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# ROLE OF THE CELLULAR STRESS RESPONSE IN THE BIOGENESIS OF REDOX-ACTIVE ASTROCYTIC INCLUSIONS IN THE AGING NERVOUS SYSTEM

by

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# Cellular stress and the biogenesis of astrocytic inclusions

## ABSTRACT

In aging vertebrates, subpopulations of limbic and periventricular astrocytes accumulate peroxidase-positive cytoplasmic inclusions distinct from lipofuscin. In rodent brain, chronic estrogenization accelerates the appearance of this senescent glial phenotype. Identical inclusions are rapidly induced in primary neuroglial cultures by cysteamine exposure. Abnormal mitochondria replete with redox-active iron and other transition metals are the subcellular precursors of the inclusions *in situ* and in cysteamine-treated cultures. The objective of this thesis was to elucidate mechanisms responsible for the biogenesis of these glial inclusions in the aging nervous system.

We determined that the accumulation of astrocytic inclusions in cysteamine-treated rat glial cultures occurs in the context of an antecedent cellular stress response characterized by (i) the upregulation of heat shock proteins (HSP) 27, 72, 90, ubiquitin and heme oxygenase-1, and (ii) enhanced resistance of cysteamine-stressed astroglia to subsequent oxidative injury. Furthermore, multiple injections of cysteamine or estradiol valerate in adult male rats induced robust overexpression of stress proteins and an accretion of identical peroxidase-positive granules in GFAP-positive astroglia. Both *in situ* and in cysteamine-treated cultures, HSP27, ubiquitin, glucose-regulated protein 94 and to a lesser extent, HSP72 (but not HSP90 or αB-crystallin) exhibited immunolocalization to these astrocytic "stress" inclusions. We observed that exogenous $H_2O_2$ induces identical inclusions in cultured astroglia and that cysteamine-derived $H_2O_2$ promotes lipid peroxidation in isolated astroglial mitochondria. These data indicate that sustained oxidative stress may represent a "final common pathway" leading to the transformation of normal mitochondria to peroxidase-positive astrocytic inclusions in the aging nervous system.

The metal-dependent peroxidase activity of these glial inclusions has been shown to oxidize dopamine and other catechols to neurotoxic free radicals *in vitro*, implicating these cells in the pathogenesis of parkinsonism and other free radical-related

neurodegenerations. Since peroxidase-positive astroglia have been identified in aging human striatum, the findings presented here suggest that antioxidant therapy coupled with pharmacological inhibition of metal sequestration by "stressed" astroglial mitochondria may prove useful in the management of Parkinson's disease and other age-associated neurodegenerative afflictions.

## RÉSUMÉ

Chez les vertébrés vieillissants, des sous-populations d'astrocytes limbiques et periventriculaires accumulent des inclusions cytoplasmiques peroxydaso-positives différentes de la lipofuscine.

L'administration chronique d'oestrogène chez le rat accélère l'apparition du phénomène de vieillissement des cellules gliales. L'accumulation de semblables inclusions est également induite par l'ajout de cystéamine en milieu de cultures primaires d'astrocytes. Ces inclusions sont constituées de mitochondries altérées qui accumulent le fer oxydoréduit. Cette thèse a pour but de déterminer les mécanismes responsables de la genèse, dans le système nerveux vieillissant, de ces inclusions gliales.

Nos résultats montrent que l'accumulation de ces inclusions dans les astrocytes de rat en culture traités à la cystéamine est précédée d'une réponse de stress cellulaire caractérisée par une augmentation (i) des protéines de choc thermique ("Heat Shock Protein"; HSP) 27, 72, 90, de l'ubiquitine et de l'oxygenase-1 de l'héme et (ii) de la résistance des astrocytes traités à la cystéamine face à un stress oxydatif subséquent. De multiples injections de cystéamine ou de valérate d'estradiol chez le rat mâle adulte induit en outre une augmentation importante des "HSPs" et des inclusions cytoplasmiques peroxydaso-positives dans les astrocytes immunoréactifs à l'acide fibrillaire gliale ("GFAP"). Dans ces inclusions se retrouve une immunoréactivité à l'HSP27, à l'ubiquitine, à la protéine 94 régulée par le glucose et, à un moindre degré, l'HSP72. Cependant, aucune immunoréactivité à l'"HSP90" ni à l'αB-crystalline n'a été observée.

Nous observons également que l'administration de peroxyde d'hydrogène ($H_2O_2$) en milieu de culture d'astrocytes induit la formation d'inclusions identiques. Par ailleurs, lorsqu'est inhibé l'$H_2O_2$, résultant de l'auto-oxydation de la cystéamine, la peroxydation des lipides mitochondriaux est abolie. Ensemble, ces données indiquent qu'un stress oxydatif soutenu est responsable de la transformation des mitochondries normales en

inclusions peroxydaso-positives dans les astrocytes du système nerveux vieillissant.

L'activité peroxydante des inclusions, dépendante de métaux de transition, peut oxyder la dopamine et d'autres catéchols, entraînant ainsi la formation de radicaux libres potentiellement neurotoxiques. Puisque de telles inclusions sont observées au niveau du corps strié de personnes âgées, il se peut qu'elles soient impliquées dans certaines maladies neurodégénératives telles que le Parkinson. Il s'ensuit que des traitements élaborés à partir d'antioxydants combinés à des chélateurs de métaux réduisant leur incorporation au niveau des mitochondries d'astrocytes, pourraient s'avérer utiles dans le traitement de la maladie de Parkinson ou d'autres maladies neurodégénératives associées au vieillissement.

## PREFACE TO THE THESIS

The structure of this thesis conforms to the manuscript-based option permitted by McGill University. In accordance with the Guidelines for Thesis Preparation, the following text is to be reproduced in the preface of this thesis:

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In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the

authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis".**

The experimental work described in Chapters 2 through 4 have been published in manuscript form and that of Chapter 5 is presently in press. The material presented in the Appendix represents a component of a larger collaborative manuscript which is also in press. The following is a list of these publications.

**Chapter 2:** Mydlarski MB, Liang J-J, Schipper HM (1993) Role of the cellular stress response in the biogenesis of cysteamine-induced astrocytic inclusions in primary culture. J Neurochem 61:1755-1765.

