. Evidence for increased extracellular glutamate in brain following portacaval anastomosis has also been provided using in vivo preparations such as the "cortical cup" technique (Moroni et al. 1983) and cerebral microdialysis (Tossman et al. 1987).

Loss of glutamate reuptake and subsequently increased concentrations of extracellular glutamate in PSE would be expected to result in changes in postsynaptic glutamate binding sites. Studies in portacaval shunted rats (Peterson et al. 1990) and in dogs with congenital portal-systemic shunts (Maddison et al. 1991) provide evidence of reduced densities of postsynaptic glutamate binding sites.

SUMMARY

Portal-systemic encephalopathy is a major neuropsychiatric complication of alcoholic liver disease. Neuropathological studies demonstrate astrocytic changes in both human and experimental PSE. Such changes consist of morphological alterations including mitochondrial swelling and proliferation, increased densities of binding sites for "peripheral-type" (mitochondrial) benzodiazepine receptors, loss of GFAP immunostaining as well as reduced activities of glutamine synthetase, increased MAO_B activities, and reduced capacity for astrocytes to uptake glutamate. Reductions in capacity for ammonia removal, compromised neuron-astrocyte metabolic interactions, and glutamate synaptic dysregulation could have serious adverse consequences for cerebral function. Such mechanisms could be implicated in the pathogenesis of PSE in alcoholic liver disease.

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Chapter 12

^{1}H Magnetic Resonance Spectroscopic Imaging Separates Neuronal From Glial Changes in Alcohol-Related Brain Atrophy

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The neuronal loss that characterizes neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, human immunodeficiency virus (HIV)–associated dementia, and alcoholic dementia occurs in the context of glial hyperplasia (gliosis). X-ray computerized tomography (CT) and magnetic resonance imaging (MRI) measures of ventricular dilation and sulcal widening have been used as estimates of the extent of tissue atrophy in these various neurodegenerative diseases. However, because neuron loss produced by neurodegenerative disease is accompanied by gliosis, tissue atrophy is an insensitive marker of neuronal loss (either neuronal death or reductions in neuronal size), underestimating its extent. Therefore, the need for a neuron-specific marker which could be detected by a neuroimaging technique has long been recognized. Such a marker could separate neuronal from glial changes.

N-acetylaspartate (NAA) is the second most plentiful amino acid in human brain (glutamate is the most plentiful) and is localized exclusively in the nervous system (Tallan et al. 1956). Considerable evidence suggests that NAA is a specific marker for neurons: (1) NAA is absent in glial tumors (Nadler and Cooper 1972); (2) chemical measurements from astrocyte tumors and pure fetal astrocyte cultures failed to detect NAA (Peeling and Sutherland 1992); (3) NAA is found in fetal granular cell neuronal cultures, but not in mature oligodendrocyte or astrocyte cultures (Urenjak et al. 1992); and (4) immunocytochemical studies of NAA within rat brain show NAA reactivity only to be in neurons and not in glia (Moffett et al. 1991).

NAA appears to be a neuron-specific marker which can be detected by magnetic resonance spectroscopy (MRS) and magnetic resonance spectroscopic imaging (MRSI). Animal

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studies in our laboratory have been performed to test the hypothesis that NAA is reduced when neurons are injured (Auer and Siesjo 1988). Global ischemia was produced using the bilateral carotid occlusion method plus hypotension. Twenty-four hours after a global insult, NAA was reduced in cortex, hippocampus, and thalamus, but not in brain stem and cerebellar regions, which were not ischemic in this model (Higuchi et al. 1991). Studies were also performed 24 hours after kainate-induced status epilepticus, which has previously been shown to produce selective neuronal injury (Auer and Siesjo 1988). NAA was reduced in the amygdaloid nucleus, hippocampus, and cortex (Ebisu et al. unpublished manuscript); these areas are known to be specifically vulnerable to epileptic damage. Both sets of studies support the hypothesis that NAA reductions are a sensitive marker of neuronal death.

A number of human MRS and MRSI studies performed in our and other laboratories have also indicated that NAA is reduced in conditions associated with decreased neuronal density. These conditions include cerebral infarctions (Dujn et al. 1992), amyotrophic lateral sclerosis (Pioro et al. 1993), Huntington's chorea, Alzheimer's disease (Meyerhoff et al. in press; Miller et al. 1993), and HIV-associated dementia (Meyerhoff et al. 1993).

There are a few reports in the literature suggesting that NAA concentrations in viable neurons might change in a reversible fashion. The best evidence for this is from studies of multiple sclerosis, where NAA in multiple sclerosis plaques has been shown to decrease and subsequently increase (Arnold et al. 1990). These findings raise the possibility that brain NAA levels are affected by factors other than neuronal density. Therefore, even though NAA appears to be a neuronal marker, decreases of NAA measures do not in all instances necessarily indicate neuronal loss. To the degree that NAA is present in dendrites, it may be a potential marker of the loss of dendritic spines and dendritic rearborization.

The range of functions of NAA in the central nervous system (CNS) is not clear. In addition to whatever its role may prove to be in neurons, it has been suggested that NAA may participate in myelin lipid synthesis or may act as an osmolyte. Canavan's disease, a rare neurodegenerative disorder of children associated with impaired myelination, appears to result from deficiency of the NAA degradative enzyme aspartoacylase II (Matalon et al. 1989). The incorporation of the acetyl group of NAA into brain fatty acids (Burri et al. 1991) also supports the hypothesis that NAA is used by oligodendrocytes in myelin synthesis. Thus, although NAA is made by neurons, and its absence suggests that neurons are not viable, research suggests that NAA may be taken up and metabolized by glia. It also has been suggested, because of NAA's relatively high concentration and its reduction in chronic hyponatremia, that NAA may function as an osmolyte (Lee and Ross 1993; Sterns et al. 1993).

In summary, although the variables that may potentially affect MRS NAA measurements have not been definitively established, there is clear and convincing evidence that MRS NAA measurements are of value as a specific marker of neuronal changes in degenerative brain diseases. We report here parts of our initial investigation of NAA changes associated with chronic alcohol abuse.

CNS TOXICITY OF CHRONIC ALCOHOL CONSUMPTION

The evidence for significant CNS toxicity of chronic alcohol consumption derives from neu-

ropsychological, neuroimaging, and neuropathological examinations. On brain CT studies, chronic alcoholics show evidence of cerebellar atrophy, dilation of the cerebral ventricles, and increased sulcal width, particularly in the frontal cortex and in the frontal-parietal-temporal area (Jernigan et al. 1986; Wilkinson 1985). Sulcal widening has been found fairly consistently in alcoholics of all ages, whereas ventriculomegaly is found more frequently in older alcoholics. Cerebral blood flow and glucose metabolism studies have found (a) a significant negative correlation between hemispheric gray matter blood flow and total alcohol consumption and (b) decreased glucose utilization, especially in the frontal area in chronic alcoholics (Risberg and Berglund 1987; Rogers et al. 1983; Samson et al. 1986). Neuropathological examinations, in addition to confirming the neuroradiological findings of enlarged sulci, dilated ventricles, and cerebellar degeneration, have revealed patchy loss of neurons, axons, and dendrites, as well as hemorrhagic lesions (Courville 1955; Victor et al. 1971). Cortical neuronal counts demonstrate loss of neurons from the superior frontal cortex (Harper et al. 1987). There is no loss of neurons from motor, anterior cingulate, or middle temporal gyri (Kril and Harper 1989), although there is a shrinkage of the mean area of neuronal cell bodies in the superior frontal, cingulate, and motor areas. Neuropsychological studies also suggest that the frontal lobes are more susceptible to the deleterious effects of alcohol on brain function than are other cortical regions (Fein et al. 1990; Walsh 1983; Waugh et al. 1989).

Animal studies (Walker et al. 1981) and human studies (Harper et al. in press) strongly suggest that chronic ethanol treatment leads to learning deficits, loss of dendritic spines, reduction in dendritic branching, and cell death in vulnerable areas of the brain, and that these are toxic effects of ethanol rather than secondary nutritional effects. A general model proposed by Harper and Kril (1990) is that alcohol-related cortical brain damage falls into two different classes. The first class includes loss of dendritic arbor and shrinkage of neuronal body size. These changes are potentially reversible and occur in many brain regions. The second class is actual neuron death, which is irreversible and has been demonstrated primarily in frontal cortex. Moreover, the reversibility of neuronal changes appears to be greater in women and in younger individuals, suggesting that alcohol-related neuron cell death is most likely to occur in elderly male alcoholics (Harper et al. in press; Pfefferbaum et al. 1992).

In this first MRSI investigation, we wanted to examine a sample that we thought most likely to exhibit neuronal loss secondary to chronic alcoholism. To maximize our chances of finding permanent neuronal changes secondary to chronic alcohol abuse, we studied elderly male chronic alcoholics. To ensure that any effects studied were not transient phenomena (i.e., potentially reversible) or phenomena related to concurrent alcohol use or to the CNS effects of withdrawal, we recruited subjects at least 3 months abstinent. Our hypothesis was that, compared with elderly control subjects, the elderly male chronic alcoholics would have (1) significant brain atrophy on MRI and (2) a reduction in frontal cortex NAA in the remaining brain tissue, reflecting neuronal loss that is invisible on MRI because of glial hyperplasia.

METHODS

Subjects

Twenty male subjects participated in the combined MRI and ^{1}H MRSI study. Eleven

abstinent chronic alcoholic subjects (mean age: 60.6 ± 7.2 years, range: 50–73 years; years of education: 15.3 ± 2.7) were recruited from residential and outpatient treatment centers specializing in the treatment of the elderly alcoholic; nine male healthy controls (mean age: 64.9 ± 9.2 years, range: 49–75 years; years of education: 16.6 ± 2.4 years) were recruited from the community. All subjects were screened to exclude those with histories of significant drug use other than alcohol, those with histories of a major psychiatric or neurological disorder other than those secondary to chronic alcohol abuse (e.g., a history of alcohol withdrawal seizures), and those on medications with CNS side effects. Episodes of alcohol use–associated head trauma with loss of consciousness not serious enough to require hospitalization did not result in exclusion. Also, in the context of other ongoing studies in our laboratory, all subjects were confirmed to be HIV negative by polymerase chain reaction (PCR). Prior to the studies, all subjects signed a consent form which thoroughly described all procedures and their possible risks.

The self-report measure of alcohol consumption was an estimate of current and past intake. One drink was considered a glass of wine, a beer, or a shot of hard liquor, all averaging approximately 10 g of ethanol. According to this self-report, two of the recovering alcoholics had been binge drinkers for the last 30 and 32 years (drinking to unconsciousness on a regular, more than monthly basis); the rest had abused alcohol continuously and heavily (> 120 drinks per month) for 10–40 years before cessation (average years of abuse: 25.6 ± 8.8 years). At the time of study, 9/11 alcoholics had been abstinent for a period of 3–11 months (6.0 ± 3.1 months, mean ± SD) and the other 2 had been abstinent for 18 and 24 months (average duration of abstinence for entire patient cohort: 8.7 ± 6.8 months). All 9 control subjects reported having always drunk less than 60 drinks per month, with 7/9 control subjects drinking between 0 and 8 drinks per month.

All subjects underwent neuropsychological testing to assess a wide range of cognitive skills, including attention, concentration, memory, verbal language, problem solving, visuomotor and visuospatial skills, and fine motor ability. The battery included the following tests: WAIS-R Digit Symbol and Digit Span (Wechsler 1981), Shipley Institute of Living Scale (Vocabulary and Abstract Reasoning) (Zachary 1986), Finger Tapping (Halstead 1947), Grip Strength (Halstead 1947), Luria 99 (from the Luria-Nebraska) (Golden 1981), Wechsler Memory Scales (Logical Memory Immediate and Delayed, Design Reproduction Immediate and Delayed) (Wechsler 1987), FOME-15 (Davenport et al. 1988), Short Categories Test (Wetzel and Boll 1987), Controlled Oral Word Association (Benton and Hamsker 1976), Trail Making A and B (Davis 1968), and Hooper Visual Organization Test (Hooper 1958).

Each test was scored according to its norms. Each test score was ranked on a scale from 0 to 2, depending on the severity of impairment. A subject received a 0 if he scored above the 15th percentile for the test, a 1 if he scored between the 8th and 15th percentile (or from 1 to 1.65 SD below the mean), and a 2 if he scored below the 8th percentile (or more than 1.65 SD below the mean). Memory retention on the Wechsler Memory Scales was ranked as 1 if retention was between 51 and 70 percent, and 2 if retention was below 51 percent. Points earned on all tests were then summed to yield a global impairment score. The global impairment score was considered to indicate unimpaired performance if it was between 0 and 1, mildly impaired perfor-

mance if between 2 and 5, moderately impaired performance if between 6 and 9, and severely impaired performance if 10 or more.

The Shipley Institute of Living Scale uses a recognition vocabulary test, which can be used as an estimate of premorbid level of cognitive functioning. According to this measure, the average T-score for controls and alcoholics was not significantly different (62.6 ± 4.8 vs. 57.7 ± 9.5; $t = 1.38$, $p = 0.18$). All subjects except one alcoholic subject scored within one standard deviation of the norm. The one who did not was nevertheless included because socioeconomic factors suggested that lack of education was the most likely explanation for his low premorbid IQ estimate; on the abstract reasoning portion of the Shipley scale, which does not depend on education, he scored within the normal range.

Magnetic Resonance Procedures

We performed MRI and MRSI studies on a whole-body Philips 2-tesla MRI/MRS system; the studies took less than 2 hours in total. The following sections describe the MRI measurement of brain atrophy and the MRSI measurement of NAA.

MRI Measurement of Brain Atrophy

A sagittal T_1-weighted study (repetition time [TR] = 450 ms, echo time [TE] = 30 ms) was used for determination of the canthomeatal plane. This was followed by a spin-echo sequence (TR = 3,000 ms, TE = 30 ms and 80 ms) performed parallel to the canthomeatal plane and covering the entire brain from the cerebellum to the vertex. The spin-echo sequence yielded a set of 19–23 proton density–weighted (the 30-ms echo) and T_2-weighted (the 80-ms echo), 5.1-mm-thick (with a 0.5-mm interslice gap) axial images. One subject had difficulty tolerating the procedure and, to shorten the acquisition time, the TR was shortened to 2,068 ms and only 13 slices were acquired (these included all of the slices needed for the analyses presented here).

Computer-assisted segmentation of the MRI into white, gray, sulcal cerebrospinal fluid (CSF), and ventricular CSF volumes was performed on sequential slices beginning with the slice on which the red nucleus appeared and ending one slice above the top of the MRSI volume of interest (VOI). Details of the MRI segmentation procedures are described in an unpublished manuscript (Di Sclafani et al.). Figure 1 displays a representative subject's MRI data together with the segmentation results for a slice through the lateral ventricles and a slice through the center of the MRS VOI.

Preprocessing involved two steps. First, the skull was stripped off the image using a modification of the algorithm developed by Lim and Pfefferbaum (1989). The resulting image, which was subject to all remaining analyses, was limited to the boundaries of the intracranial vault. The second step was estimation and removal of radiofrequency (RF) field inhomogeneity via digital filtering. The inhomogeneity-corrected images were then processed independently by two operators, both blind to subject identity.