**Chapter 3:** Schipper HM, Mydlarski MB, Wang X (1993) Cysteamine gliopathy *in situ*: a cellular stress model for the biogenesis of astrocytic inclusions. J Neuropathol Exp Neurol 52:399-410.

**Chapter 4:** Mydlarski MB, Schipper HM (1993) Stress protein co-localization to autofluorescent astrocytic inclusions in situ and in cysteamine-treated glial cultures. Brain Res 627:113-121.

**Chapter 5:** Mydlarski MB, Liberman A, Schipper HM (1995) Estrogen induction of glial heat shock proteins: implications for hypothalamic aging. Neurobiol Aging in press.

**Appendix:** Manganaro F, Chopra VS, Mydlarski MB, Bernatchez G, Schipper HM (1995) Redox perturbations in cysteamine-stressed astroglia: implications for inclusion formation and gliosis in the aging brain. Free Rad Biol Med in press.

The candidate helped design and performed all of the research presented in this thesis. Contributions of others to this work include the following: In Chapter 2, Jin-Jun Liang generated the Coomassie Blue-stained preparation shown in Fig. 2b. HPLC analysis was

performed by William Lubenskyi. In Chapters 3 and 5, Xudong Wang and Adrienne Liberman, respectively, assisted with the intracardiac perfusions of the animals and subsequent brain sectioning. In the Appendix, Fortunato Manganaro assisted with the cytochrome C oxidase assay for determination of mitochondrial purity.

## CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The work presented in this thesis constitutes an original contribution to the knowledge of the subcellular constituents of redox-active astroglial inclusions and the mechanisms governing their biogenesis. The results presented herein are published, or in press, and have been presented in platform or in poster format at the 1992 Altschul symposium on Biology and Pathology of Astrocyte-Neuron Interactions (Saskatoon), the 1993 meeting of the American Academy of Neurology (New York), the 1993 International Symposium on Reactive Oxygen Species (Bethesda), and the 1993 (Washington) and 1994 (Miami) meetings of the Society for Neuroscience. Specific contributions to original knowledge include the following:

1. Exposure of primary astrocyte cultures to CSH provokes a robust cellular stress response characterized by the overexpression of HSPs 27, 72, and 90, HO-1 (Chapter 2) and Ub (Chapter 4), long before increases in numbers of DAB-positive cytoplasmic granules become apparent at the LM level. Relative to control cultures, CSH-exposed astrocytes are more resistant to subsequent mechanical and oxidative injury, providing physiologic evidence of an antecedent stress response in the CSH-treated cells (Chapter 2). These results indicate that formation of peroxidase-positive astrocytic inclusions in CSH-treated cultures occurs in the context of a generalized cellular stress response.

2. Systemic administration of CSH to adult rats significantly enhances astroglial stress protein expression and accelerates the aging-related accumulation of DAB-positive astroglial inclusions in hippocampus, striatum, corpus callosum and periventricular brain regions. As *in vitro*, the biogenesis of peroxidase-positive astrocyte granules *in situ* occurs in the context of a cellular stress response (Chapter 3).

3. Repeated, systemic injections of CSH elicits robust astroglial hypertrophy (gliosis) in the corpus callosum, ventral hippocampal commissure and striatum of adult male rats. In contrast, there is no evidence of neuronal damage or demyelination in Nissl-, myelin-,

or silver-stained sections derived from rats subjected to long-term CSH exposure. Taken together, these results indicate that CSH provokes a unique "gliopathy" independent of antecedent or concomitant neuronal injury (Chapter 3).

4. EV treatment induces HSP expression followed by astrocyte granulation in estrogen receptor-rich brain regions (Chapter 5), supporting our contention that astrocytes must engage in a cellular stress response prior to accumulating redox-active cytoplasmic inclusions. To our knowledge, this study is the first to demonstrate estrogen-related induction of HSPs in astrocytes.

5. In contrast to CSH, the EV-induced enhancement of astrocyte granulation is not accompanied by any apparent increase in GFAP expression (Chapter 5). This finding indicates that accumulation of peroxidase-positive cytoplasmic inclusions may occur within the context of, or entirely independent of, classical astrocyte hypertrophy (gliosis).

6. Colocalization of Ub, HSP27, GRP94 and, to a lesser extent, HSP72 to the astroglial inclusions, both in CSH-treated primary cultures and in the intact adult rat brain, indicates that Gomori-positive astrocyte granules are "stress inclusions" (Chapter 4). Moreover, this finding greatly extends previous histochemical and morphological data underscoring the identical origin of CSH-induced astroglial inclusions *in vitro* and those which spontaneously accumulate in the aging periventricular brain.

7. CSH induces lipid peroxidation in isolated astroglial mitochondria (Appendix). This CSH effect is abolished by catalase coincubation, attesting to the role of CSH-derived $H_2O_2$ in the development of oxidative mitochondrial injury and subsequent astrocyte granulation. Direct administration of $H_2O_2$ to primary astrocyte cultures results in a robust accretion of peroxidase-positive cytoplasmic inclusions (Chapter 2). Taken together with the fact that both CSH and EV induce expression of redox-sensitive glial HSPs, these results indicate that intracellular oxidative stress may represent a "final common pathway" leading to the biogenesis of redox-active astroglial inclusions in the aging nervous system.

## ACKNOWLEDGMENTS

I wish to express my sincerest and most heartfelt gratitude to my Graduate Supervisor, Dr. Hyman M. Schipper, for his patience, guidance and support throughout the years of my graduate studies. I consider myself very fortunate to have had the opportunity to learn from Dr. Schipper who has transmitted to me a thirst for knowledge and scientific discovery.

I gratefully acknowledge Ms. Adina Mateescu and Mrs. Adrienne Liberman for their excellent technical assistance and Mr. René St-Jacques for help with translation of the abstract. I also thank all the past and present members of Dr. Schipper's laboratory and my colleagues at the Bloomfield Centre for Research in Aging for providing a stimulating work environment.

I also wish to extend my appreciation to the Gertrude and Charles Clark Fellowship of the Jewish General Hospital for generous financial support between 1992 and 1994.