On a slice-by-slice basis, the operators set a threshold for subtraction images (i.e., the difference image between the T_2-weighted and proton density–weighted images) to specify conservative CSF and non-CSF samples. These samples were chosen to represent tissue that definitely belonged to the correct (i.e., CSF or non-CSF) classification and left a sizable amount of tissue unclassified. For each slice, the conservative CSF and non-CSF samples were used as training sets for a discriminant analysis, which used the subtraction image pixel intensities to classify each pixel as either CSF or non-CSF. Each operator then separated

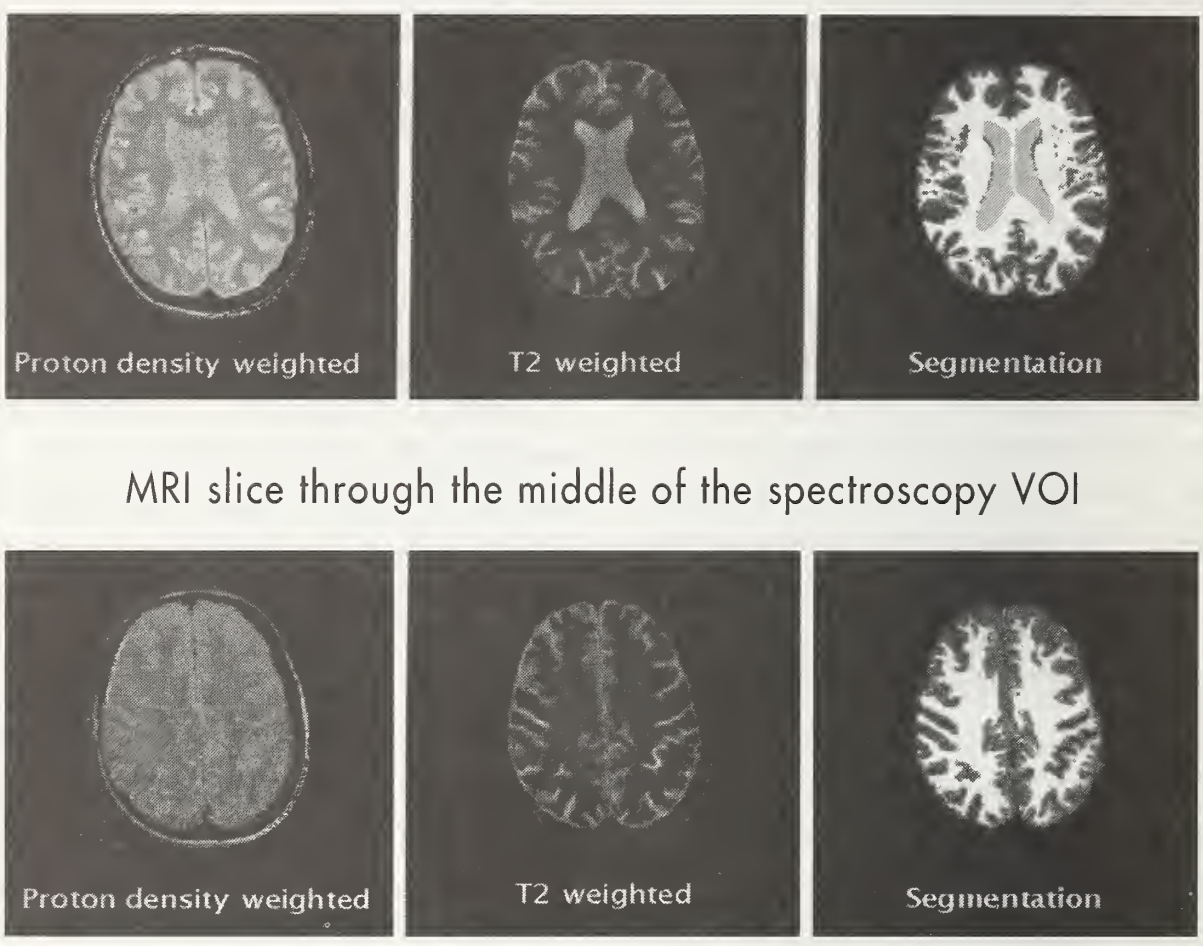

Figure 1. Proton density–weighted and T_2-weighted magnetic resonance images, together with the computer-aided tissue segmentation results, are displayed for representative slices through the middle of the lateral ventricles and through the middle of the MRSI volume of interest.

ventricular from sulcal CSF by delineating the anatomical boundaries of the ventricular system on each slice. Ventricular and sulcal CSF volumes were computed as a percent of total intracranial vault volume on the slices analyzed to correct for the variation due to head size. There was excellent agreement between the operators, with the between-operator correlation greater than 0.99 for both percent ventricular CSF and percent sulcal CSF.

A further segmentation of the non-CSF pixels into gray and white matter was performed. This was accomplished in a similar manner to the CSF versus non-CSF discrimination. On a slice-by-slice basis, the operators set a threshold for the proton density–weighted images to specify conservative white matter and gray matter samples. These samples were chosen to represent tissue that definitely belonged to the correct (i.e., white vs. gray) classification and left a sizable amount of tissue unclassified. For each slice, the conservative tissue samples were used as training sets for a discriminant analysis, which used both the proton density–weighted and the T_2-weighted pixel intensities to classify all non-CSF pixels as either gray or white matter. The white matter and gray matter volumes were then computed as a percent of total in-

tracranial vault volume on the slices analyzed. The between-operator correlations for percent white and percent gray matter were 0.82 and 0.66, respectively.

MRSI Measurement of NAA

A 17-mm-thick VOI was selected for ^{1}H MRSI. In figure 2 the VOI is indicated as a white box on the transverse (summed) magnetic resonance image.

Gradient phase encoding was applied over the VOI in two dimensions (24 x 24 phase encoding steps) to yield spectra with an effective voxel size of 2.1 mL. Details of the MRSI measurements are described by Meyerhoff and colleagues (in press). The summed magnetic resonance image (composed of three 5.1-mm-thick MRI slices) was used for selection of the MRSI voxels to be analyzed. Four voxels were selected in left and right frontal and parietal association cortices. Voxel size and the typical location of the voxels selected for analysis are illustrated on the transverse summed MRI shown in figure 2. Spectra from these voxels were extracted from the MRSI dataset for analysis. Three peaks were fitted to the three major resonances in the spectra, originating from choline-containing metabolites (Cho), from the sum of creatine and phosphocreatine (Cr), and from *N*-acetyl groups, predominantly NAA. These metabolite peak areas are generally considered to be directly proportional to metabolite concentrations. An illustrative spectrum from a single voxel is displayed in figure 3.

The primary hypothesis of a NAA reduction in frontal cortex of recovering alcoholics was tested by evaluating the group (alcoholic vs. control) by location (frontal vs. parietal) interaction effect in a repeated-measures analysis of variance (ANOVA) design where each subject had four repeated measures from the four selected MRSI voxels. The peak area ratio of NAA to Cr (NAA/Cr) was chosen for this primary analysis because the Cr peak was not expected to be affected by alcohol. The Cho peak