*To my parents, for their love and support.*

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## TABLE OF COMMONLY USED ABBREVIATIONS

| | |
|---|---|
| 3VS: | third ventricular subependymal zone |
| AD: | Alzheimer's disease |
| ARC: | arcuate nucleus of the hypothalamus |
| CA: | corpora amylacea |
| CA1,2,3,4: | cornu ammonis, regions 1,2,3,4 |
| CAH: | chrome alum hematoxylin |
| CC: | corpus callosum |
| CNS: | central nervous system |
| CP: | caudate-putamen |
| CSH: | cysteamine; 2-mercaptoethylamine |
| DAB: | 3,3'-diaminobenzidine |
| DIV: | days in vitro |
| EM: | electron microscopy |
| ER: | endoplasmic reticulum |
| EV: | estradiol valerate |
| FITC: | fluorescein isothiocyanate |
| GFAP: | glial fibrillary acidic protein |
| GPA: | Gomori-positive astrocytes |
| GRP: | glucose regulated protein |
| GSH: | reduced glutathione |
| HD: | Huntington's disease |
| HO: | heme oxygenase |
| HSE: | heat shock element |
| HSF: | heat shock factor |
| HSP: | heat shock protein |
| LM: | light microscopy |
| MAO: | monoamine oxidase |
| MnSOD: | manganese superoxide dismutase |
| MPTP: | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |

| | |
|---|---|
| MS: | multiple sclerosis |
| PD: | Parkinson's disease |
| PE: | persistent estrus |
| peri-III: | peri-third ventricular region |
| Ub: | ubiquitin |

# CHAPTER 1

# INTRODUCTION

## 1. NEUROGLIA

The mammalian central nervous system exhibits remarkable cellular heterogeneity, being comprised of neurons, endothelial cells, meningeal cells, ependymocytes, choroid plexus epithelial cells, pineal cells and neuroglia. The term "neuroglia", meaning "nerve glue" was coined by Rudolf Virchow (1860) to describe what he considered an interneuronal connective-tissue-like matrix in which stellate and fusiform cells appeared suspended. This view remained popular until electron microscopic ultrastructural examination of fixed brain tissue revealed that the interstitial material was composed entirely of the cell bodies and processes of neuroglial cells (Peters et al., 1976). On the basis of light microscopic studies using metallic impregnation techniques developed by Camillo Golgi in the mid-1870's (Golgi, 1882-1885) and later, by Santiago Ramón y Cajal (1913, 1916) and Pio del Río Hortega (1919, 1921), the neuroglia became divided into two main categories, macroglia and microglia. The macroglia, like neurons, are ectoderm-derived and have been further subdivided into astrocytes (astroglia) and oligodendrocytes (oligodendroglia). The ectoderm-derived ependymal cells which line the ventricles and spinal canal, as well as the pituicytes of the neurohypophysis and the retinal Müller cells exhibit many features characteristic of astrocytes (Fedoroff and Vernadakis, 1986) and may be considered forms of neuroglia (Carpenter and Sutin, 1983). In contrast, the developmental origin of microglia, which are relatively few in number in healthy brain tissue, remains controversial. Most often these cells have been described as derivatives of blood-borne monocytes which seed the brain early in its ontogeny (Lasser, 1983; Ling et al., 1980). However, other data appear to suggest that they develop from neuroectoderm, akin to macroglia (Hao et al., 1991; Kitamura et al., 1984; Richardson et al., 1993).

## 2. ASTROCYTES

Examination of brain tissue sections prepared for light microscopy with metal stains reveals star-shaped cells sending numerous processes into the adjacent neural tissue. In 1895, von Lenhossek first used the term "astrocyte" to distinguish this particular subset of stellate neuroglia (Tower, 1988). Astrocytes, the most numerous cellular constituent of the brain, account for approximately 50% of the total brain neuroglial population, are 10 times more plentiful than neurons, and constitute from 20 to 50% of the volume in various brain regions (Hansson, 1988; Tower, 1988).

### 2.1 Embryogenesis of Astrocytes

In the embryo, the neural tube initially consists of proliferating, process-bearing columnar epithelial cells. Until embryonic day 12 (E12) in the rat, the telencephalic border is almost completely composed of these cells. At this point, the hemispheric outlines and lateral ventricles can be identified. The radially-arranged columnar cells project to the subpial surface from the ventricular surface thereby forming an early cerebrum to be used as a scaffolding for neuroblast migration. As postmitotic neuroblasts are produced, they detach from the adjacent columnar cells and begin migrating toward the pial surface to form the cortical plate. During this process, multipotential stem cells originating in the basal ventricular zone of the columnar epithelium give rise to glioblasts, the mitotic progenitor cells committed to form radial glia, ependymal cells and other neuroglial elements. At this stage, glioblasts lack intermediate filaments. The first astroglial precursors are characterized by the intermediate filament protein, vimentin and are designated proastroblasts. Houlé and Fedoroff (1983) determined that at E9, the radial processes of cells in the mouse neural tube contain vimentin. At E15, these radial glia co-express another intermediate filament protein, glial fibrillary acidic protein (GFAP) (Fedoroff et al., 1983) which is restricted to astrocytes (see below) and processes of ependymal tanycytes in the mature CNS. With continued CNS maturation, the radial glia accumulate intracellular organelles and lose vimentin in favor of GFAP. These cells, also

referred to as astroblasts, represent the penultimate astrocytic developmental stage, differentiating into mature astrocytes once neuronal migration has occurred.

2.2 Ultrastructural Features of Astrocytes *In Situ*

Classically, astrocytes have been divided into two subtypes, fibrous and protoplasmic, based on their morphology and localization to white or gray matter, respectively. Viewed with transmission electron microscopy (EM), both types of astrocytes exhibit pale cytoplasm, few profiles of rough endoplasmic reticulum, scattered free polysomes and Golgi complexes, glial fibrils, mitochondria, occasional opaque glycogen granules, and a spherical or ovoid euchromatic nucleus (King and Schwyn, 1970; Maxwell and Kruger, 1965; Palay, 1958; Peters et al., 1991). Both astrocytic types form perivascular end-feet on the surfaces of capillaries. There are certain ultrastructural features which tend to differentiate fibrous from protoplasmic astrocytes. Fibrous astrocytes contain many 8 to 9 nm-diameter filaments throughout the cytoplasm. Numerous processes stem from the fibrous cell body in a radial fashion to branch and form smaller, fibril-filled, cylindrical projections which interdigitate with the surrounding neuronal constituents. In protoplasmic astrocytes, there are fewer fibrils pervading the cytoplasm, and those that occur tend to be arranged in tightly-packed bundles. Moreover, the fibril content of protoplasmic astrocytes varies considerably among different brain regions. Certain protoplasmic astrocytes in the cerebellum (Palay and Chan-Palay, 1974) and cerebral cortex (Peters et al., 1991) contain very few fibrils, whereas others in the gray matter of the inferior olive are so replete with fibrils that certain authors regard them as fibrous astrocytes (Scheibel and Scheibel, 1955; Walberg, 1963). In contrast to the fibrous variety, the perikarya and freely-branching processes of protoplasmic astrocytes are irregularly-shaped and appear to be defined by the profiles of the dendrites and axons in the surrounding neuropil. Smaller protoplasmic processes tend to be sheet-like in appearance and often ensheath synapses. Notwithstanding the above, it has become increasingly clear that distinguishing between types of astrocytes solely on the basis of gliofibril content and residence within either white or gray matter tends to overlook the rich heterogeneity which exists for these cells.