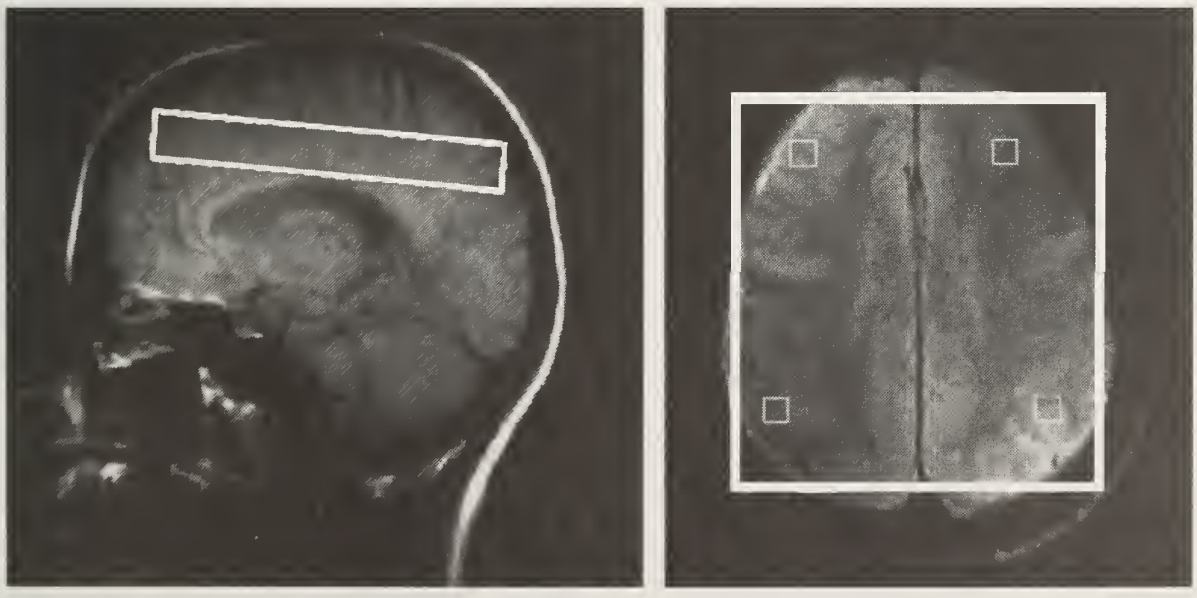

Figure 2. Sagittal and transverse magnetic resonance images of a recovering elderly chronic alcoholic. The white boxes indicate placement of the proton MRSI volume of interest, and the white squares on the transverse image show the typical locations from which cortical spectra were extracted.

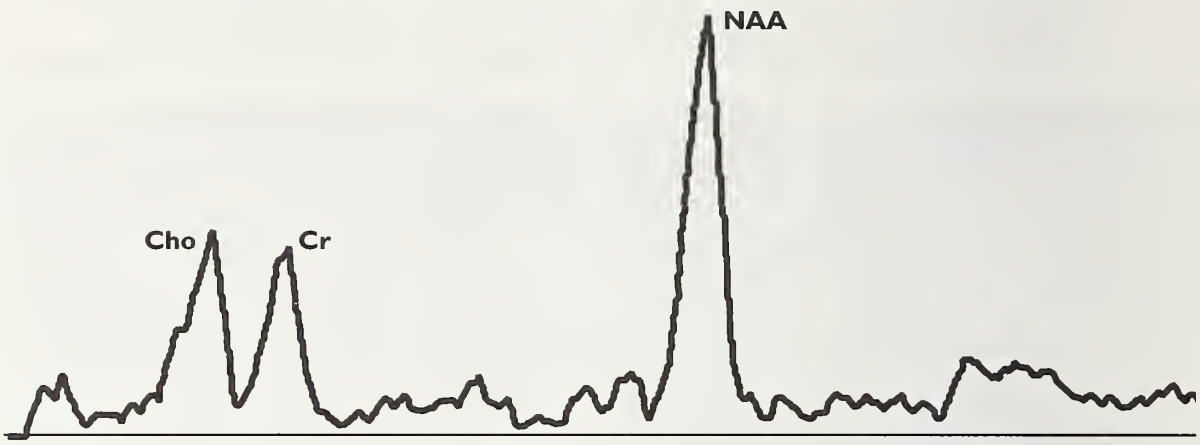

Figure 3. A typical proton MRSI spectrum obtained from the frontal cortex of an elderly recovering chronic alcoholic. Resonance peaks are from *N*-acetylaspartate (NAA), choline-containing metabolites (Cho), and the sum of creatine and phosphocreatine (Cr).

consists of resonances from compounds that are involved in membrane lipid synthesis and breakdown and may conceivably be affected by alcohol use. Therefore, the NAA/Cho ratios were evaluated in a secondary analysis. The test for specific frontal cortex reductions in NAA/Cr was evaluated within a repeated-measures model using parietal control areas and testing for a group (alcoholic vs. control) by location (frontal vs. parietal) interaction. This model increased the power of the study by allowing evaluation of the experimental hypothesis in the context of the within-subject variation in NAA/Cr rather than the between-subject variation in NAA/Cr. All values were expressed as mean ± 1 SD, and $p < 0.05$ was considered statistically significant.

RESULTS

Neuropsychological Assessments

There was a significantly greater level of cognitive impairments in the recovering alcoholics compared with the control sample. All the recovering alcoholics evidenced some degree of cognitive impairments: three were mildly impaired, four were moderately impaired, and four were severely impaired. Of the controls, three were unimpaired, four had mild impairment, and two had moderate impairment. The impairment scores for controls ranged from 0 to 6, with an average of 2.7; for the alcoholics, the range was from 4 to 18, with an average of 9.27 ($t_{14.3} = 3.80$, $p = 0.002$; note that the degrees of freedom are adjusted for differences in variance between the groups).

MRI Measures of Brain Atrophy

There were no significant differences between groups for either the ventricular ($t_{18} = 1.22$, $p = 0.24$) or the sulcal ($t_{18} = 0.09$, $p = 0.93$) atrophy measure. The ventricular and sulcal atrophy data are presented in figure 4.

Although there was substantial overlap between groups, there were four recovering alcoholic patients who had larger ventricles than any of the controls, with two of these having ventricles 40 percent and 80 percent larger than the largest ventricle in the control group. These two recovering alcoholics also had the largest sulci, although the overlap between alcoholics

and controls was greater for the sulcal compared with the ventricular atrophy measure. Further, there was no significant difference between the groups on the percent gray matter (t_{17} = 0.70, p = 0.49) or percent white matter (t_{17} = 0.43, p = 0.68).

MRSI Measures of NAA

Metabolite ratios from the bilateral frontal and bilateral parietal voxels for the recovering alcoholics and the controls are depicted in table 1. For NAA/Cr, there was a significant location-by-group interaction ($F_{1,18}$ = 4.36, p = 0.04). This interaction indicated that, compared with controls, the alcoholics had a reduction in frontal NAA/Cr relative to parietal NAA/Cr. The actual reduction in frontal NAA/Cr in recovering alcoholics compared with controls was 11 percent.

Figure 5 displays the difference measure between frontal and parietal NAA/Cr for the two groups. A similar pattern of differences in mean ratios was apparent for NAA/Cho. With a smaller effect (a 6-percent reduction in NAA/Cho in alcoholics compared with controls) and larger standard errors, the NAA/Cho effect did not reach statistical significance.

Figure 6 displays, separately for alcoholics and controls, the scatterplot of the difference between frontal and parietal NAA/Cr versus percent ventricular CSF. In the alcoholics only, there was a significant correlation (r = -0.69, p = 0.02), indicating greater relative reductions in frontal NAA/Cr in those alcoholics with the greatest brain atrophy (this was true also for percent sulcal CSF: r = -0.73, p = 0.01). This difference in regression slopes is reflected in a significant interaction term ($F_{1,16}$ = 6.78, p = 0.019) from a covariance analysis where group is the dependent variable, the relative frontal reduction in NAA/Cr is the covariate, and the ventricular atrophy measure is the dependent variable. This association in alcoholics between the frontal (relative to parietal) cortex reduction in NAA/Cr ratios and the amount of ventricular

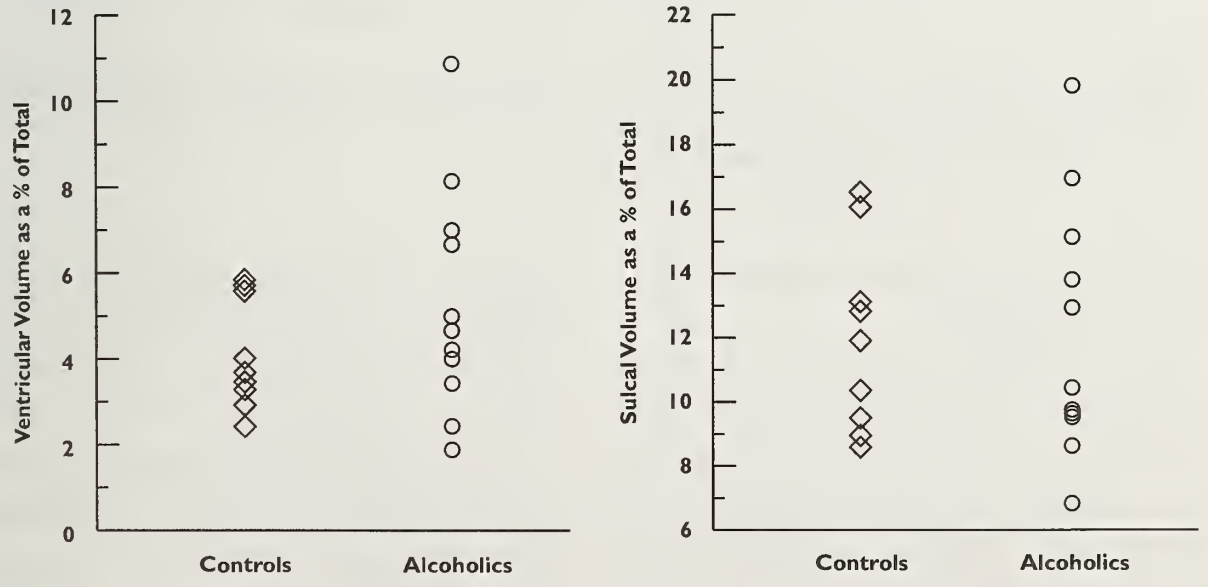

Figure 4. Distributions of ventricular and sulcal volume, each as a percent of total brain volume, for the controls and alcoholics. Although there were four alcoholics beyond the range of the controls for ventricular volume, the distributions for the two groups were not significantly different for either atrophy measure.

Table 1. Metabolite Ratios Obtained From Bilateral Spectra From Frontal and Parietal Cortices in Abstinent Elderly Alcoholics and Age-Matched controls (mean ± SD)

Ratio	Cortex	Controls (n = 9)	Alcoholics (n = 11)
NAA/Cr*	frontal	2.89 ± 0.39	2.56 ± 0.51
	parietal	2.57 ± 0.29	2.72 ± 0.26
NAA/Cho	frontal	2.42 ± 0.51	2.26 ± 0.53
	parietal	2.49 ± 0.49	2.60 ± 0.53

*p = 0.04, location-by-group effect, i.e. controlling for changes in parietal voxels.

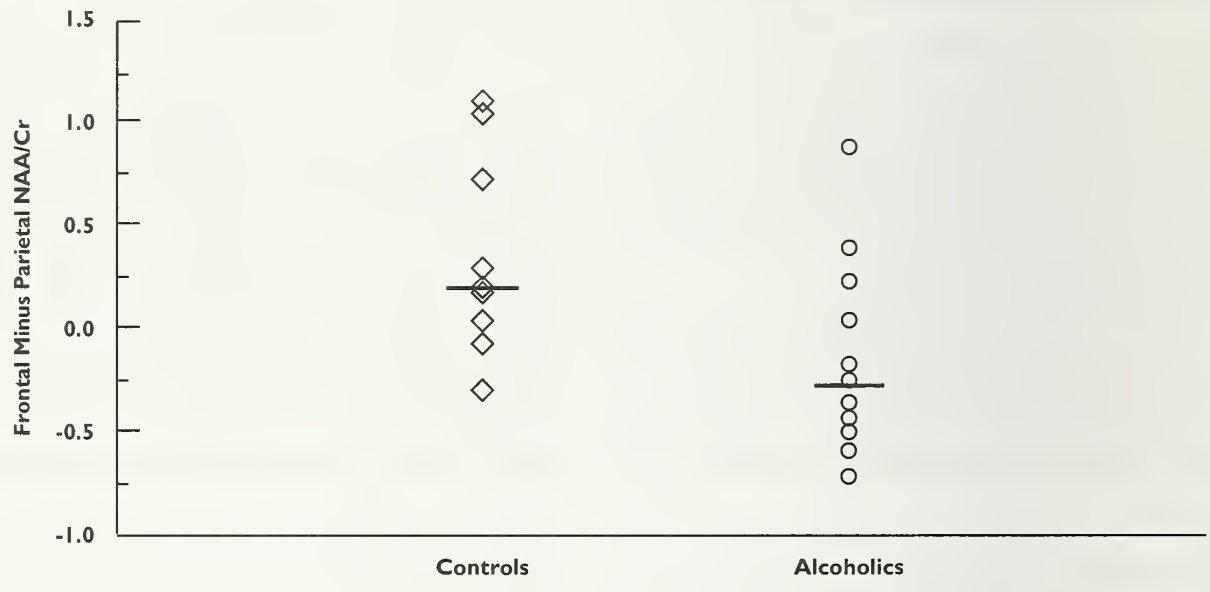

Figure 5. Distribution of frontal minus parietal NAA/Cr ratio for the controls and alcoholics, with horizontal lines representing the median ratio. The frontal relative to parietal NAA/Cr ratio was lower in alcoholics compared with controls (p = 0.04).

dilation reflected a frontal cortex (and not a parietal cortex) effect.

Figure 7 displays, separately for alcoholics and controls, the scatterplot of frontal NAA/Cr versus percent ventricular CSF. As in Figure 6, in the alcoholics only, there was a significant correlation (r = -0.70, p = 0.02), indicating lower frontal NAA/Cr in those alcoholics with the greatest brain atrophy.

Because of the slice selection used for MRSI, there was a chemical shift offset such that the exact tissue sampled for the various metabolites (NAA, Cr, and Cho) was not identical. Convolving the MRSI point spread function for NAA against the segmented MRI confirmed that the NAA measurements (1) were derived from fully volumed brain tissue (with less than 1 percent of the potential signal coming from CSF) and (2) were sampled from at least as much frontal gray matter in the alcoholics as in the controls. Thus, the NAA/Cr findings were not an artifact of different tissue sampling within the alcoholic and control groups.

Finally, no significant associations were found between NAA/Cr measures and the neuropsychological test global impairment scores.

DISCUSSION

The primary finding of this study is a reduction in the NAA/Cr ratio in frontal cortex relative to that of parietal cortex in recovering elderly alcoholics compared with age-matched controls. In the small study samples reported here, reduced frontal NAA/Cr (relative to parietal NAA/Cr) in the absence of significant atrophy on MRI suggests frontal cortex neuronal loss associated with glial hyperplasia in elderly alcoholics compared with age-matched controls. This suggestion is tempered by the limitations of NAA as a neuronal marker, as discussed below. Convolving the MRSI point spread function with the MRI segmentation results suggests that the NAA/Cr findings were not an artifact of sampling different mixes of tissue within the alcoholic and control groups.

Because of our sampling design, it is possible that the effect that we observed underrepresents the effect in the elderly male alcoholic population. Our inclusion criterion—elderly male alcoholics who had remained abstinent for at least 3 months—resulted in the study of a potentially biased sample of elderly male alcoholics. We have underestimated CNS morbidity in the elderly male alcoholic population to the degree that the ability to remain sober is associated with less severe alcohol-related CNS morbidity. Frontal cortex NAA/Cr reductions in elderly male chronic alcoholics may prove to be even larger if studied in samples more representative of the entire elderly male alcoholic population.