## 2.3 Molecular Markers for Astrocytes

In the past few years, an astrocyte lineage classification scheme has evolved based on differential expression of cell type-specific markers. This process began in the early 1970's, when Eng et al. (1971) isolated GFAP, a major constituent of the intermediate gliofilaments, from fibrous astrocytes. Because GFAP is a specific marker for CNS astrocytes, detailed, molecular examination of *in vitro* preparations of GFAP-positive astrocytes became possible. Vimentin, another intermediate filament protein, is expressed in astrocytic progenitor cells (described above) and in "reactive" astrocytes under various pathological conditions in the mature CNS (Eng, 1988; Malhotra et al., 1990; Petito et al., 1988; Reier, 1986). Unlike GFAP, vimentin expression is not restricted to astroglia; it is an abundant cytoskeletal protein in fibroblasts and other non-glial cell types (Dahl et al., 1981). Astrocytes are also characterized by the presence of glial-specific enzymes such as glutamine synthetase (D'Amelio et al., 1990; Norenberg and Martinez-Hernandez, 1979; Tansey et al., 1991) and glutathione-S-transferase subtype Yb (Cammer et al. 1989), and the calcium-binding protein, S-100 (Boyes et al. 1986).

## 2.4 Types of Astrocytes *in Vitro*

Evidence for the existence of heterogeneous astrocyte lineages was obtained in 1983 by Raff and colleagues (Raff et al., 1983a). In cell cultures derived from rat optic nerve, two types of GFAP-positive astrocytes, with morphologic and antigenically distinct properties, were isolated. Astrocytes which characteristically appeared as stellate, process-bearing cells and were immunolabeled with the monoclonal antibody A2B5 were designated type 2 astrocytes. Type 1 astrocytes, which appeared as large, flat, polygonal, non-process-bearing cells, were A2B5-negative. Additionally, type 1, but not type 2, astrocytes exhibit immunoreactivity for the surface glycoprotein Ran-2 (Bartlett et al., 1980). Raff and coworkers (1983b) further demonstrated that type 2 astrocytes and oligodendrocytes, but not type 1 astrocytes, are derived *in vitro* from a common bipotential progenitor cell termed the O-2A (oligodendrocyte-type 2 astrocyte) progenitor. There are numerous antibody markers specific for both type 2 astrocytes and the O-2A progenitor which do not immunoreact with type 1 astrocytes (for reviews see Levison and McCarthy, 1991;

Miller et al., 1989).

2.5 The *in Situ* Counterparts of Type 1 and 2 Astrocytes

It has been difficult to determine whether astrocytes in the intact brain correspond to either of the two types observed in culture. Based on immunoreactivity for A2B5 in sections of rat optic nerve, Miller and Raff (1984) originally suggested that type 1 and type 2 astrocytes represent the *in vitro* equivalents of protoplasmic and fibrous astrocytes *in situ*, respectively. However, subsequent immunohistochemical studies have revealed that A2B5 is not entirely consistent in its labeling of type 2 rat optic nerve astrocytes and the authors no longer maintain this hypothesis (Miller et al., 1989). Others have since demonstrated that a subpopulation of type 1 astrocytes in the mouse are immunoreactive for the A2B5 antibody (Magoski et al., 1992). Whether the O-2A lineage classification is applicable to the *in vivo* situation also remains controversial (Cameron and Rakic, 1991). Evidence for GFAP-positive cells co-expressing ganglioside GD3, an oligodendrocyte-specific marker, has not been obtained *in situ*.

In culture, a variety of factors induce the transformation of astrocytes with type 1 antigenic and morphologic characteristics into stellate-shaped cells. These include co-culturing with neurons (Hatten, 1984), addition of hydrocortisone to culture medium (Morrison et al., 1985) and raising intracellular levels of cyclic AMP (Pollenz and McCarthy, 1986). Based on the above, some authors have inferred that both fibrous and protoplasmic astrocytes correspond to type 1 astrocytes *in vitro*, and that type 2 astrocytes may not have correlates in the developing nervous system. Type 2 astrocytes may merely represent the high degree of morphologic plasticity exhibited by astroglia in culture (Levison and Goldman, 1993). Similarly, the distinct morphologic and functional features characterizing various astroglial subpopulations *in situ* may be influenced, in part, by their location in the nervous system (Fedoroff and Vernadakis, 1986).

## 2.6 Adaptive Roles of Astrocytes

### 2.6.1 Structural and Trophic Support

As early as 1895, it had been suggested that astrocytes and other neuroglial elements which occupy the interneuronal space confer structural support to the CNS (Weigert, 1895). Indeed, the extensive network of intermediate filaments within astrocytes likely provide important mechanical support to the surrounding neuropil. Astrocytes, specifically the radial glia, play a key role in embryonic brain development by providing a scaffolding for neuronal migration (see Section 2.1) (reviewed in Rakic, 1981, 1984). In the mature nervous system, astrocytic processes ensheath synapses and other neuronal membranes and prevent neuronal perikarya and dendrites from receiving nonspecific afferent terminals (Peters et al., 1991). At the ultrastructural level, astrocytes in the CNS synthesize most of the protein component of the extracellular matrix and various cell adhesion molecules, including neural cell adhesion molecule, and the basement membrane glycoproteins, laminin and fibronectin, promoters of neurite outgrowth (Chiu et al., 1991; De Vellis et al., 1986; Peters et al., 1991; Prochiantz and Mallat, 1988; Shea et al., 1992). Astrocytes in culture are known to produce other neurotrophic and neurite outgrowth-stimulating factors including nerve growth factor and S-100, respectively (reviewed in Montgomery, 1994). In response to CNS injury, astrocytes proliferate, hypertrophy and increase their GFAP content and form a scar to replace the spaces previously filled by neuronal elements, a process called reactive gliosis (Reier, 1986). If synthesis of these growth-promoting and matrix-stabilizing substances occurs during reactive gliosis, astrocytes may well play a significant reparative role in the lesioned brain.