This preliminary study suggests that two related processes involving neuronal loss are taking place in elderly male alcoholics with severe CNS involvement. These processes are

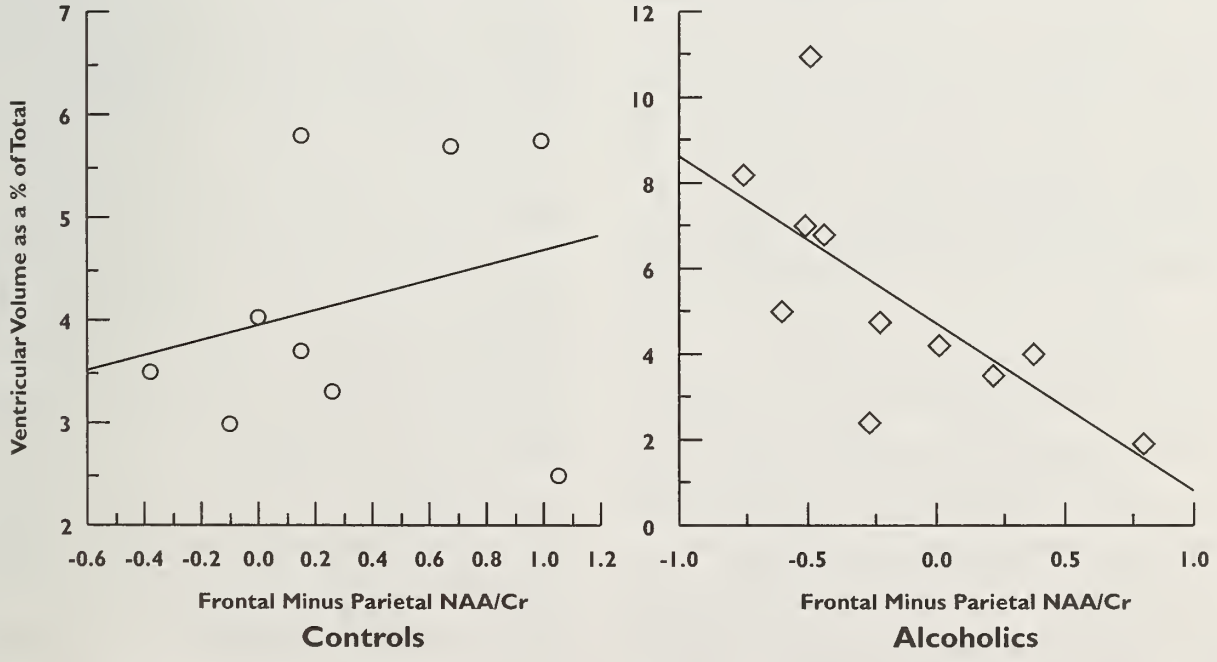

Figure 6. Percent ventricular volume plotted against frontal minus parietal NAA/Cr ratios. For the alcoholics only, higher percent ventricular volumes were associated with lower frontal minus parietal NAA/Cr ratios ($r = -0.69$, $p = 0.02$).

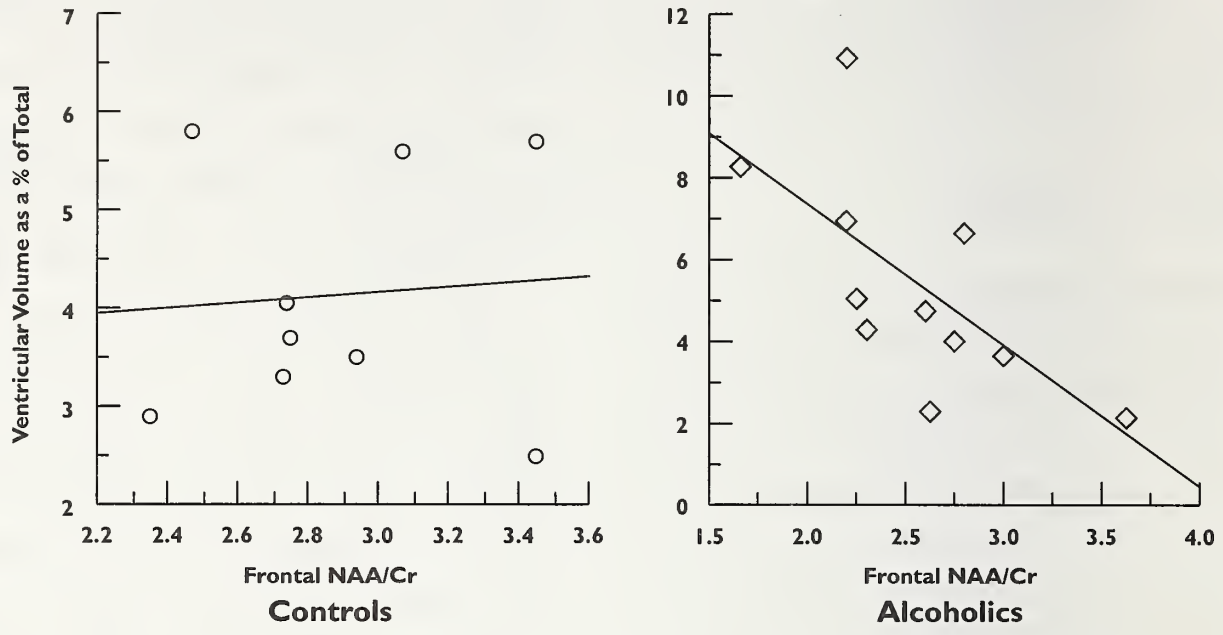

Figure 7. Percent ventricular volume plotted against frontal NAA/Cr ratios. For the alcoholics only, higher percent ventricular volumes were associated with lower frontal NAA/Cr ratios (r = -0.70, p = 0.02), indicating that the inverse relationship between ventricular volumes and frontal minus parietal NAA/Cr ratios depicted in figure 6 is due primarily to lower frontal NAA/Cr ratios.

(1) a global loss of brain tissue with corresponding increases in the CSF volume and (2) an additional loss of neurons in the remaining frontal brain matter, with this neuronal tissue replaced by proliferating glial cells. The reduction in frontal NAA (inferred from the reduced NAA/Cr ratios) in the elderly male alcoholics reflects that part of neuronal loss that is in addition to that reflected by atrophy. An appropriate NAA-related estimate that would reflect total neuronal loss (in either the whole brain or a specific brain region) in elderly male alcoholics would be the integrated brain NAA signal corrected for head size. This measure would reflect loss of NAA both from brain atrophy and from neuronal loss with compensatory gliosis.

Although we do not have neuroimaging data from our study subjects earlier in their sobriety, one could conjecture that they initially evidenced greater amounts of ventricular and sulcal enlargement which partially resolved with continued abstinence. Such a pattern has been reported in many previous MRI studies of chronic alcoholics (Carlen et al. 1984; Schroth et al. 1988; Zipursky et al. 1989). An interesting question is whether the frontal cortex NAA reductions we see after considerable abstinence would have been of the same magnitude if we had examined the subjects earlier in their abstinence. To the degree that the finding of reduced NAA reflects neuronal loss, one would hypothesize that it would be unchanged with abstinence. To the degree that the finding of reduced NAA reflects a factor such as loss of dendritic arborization and cell body shrinkage, which may (at least partially) reverse with continued abstinence, the NAA signal may (partially) recover with abstinence. The answer to this question is potentially important. To the degree that reduced frontal cortex NAA in (elderly) alcoholics reflects permanent neuronal loss, it may be, early in abstinence, a better diagnostic procedure than MRI for estimating long-lasting alcohol-related CNS morbidity in

specific patients. In other words, early in abstinence NAA may more strongly reflect permanent alcohol effects on brain structure while MRI measures of atrophy may more strongly reflect transient effects. It may be that relatively permanent alcohol effects on the brain are related to the patient's probability of remaining abstinent, and knowledge of the presence of such phenomena may be helpful in long-term treatment planning.

It is important to note that this study is only a preliminary ^{1}H MRSI study of the effects of chronic alcoholism. Both men and women need to be studied, as do alcoholics of different ages and with minimal versus substantial periods of abstinence. Furthermore, studies of larger samples with more extensive sampling of NAA from greater volumes of brain tissue are needed. This would allow evaluation of frontal cortex changes without relying on a parietal control area. We note the small differences between alcoholics and controls in parietal mean ratios (opposite in direction to the differences in frontal cortex and slightly less than half their size) and the inability in the present study to fully separate the frontal from parietal phenomena.

Regarding the MRSI methodology, direct quantitation of NAA concentrations would be helpful, removing the necessity to examine metabolite ratios and to rely on the assumption that Cr is unaffected by chronic alcohol use. A reduction in the NAA signal could result from either a reduction in NAA concentration or a change in the NAA T_1 and T_2 relaxation times. Tedious NAA relaxation time measurements are needed to determine whether relaxation time changes contributed to our findings. Finally, it is possible that other currently unknown factors cause a decrease of NAA concentrations in viable neurons.

We wish to emphasize that MRS and MRSI are in their infancy. Great improvements are expected as magnetic resonance, computer, and data analytic technologies mature. Furthermore, the MRS and MRSI technologies will eventually be more readily available on current generation MRI instruments in clinical settings, and may be the neuroimaging technique that will provide a neuron-specific marker which could separate neuronal from glial changes. Such measurements may prove to be invaluable in the study and clinical management of chronic alcoholism.

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Chapter 13

Alcohol and Glial Cells: Areas for Future Research

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The authors of the preceding chapters in this research monograph discuss a wide variety of activities observed in normally functioning astrocytes, oligodendroglia, and microglia and present evidence that alcohol disturbs many of these activities. The influence of ethanol exposure on adult and developing cells is discussed, although many of the research techniques utilized to study glial cells require the use of cultures that are started with developing cells. Thus, the need remains for research on glial cells in situ, in developing and in older individuals. Noninvasive techniques are currently being developed to make evaluations of glial cell activity possible in live animals and human beings in the near future (see chapter 12). In the meantime, the effects of ethanol on glial cells are studied predominantly in cultures from the brains of animals that were exposed to ethanol while living, and through the exposure of cultured cells to ethanol.

Historically, more research has been available on the effects of ethanol on oligodendroglia and myelin (see chapter 2) than on astrocytes and microglia. Ethanol-induced delays of oligodendroglial maturation, myelination, and altered radial glial maturation and transformation to astrocytes have been observed in conjunction with a transient astrogliosis (see chapter 4). This research continues, and new functions postulated for oligodendroglia will provide even greater interest in this field.

The majority of the chapters in this monograph address the effects of ethanol on astrocytes, reflecting increased interest in these cells now that advances in technology have made their study more feasible. Even before these advances in research techniques were available, much documentation of gliosis in response to ethanol exposure was available. Proliferation of glial cells and glial scarring were perceived to be pathological. New information presented in these chapters suggests that the proliferative responses of glial cells to cell injury and infection may serve as a reaction to protect the nervous system from injury. For example, astrocytes produce interleukin-6, a mitogen for

astrocytes, in response to tumor necrosis factor α, interferon gamma, virus, and calcium ionophores. The resulting reactive gliosis can inhibit or promote neurite outgrowth, depending on the circumstances. In many cases, the pathological effects associated with gliosis may be related to the swelling of astrocytes and consequent alteration of their normal functions (see chapter 1). Proliferation of astrocytes has also been observed in animals exposed to ethanol during early postnatal brain development, evidenced by altered gene expression resulting in increased glial fibrillary acidic protein (GFAP) messenger RNA (mRNA) (see chapter 6). Chronic prenatal ethanol exposure also increases the numbers of reactive microglia in the brain (see chapter 3).

Proliferation and advanced maturation of microglia, and enhanced development of brain vasculature in response to prenatal ethanol exposure, support the hypothesis that microglia are reacting to ethanol exposure in a way designed to increase the chances of neuronal survival and growth. However, there is the chance that the developing nervous system could be damaged by excessive numbers of microglial cells producing not only reparative neurotrophins, but also excessive neurotoxic oxygen species such as nitric oxide. Developing individuals exposed to ethanol may be compromised in their ability to deal with excessive levels of ammonia and free radicals due to ethanol-induced decreases in astrocyte production of glutamine synthetase and superoxide dismutase (see chapters 7 and 11). It has also been reported that although proliferation of astrocytes occurs, some astrocytes are damaged, resulting in the loss of their critical functions; these functions include production of neurotrophic factors (see chapter 9), ability to transport glucose and remove glutamate from synapses (see chapter 5), and immune responses (see chapter 8). Loss of these functions may lead to a damaging buildup of ammonia and free radicals, excitotoxicity (see chapter 10), and increased vulnerability to infections (see chapter 8). It is hoped that diagnostic tests and treatments will be available to intervene in these disorders in the near future.

Two suggestions for treatment and prevention of fetal alcohol damage are presented in chapter 7. Manganese added to the diet of mothers drinking ethanol prevented the previously observed ethanol-induced decreases in enolase and glutamine synthetase. A plant extract from grapes also had a positive effect on the development of glial cells cultured from the brains of offspring of ethanol-treated rats. Enolase and glutamine synthetase were increased in these cells treated with grape extract following ethanol exposure in utero, compared with decreases observed in those without grape extract treatment. Although the exact nature of this grape plant extract is not provided in chapter 7, a recent article reported that grape skin extract (ground-up skins of white or red grapes in distilled water), and not ethanol, causes relaxation of aortic rings due to nitric oxide content (Fitzpatrick et al. 1993).

Another new and exciting possibility for treatment of ethanol-induced brain damage is presented in chapter 9. Transplantation of activated astrocytes into the brains of ethanol-damaged rats improved cognitive performance and restored working and reference memory. Further research is needed to determine not only the influence of astrocytes in repairing ethanol-induced brain damage in developing individuals and adults, but also the role of astrocytic neurotrophins in normal cognitive function.

GLIAL CELLS, NEUROSTEROID PRODUCTION, AND GENDER DIFFERENCES IN THE BRAIN

Neurosteroid production is an important function of glial cells which is not addressed by the chapters in this monograph (see Lancaster in press for review). Neurosteroids are synthesized in glial cells from cholesterol, or from peripherally synthesized steroids (Majewska 1992; Paul and Purdy 1992). Cytochrome P-450-scc, a mitochondrial enzyme which is active in producing neurosteroids, is found predominantly in glial cells. Glial cells, in contrast to neurons, have the enzymes that produce the neurosteroid pregnenolone from cholesterol or mevalonolactone (Akwa et al. 1991). Pregnenolone is converted by astrocytes into the steroid progesterone and a neuromodulatory steroid, tetrahydroprogesterone (THP). Neurons lack the enzymes required for progesterone synthesis (Kabbadj et al. 1993).

Oligodendroglia can also convert pregnenolone to progesterone, and in turn convert progesterone to THP. THP can be formed de novo by oligodendroglia, and can be formed by neurons by utilizing the progesterone provided by glial cells. The neurosteroid dehydroepiandrosterone (DHEA) is formed from pregnenolone and can be converted to dehydroepiandrosterone sulfate (DHEAS); DHEA can also be converted to androstenedione, which can be reduced to androsterone. Sulfate forms of neurosteroids (pregnenolone sulfate and DHEAS) have antagonistic action on $GABA_A$ receptors (Demirgoren et al. 1991), while THP, androsterone, and tetrahydrodeoxycorticosterone (THDOC) formed from corticosterone act as allosteric agonists of the $GABA_A$ receptor (Costa and Guidotti 1991; Puia et al. 1990). Pregnenolone sulfate enhances NMDA currents and inhibits the activity of GABA and glycine receptors (Bowlby 1993; Maione et al. 1992; Wu et al. 1991). Neurosteroids bind at a recognition site on the $GABA_A$ receptor that is unique and separate from binding sites for barbiturates and benzodiazepines (Lan et al. 1991).