### 2.6.2 Angiogenesis and Induction of the Blood-Brain Barrier

Astrocytes may also provide angiogenic support to the injured brain. In studies of co-cultures containing astrocytes and endothelia, contact between the two cell types induced formation of capillary-like structures (Laterra and Goldstein, 1991; Laterra et al., 1990). In the past, some held that the physical construct of the blood-brain barrier (BBB) was

provided by the astrocytic end-feet investing the brain capillaries. It is now clear, however, that the properties of the BBB are conferred by capillary endothelial cells which form selectively permeable tight junctions. There is evidence that astrocytes may release factors which induce the formation of the endothelial cell tight junctions, thereby playing a primary role in the establishment and maintenance of the BBB (Beck et al., 1984, 1986; Brightman and Tao-Cheng, 1988; Janzer and Raff, 1987; Stewart and Coomber, 1986). Perivascular astrocytes may also enhance blood flow to critically active brain regions by releasing nitric oxide, a vascular relaxing factor, within discrete cerebrovascular beds (Murphy et al., 1993).

### 2.6.3 Housekeeping Functions and Neurotransmission

Astrocytes contribute to the brain's ionic homeostasis and prevent aberrant neuronal discharge by taking up potassium and other ions released into the extracellular space by active neurons (Berwald-Netter et al., 1986; Hertz, 1992; Ransom and Carlini 1986; Walz, 1989). Carbonic anhydrase has been localized to astrocytes (in addition to oligodendrocytes) where it plays a role in maintaining the pH of the brain (Cammer and Zhang, 1991; Jeffrey et al., 1991). Astroglia also participate in the modulation of neurotransmission, via the sequestration and metabolism of various transmitter molecules. The excitatory and, to a lesser extent, inhibitory neurotransmitters, glutamate and gamma-aminobutyric acid (GABA) respectively, are removed from the synaptic cleft by astrocytes where they are metabolized into glutamine. This amino acid can be used by neurons to resynthesize both GABA and glutamate. Furthermore, astrocyte-mediated uptake and metabolism of glutamate prevents buildup of this excitotoxin, which at high concentrations, is injurious to neurons (Rosenberg and Aizenman, 1989). Dopamine, a catecholamine, is also taken-up by astrocytes where it undergoes oxidative deamination by monoamine oxidase-B (MAO-B) (Levitt et al., 1982).

### 2.6.4 Neuroactive Substances and their Receptors

Astrocytes contain numerous receptors for neurotransmitters, biogenic amines and neuroactive peptides (reviewed in Krisch and Mentlein, 1994). These include receptors specific for ACh (M1), histamine, serotonin, glutamate, GABA (Kettenman et al., 1988), benzodiazepine (peripheral type; Bender and Hertz, 1988), norepinephrine, angiotensin II, substance P, prostaglandins E1 and E2, VIP, CRH, ACTH, the natriuretic peptides ANP, BNP and CNP, oxytocin, insulin, insulin-like growth factors I and II, enkephalin and somatostatin . In addition, astrocytes in various brain regions also possess receptors for the steroid hormone, estrogen (Langub and Watson, 1992).

Although the functional significance of this plethora of receptors is somewhat unclear, their activation, in most cases, enhances the rate of formation of cAMP and cGMP, or alternatively, increases hydrolysis of phosphatidylinositol (Krisch and Mentlein, 1994). In many cell types, including astrocytes, increased levels of cAMP initiates protein phosphorylation. Other receptor-mediated astrocytic actions that have been proposed include (i) altering of membrane channel proteins to modify the ionic properties and pH of the brain's microenvironment, (ii) enhanced glycogen breakdown, (iii) increased synthesis of neurotrophic substances, (iv) modification in the rates of neurotransmitter uptake and metabolism and (v) alteration of the degree to which neuronal processes are covered or contacted by astroglia (reviewed in Krisch and Mentlein, 1994 and Montgomery, 1994). With respect to the last proposal, it is known that changes in hormonal state associated with lactation and estrous cyclicity induce astroglial process remodelling and GFAP redistribution (Hatton et al., 1984; Salm et al., 1985).

mRNA transcripts for a number of neuropeptides as well as the synthesis of a wide spectrum of neuroactive compounds, including adenine nucleotides, angiotensinogen, eicosanoids, nitric oxide, enkephalin peptides, GABA, glutamate, insulin-like growth factors I and II, nerve growth factor, somatostatin, taurine, and thyroid hormone have been demonstrated in astrocytes, both *in vitro* and *in situ* (reviewed in Martin, 1992). In

addition, the release of many of these substances by astroglia has been demonstrated in primary culture (*ibid*). Taken together, these findings indicate that astrocytes may participate in a host of bi-directional, intercellular, chemically-mediated interactions within the CNS. Indeed, some have postulated that certain astroglial receptors may mediate the generation of intracellular calcium waves as forms of astro-astrocytic (Charles et al., 1992; Finkbeiner, 1992) and astro-neuronal communication (Nedergaard, 1994; Smith, 1994).

### 2.6.5 Protective Roles

Astrocyte-mediated protection of the CNS against excitotoxin buildup has been discussed (see above). Astroglia also appear to play a central role in ammonia detoxification as surmised by the localization of glutamine synthetase, which is virtually restricted to these cells in the brain (Benjamin and Quastel, 1975; Martinez-Hernandez et al., 1977). Astrocytes may sequester and prevent the toxic accumulation of lead in the neuropil (Holtzman et al., 1984; Tiffany-Castiglioni et al., 1989), and by virtue of the presence of metal-binding proteins such as metallothionein, may serve as "sinks" for a number of heavy metals (Young et al., 1991). Astrocyte-derived glutathione may also subserve an important anti-oxidant defense mechanism in the CNS (Pileblad et al., 1991).

### 2.6.6 Immune Functions

Astrocytes in culture act as antigen-presenting cells to immunocytes (Fontana et al., 1984), although this role has not been definitively established *in situ* (Eddleston and Mucke, 1993). Whereas human astrocytes appear to present antigens to T lymphocytes, they do not appear capable of inducing further antigen-dependent T-cell proliferation (Weber et al., 1994). Nevertheless, following exposure to certain viruses, interferon (IFN)-$\gamma$, or IFN-$\gamma$ together with tumor necrosis factor (TNF)$\alpha$, cultured astrocytes can express class I and/or II of the major histocompatibility complex (MHC) (Liu et al., 1989; Richt and Stitz, 1992; Takiguchi and Frelinger, 1986; Vidovic et al., 1990). Similarly, astrocytes can be induced to express and secrete various inflammatory mediators including the

cytokines, interleukin (IL)-1, IL-3 and IL-6, IFN α and β, and TNF, as well as prostaglandins and intercellular adhesion molecule (ICAM)-1, (Fierz and Fontana, 1986; Fontana et al., 1988; Frei et al., 1988; Lieberman et al., 1989; Satoh et al., 1991; Sawada et al., 1989, 1992). Expression of ICAM-1 may serve to attract lymphocytes into the CNS, or it may act as a ligand promoting astrocyte-lymphocyte association (Satoh et al., 1991). However, as discussed in the following section, the immunologic roles ascribed to astrocytes may also predispose the CNS to immunopathologies such as multiple sclerosis (MS) and animal models of this demyelinating disorder (Fontana et al., 1984).

## 2.7 Dystrophic Effects of Astrocytes

### 2.7.1 Neoplasia, Ionic Imbalance and Scarring

Disrupted control of astrocyte proliferation and/or apoptosis may lead to neoplastic transformation and malignant behavior. Indeed, astrocytomas (gliomas) are among the most common and clinically aggressive of the human CNS neoplasms. Astroglia may also play an important role in the pathogenesis of epilepsy. Pollen and Trachtenberg (1970) have proposed that disrupted astrocyte buffering of potassium may lower the neuronal threshold for propagation of action potentials, resulting in the hyperexcitable epileptic state. Furthermore, in various forms of epilepsy, glial scarring is prevalent and may contribute to the pathogenesis of these disorders by serving as a nidus for epileptogenic discharges (Grisar et al., 1988). Whether glial scarring is an adaptive or dystrophic response to CNS injury remains controversial (reviewed in Reier et al., 1989). Although, as previously mentioned, reactive gliosis may serve to replace lost neuronal tissue and prevent infiltration of non-neural elements into the necrotic area, glial scarring may also impede axonal regrowth (Reier, 1986; Reier et al., 1983). In an *in situ* model for glial scarring, reactive astrocytes express molecules which have been shown to hinder the outgrowth of neurites in culture (McKeon et al., 1991). However, it has been suggested that other cellular constituents of glial scars, including microglia, oligodendroglia and immune cells, may be responsible for the failure of axonal regeneration following CNS injury (reviewed in Hatten et al., 1991).

### 2.7.2 Immunosensitization

As discussed above, it is unclear whether astrocytes in the intact CNS function as major antigen-presenting cells. However, there is evidence that in lesions characteristic of MS (demyelinating plaques) and in experimental allergic encephalomyelitis (EAE), an animal model of MS, astroglia express MHC class II antigens (Fierz and Fontana, 1986; Frohman et al., 1989; Matsumoto et al., 1992). In other studies of experimentally-induced demyelination, astrocytes from susceptible, but not demyelination-resistant, mouse and rat strains readily express MHC II antigens in culture (Borrow and Nash, 1992; Massa et al., 1987). Additionally, reactive astrocytes in MS plaques express the cytokine TNFα (Hofman et al., 1989; Selmaj et al., 1991a) which is cytotoxic to the myelin-producing oligodendroglia *in vitro* (Robbins et al., 1987; Selmaj and Raine, 1988). At later stages of the disease, astrocytes (and microglial cells) participate in phagocytosis of myelin units (Raine, 1982).

### 2.7.3 Excitotoxicity and Aluminum-related Neural Injury

Overexposure to glutamate, aspartate and certain sulfur-containing amino acids, results in excitotoxic neuronal injury. Normally, astrocytes sequester excess glutamate released at excitatory synapses within the CNS. However, under ischemic conditions, the astrocytic glutamate transporter protein may release glutamate back to the extracellular space (Nicholls and Attwell, 1990) and thereby contribute to the neuronotoxicity observed in energy deficient states (Choi and Rothman, 1990; Meldrum et al., 1991). Abnormal astrocytic uptake of glutamate has also been implicated in Alzheimer's disease (AD) (reviewed in Hertz, 1989) and Huntington's disease (HD) (Perry and Hansen, 1990). In the mammalian CNS, oligodendroglial cytotoxicity and excitotoxin-related neuronal injury may be mediated, in part, by nitric oxide (Dawson et al., 1991, 1993; Park et al., 1994). Nitric oxide is a gaseous intra- and extracellular messenger which has been implicated in the modulation of long-term potentiation (Schuman and Madison, 1991). This neuroactive substance is constitutively present in cultured astrocytes and is rapidly inducible in these cells by various treatments (Murphy et al., 1993; Park et al., 1994; Galea et al., 1992).

In cultured astroglia, induction of nitric oxide synthesis engenders deficiencies of mitochondrial oxidative phosphorylation (Bolaños et al., 1994). However, astroglia exposed to inhibitors of the mitochondrial respiratory complex do not exhibit increased cytotoxicity, apparently because of their ability to maintain their energy requirements via enhanced anaerobic glycolysis (*ibid*). Since neurons and oligodendroglia appear to be more dependent on aerobic respiration for their survival, production and release of nitric oxide by astroglia, if corroborated *in situ* (Murphy et al., 1990; Agulló and García, 1991), could further implicate these cells in ischemic brain injury, demyelinating disorders and a host of other neuropathologies (Kirino, 1982; Galea et al., 1992). Astrocytes may also play an important role in the pathophysiology of HD. Astrocytes contain the enzymes 3-hydroxyanthranilic acid oxygenase and quinolinic acid phosphoribosyltransferase required for synthesis and degradation of quinolinic acid, respectively (Schwarcz et al., 1988; Selmaj et al., 1991b). Quinolinic acid is a metabolic derivative of tryptophan which has been shown to be toxic to certain glutamate-receptor-positive neurons. Activity of 3-hydroxyanthranilic acid oxygenase is augmented in the striatum of patients with HD and may promote overproduction of quinolinic acid with preferential killing of neurons affected in this neurodegenerative disorder (Schwarcz et al., 1988; Selmaj et al., 1991b; Whetsell et al., 1988).