Glial synthesis of neurosteroids is triggered through stimulation of benzodiazepine receptors concentrated on the outer mitochondrial membranes of astrocytes (Anholt 1986). These mitochondrial benzodiazepine receptors (MBR's) are also referred to as "peripheral-type benzodiazepine receptors" (PTBR's) (see chapter 11). Activation of MBR's facilitates the influx of cholesterol through mitochondrial membranes, promoting the formation of pregnenolone and other neurosteroids. MBR's can be stimulated by diazepam binding inhibitor (DBI), or by two of its metabolites, octadecaneuropeptide (ODN) and triakontatetraneuropeptide (TTN) (Costa and Guidotti 1991).

DBI concentration is increased in the cerebrospinal fluid (CSF) of patients with portal-systemic encephalopathy. Furthermore, researchers have demonstrated that portacaval anastomosis and associated increases in ammonia levels in the brain lead to increased ODN-immunolabeling in astrocytes of several brain regions (Butterworth et al. 1991). These results are complemented by studies showing increased densities of PTBR's in the frontal cortex and caudate nuclei of alcoholic cirrhotic patients who died in hepatic coma (Lavoie et al. 1990). Thus, chronic hyperammonemia has been linked to elevation of astrocytic PTBR density, and to the loss of

glutamine synthetase activity in selected areas in the brain (see chapter 11).

Preference for ethanol and sensitivity to ethanol have been linked to DBI content in the brain. DBI content was increased in the cerebellum and hypothalamus of ethanol-preferring rats after chronic ethanol intake, and was higher in the brains of rodents genetically selected for ethanol sensitivity (Alho et al. 1987). Although DBI levels in the CSF of alcoholics and control subjects were found to be similar, a positive correlation for CSF levels of DBI and corticotropin-releasing hormone was reported (Roy et al. 1990).

Chronic alcohol abusers, with or without overt liver damage, often have altered steroid hormone levels (Mello et al. 1993). Abnormal steroid levels in these individuals have been attributed to interference of steroid metabolism by the competing demands of alcohol and steroid metabolism on the liver, by the direct influence of alcohol on the steroid-producing cells, and by the influence of alcohol on the hypothalamic-pituitary axis (Bartke 1993). Gender differences in the consequences of chronic alcohol abuse may be linked to differences in steroid hormone levels, which may in some way be responsible for the increased risk observed in women for alcohol-induced liver and brain disease (Gallant 1990). Currently, no information on gender differences in astrocytic MBR's or DBI is available, although sex differences in specific neurosteroid levels (Lanthier and Patwardhan 1986), sex differences in alcohol intake (Harford et al. 1992; Lancaster and Spiegel 1992; Li and Lumeng 1984), and increased vulnerability of females to alcohol-induced liver damage (Norton et al. 1987) and brain damage (Harper et al. 1990) support the need for investigation in this area.

Circulating levels of peripherally synthesized steroids influence the production of neurosteroids by glial cells (Majewska 1992). Gender differences in $GABA_A$ and NMDA responses may be modulated by variations in levels of neurosteroids formed de novo in the brain, and as metabolites of circulating gonadal and adrenal steroids. Supporting this hypothesis is the evidence for higher levels of the neurosteroids DHEA and DHEAS in women compared with men (Lanthier and Patwardhan 1986).

Elevated levels of corticosterone, released in response to stress (Eskay et al. 1993) and alcohol or drug use (Wand 1993), are capable of stimulating formation of neurosteroids, which have the ability to increase GABA activity (Puia et al. 1990), consequently influencing behavior. Thus, neurosteroid modulation of GABA activity would be expected to lead to changes in GABA-influenced behaviors, including responses to alcohol (Hunt 1982; Melchior and Allen 1992; Melchior and Ritzmann 1992).

Elevated levels of GABA decrease memory and learning by inhibiting long-term potentiation. Alcohol, by inhibiting NMDA activity and enhancing GABA activity, interferes with the neural processing that is required for memory and learning (Morrisett and Swartzwelder 1993). Since the GABA-antagonistic neurosteroid DHEAS improves memory in mice (Flood et al. 1992; Roberts et al. 1987), hypothetically, GABA-antagonist neurosteroids could be used to treat alcohol-induced memory loss. The neurosteroid pregnenolone sulfate, with its ability to antagonize GABA activity and to enhance NMDA activity (Bowlby 1993; Maione et al. 1992; Wu et al. 1991), is another potential candidate for studies on in-

tervention in alcohol-induced memory loss and learning disorders.

Neurosteroid levels change dramatically in response to variations in levels of peripherally synthesized steroids associated with conditions as diverse as menstrual cycles, stress, hyperadrenalism, steroid administration, and alcohol and drug use (Majewska 1992). Gliosis, associated with chronic alcohol abuse, hepatic encephalopathy (Norenberg 1987), and exposure to alcohol during brain development (Goodlett et al. 1993), influences both the quantity and the types of neurosteroids that are synthesized. When astrocytes are plated at high density, pregnenolone or DHEA is formed; in comparison, progesterone and androstenedione are produced at low plating density. These neurosteroids may influence the proliferative responses of astrocytes associated with brain injury and in astrocytic tumors (Akwa et al. 1993).

GLIAL CELLS AND IMAGING

To date, researchers interested in imaging the brains of alcoholics have focused on refining the technology for determining precise neuronal changes. New research presented in this monograph shows the importance of glial cell function in the brain and in response to alcohol exposure. Perhaps in the future, researchers interested in imaging the brains of alcoholics will develop new methods and refine existing technology to be capable of monitoring early changes in glial cell function as a consequence of alcohol exposure. Because of the noninvasive properties of imaging, it is reasonable to expect that researchers could develop techniques for monitoring the influence of alcohol on brain development of infants, children, and adolescents and studies to monitor differences between males and females. Until recent developments in brain imaging, the study of the responses of the human brain to alcohol was limited largely to autopsy tissues or relied upon comparisons with animal studies. Development of new noninvasive technology for monitoring the influence of alcohol on living brain will provide a wealth of information currently unavailable. Further refinement of these techniques to distinguish between glial and neuronal responses could introduce a new frontier in alcohol research.

Fein and colleagues (see chapter 12) used ^{1}H magnetic resonance spectroscopic imaging to achieve computer-assisted segmentation of the magnetic resonance image (MRI) into white matter and gray matter and to determine sulcal CSF and ventricular CSF volumes of alcoholic and control brains. These studies also used a neuronal-specific marker to subtract out the glial cell images, providing the technology for separating neuronal and glial cell changes in the brains of alcoholics. Kulisevsky and colleagues (1992) reported pallidal hyperintensity from MRI evaluation of patients with chronic liver disease. Subsequent postmortem analyses of the brains of these individuals showed a positive correlation between the MRI changes and the presence of Alzheimer type II astrocytic changes (see chapter 11). These studies support the importance and feasibility of conducting imaging analyses of white matter and glial cells, and then relating the results to changes that have been associated with the consequences of alcohol abuse. Currently, glial cell images are usually "thrown away" or subtracted to give a clearer picture of neuronal imaging, just as researchers threw away

glial cells in culture in order to study neurons in the past. Perhaps in the future, the glial cell images will be clarified and attention will be focused on them, just as a new research thrust is being directed to study the many functions of glial cells in the brain.

CONCLUSION

Improved technology has created new opportunities to study the influence of alcohol on glial cells. Perhaps the new interest in research on glial cells will lead to investigations of the interactions of alcohol and neurosteroids in producing sex-specific reactions to alcohol, alcohol-induced alterations in brain development, gliosis, and memory and learning.

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