Accumulation of aluminum in the CNS may be associated with the pathogenesis of certain neurological disorders including AD. Astrocytes appear to play an important role in aluminum-related neuropathology. Exposure to aluminum induces robust astrogliosis *in situ* (Harris, 1975; Lewis et al., 1977; Winkelman and Ricanati, 1986), cytopathologic changes in cultured astrocytes (Norenberg et al., 1989), and the release of neuroactive compounds including taurine, adenosine and glutamate. The latter compounds may, in turn, evoke derangements in neurotransmission and, in the case of glutamate, excitotoxic neuronal injury (Albrecht et al, 1991). The ability of astrocytes to protect neurons from glutamate cytotoxicity in culture has been shown to be abrogated after exposure of the former to aluminum (Sass et al., 1993).

### 2.7.4 Metabolism of β-Amyloid Protein

Astrocytes may be involved in additional aspects of AD. Reactive astrocytes *in situ* express β-amyloid precursor protein following excitotoxic injury to neurons (Siman et al., 1989). The expression of calcium-activated proteases has been noted in reactive astrocytes (reviewed in Eddleston and Mucke, 1993) and cathepsin D, a lysosomal protease, is increased in astrocytes of the AD brain (Diedrich et al., 1991). Conceivably, reactive astrocytes in the injured or diseased brain may contribute to aberrant degradation of the protease-sensitive amyloid precursor protein with ensuing aggregation of β-amyloid in senile plaques (Razzaboni et al., 1992).

### 2.7.5 MPTP-induced Neurodegeneration

Astrocytes have been implicated in the biotransformation of pro-toxins and other xenobiotics into potent neurotoxins. Perhaps the best known example is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a byproduct formed in the synthesis of meperidine-like narcotics. Astrocytes contain the enzyme, monoamine oxidase B (MAO-B), which is responsible for the oxidation of MPTP to the neurotoxin 1-methyl-4-phenylpyridinium ion ($MPP^+$) (Brooks et al., 1989; Ransom, 1988; Ransom et al., 1987). Upon its release from astrocytes, $MPP^+$ is actively taken up by nigrostriatal (dopaminergic) neurons (Javitch and Snyder, 1984) where it inhibits complex I of the respiratory chain in mitochondria (Nicklas et al., 1985; Ramsay and Singer, 1986). The latter results in bioenergetic failure which may promote or exacerbate nigrostriatal degeneration and parkinsonism in humans and experimental animals (Beal, 1992; Chan et al., 1991; Di Monte and Smith, 1988)

### 2.7.6 Hepatic Encephalopathy

As discussed above, astrocyte dysfunction resulting from initial neurotoxic insults may promote further ("secondary") CNS pathology. In some metabolic encephalopathies,

astrocytes may exhibit early and specific cytopathological changes which suggest that these cells may be the primary targets of the disease process. In hyperammonemic states such as hepatic encephalopathy (HE), astrocytes undergo an "Alzheimer type II response" characterized by the appearance of swollen, pale nuclei, glycogen deposition and peripheral chromatin, prominent nucleoli and scant, faint cytoplasm virtually devoid of GFAP immunoreactivity (Norenberg, 1986; Norenberg, 1988; Sobel et al., 1981). Whereas increased levels of GFAP occurs in many pathological conditions (Bignami et al., 1980; Eng and DeArmond, 1983), loss of GFAP is uncommon and relatively specific to HE (Kretzschmar et al., 1985; Sobel et al., 1981) and can be reproduced in culture by treating astroglia with ammonium chloride (Norenberg et al., 1990). In experimental models of hyperammonemia, increased glutamine synthetase immunoreactivity has been demonstrated in brain regions exhibiting Alzheimer type II-like astrocytes (Yamamoto et al.,1989). Upregulation of this enzyme may serve to compensate for increased levels of ammonia in the brain. However, chronic hyperammonemia, as occurs in protracted HE, may overload this adaptive astroglial response, engender bioenergetic failure (Norenberg, 1994), and interfere with crucial astrocyte housekeeping roles including neurotransmitter uptake, ionic, osmotic and acid/base regulation (reviewed in Norenberg et al., 1986) as well as glutamate metabolism (Rao and Murthy, 1992). Thus, although astrocytes may represent the initial and unique site of cytotoxicity in certain metabolic disorders such as HE, continued exposure to the causative toxin results in impaired astrocyte function which, in turn, promotes aberrant neuronal activity and the development of clinical symptomatology (Norenberg, 1994).

### 2.7.7 Aging

In contrast to fetal or neonatal astroglia, mature astrocytes are not permissive substrates for neuritic regeneration in culture and may similarly impede axonal regrowth in the injured, adult brain (Tiveron et al., 1992; Smith et al., 1990). In various mammalian species, including humans, astrocyte hypertrophy and possibly hyperplasia (reactive gliosis) is a characteristic of normal brain aging (Bronson et al., 1993; Coleman and

Flood, 1987; Flood and Coleman, 1988; Shimada et al., 1992). In the normal aging brain and in AD, increased MAO content has also been documented (Adolfsson et al., 1980; Fowler et al., 1980; Oreland and Gottfries, 1986; Robinson, 1975; Sparks et al., 1991) and, in the latter, appears to localize to reactive astrocytes (Saura et al., 1994). In an *in situ* model using basic fibroblast growth factor to induce astrogliosis, augmented MAO-B (and MAO-A) activity promoted the accelerated catabolism of dopamine (Wang et al., 1994). In addition to disrupting monoaminergic neurotransmission, enhanced MAO-B-mediated oxidative deamination of dopamine generates $H_2O_2$ and other cytotoxic oxyradicals (Spina and Cohen, 1989) which have been implicated in the pathogenesis of Parkinson's disease (PD) and other aging-related neurodegenerative conditions (Cohen, 1986; Halliwell, 1989). Thus, age-associated astrogliosis with concomitant increases in MAO-B activity may predispose the senescent nervous system to free radical-related injury as well as prevent the establishment of appropriate regenerative processes.

In various regions of the vertebrate brain, "Gomori-positive" astrocytes replete with peroxidase-positive cytoplasmic inclusions accumulate as a consequence of aging and may be associated with the development of free radical-related neurodegenerative disorders. The work described in this thesis centres on the role of the cellular stress response in the formation of these peroxidase-positive astroglial inclusions. A detailed account of this astrocyte subpopulation is presented in the following section.

## 3. THE GOMORI-POSITIVE GLIAL SYSTEM *IN SITU*

A subpopulation of neuroglia referred to as peroxidase-positive, or Gomori-positive astrocytes (GPA) has been described in limbic and periventricular brain regions of all vertebrates thus far examined, including frogs (Srebro et al., 1975), rats (Wislocki and Leduc, 1954), mice (Srebro and Lach, 1987; Srebro et al., 1971), dogs, (Diepen et al., 1954), cats (*ibid*), monkeys (Creswell et al., 1964) as well as humans (Schipper et al., 1988; Schipper, 1991; Schipper and Cissé, 1995; Srebro and Macinska, 1973). The presence of discrete cytoplasmic inclusions (described below) distinguishes these cells from the far more numerous Gomori-negative astroglia among which the former reside. Delineation of the unique biological and histochemical properties of these cells and determination of their role(s) in brain aging and neurodegeneration is a major focus of Dr. Schipper's laboratory.

### 3.1 Tinctorial and Histochemical Features

GPA were initially identified by light microscopy on the basis of histochemical staining of their cytoplasmic inclusions. The granules exhibit metachromasia in toluidine blue-stained sections (Creswell et al., 1964; Schipper, 1982) and have an affinity for the Gomori stains, aldehyde fuchsin (AF) and chrome alum hematoxylin (CAH) (Koritsánszky et al., 1967). Gomori stains were originally used to identify pancreatic β cells (Gomori, 1939). The high sulfur content of β cells (pro-insulin disulfide bonds) is thought to account for their Gomoriphilia (Srebro, 1969; Wagner and Pilgrim, 1978). Histochemical and microprobe assessments have confirmed that GPA granules are rich in sulfhydryl groups (Srebro and Cichocki, 1971; Wagner and Pilgrim, 1978). However, it soon became apparent that an affinity for Gomori stains is not restricted to the astrocytic inclusions. AF, and in some cases CAH, complexes with sulfuric acid esters, and with sulfonic, aldehyde, carboxyl and phosphate groups of neuronal and other non-astrocytic substrates (Barden, 1978; 1984; Creswell et al., 1964; Noda, 1959; Wislocki and Leduc, 1954). Furthermore, AF stains oxidized neuronal lipofuscin, neuromelanin (Barden,

1978), neuronal dense bodies, neurosecretory material of the hypothalamo-hypophyseal system (Creswell et al., 1964) and corpora amylacea (Barden, 1978). Based on these heterogeneous tinctorial properties, the Gomoriphilic astrocyte granules were initially considered analogous to the aging pigment, lipofuscin (Röhlich et al., 1965), or as phagocytosed neurosecretion (Löfgreen, 1961; Noda, 1959). However, these views were discounted in the face of studies demonstrating that: (i) Few GPA granules are present in the supraoptic nucleus where neurons replete with sulfur-rich neurosecretory material (neurophysins) abound (Cottle and Silver, 1970). (ii) In unstained sections viewed under light microscopy, there is an absence of visible pigment characteristic of lipofuscin (Goldgefter, 1976). (iii) Under transmission EM, lipofuscin exhibits heterogeneous electron-lucent and dense regions in osmicated preparations (Sohal and Wolfe, 1986), whereas osmicated GPA granules tend to be more uniformly electron-dense (Brawer and Sonnenschein, 1975; Srebro and Lach, 1987). (iv) GPA granules are not labeled with the conventional histochemical lipid markers Sudan Black, Sudan III (Goldgefter 1976) or oil red O (Schipper et al., 1990c). (v) GPA inclusions emit an unusual orange-red autofluorescence between 610 and 640 nm consistent with the presence of porphyrins (Cottle and Silver, 1970; Goldgefter et al., 1980) or oxidized flavoproteins (Duchen and Biscoe, 1992; Kohler and Fromter, 1985) and distinct from the green or yellow-orange autofluorescence (400-545 nm) typically emitted by lipofuscins (Udenfriend, 1962; Goldgefter et al., 1980).

Histochemical studies using Perl's method have revealed the presence of iron in these astrocytic inclusions (Hill and Switzer, 1984; Srebro and Macinska, 1972). Furthermore, the inclusions stain intensely with diaminobenzidene (DAB), a marker of endogenous peroxidase activity (Srebro, 1971; Keefer and Christ, 1976; Schipper et al., 1990a). However, the endogenous peroxidase activity does not appear to be mediated by enzymes such as catalase or glutathione peroxidase because DAB oxidation occurred: (i) in the presence of the catalase inhibitor, aminotriazole, (ii) after tissue boiling, and (iii) under both highly acidic and alkalinic conditions (Kumamoto, 1981; Schipper et al., 1988; Schipper et al., 1990a). These findings indicate that a non-enzymatic (or pseudo)

peroxidase reaction is responsible for DAB oxidation in these cells (Goldfischer et al., 1966).

3.2 Ultrastructural Features

Gomori-positive astrocytes, in common with other astroglia, contain rounded or ovoid, euchromatic nuclei, electron-lucent cytoplasm and bundles of gliofilaments (Brawer and Sonnenschein, 1975; Brawer et al., 1978; Casanueva et al., 1982; Srebro and Lach, 1987). Under transmission electron microscopy, the distinctive cytoplasmic inclusions range from 0.3 to 1.5 $\mu$m in diameter, appear round to angular in shape and, in contrast to lipofuscin which exhibits electron lucent and dense regions, are strongly and homogeneously osmiophilic (Brawer and Sonnenschein, 1975; Brawer et al., 1978). The inclusions are often bounded by delimiting membranes (Srebro and Lach, 1987) and may abut small microtubule-containing cisternal elements (Brawer et al., 1994a) and electron-dense profiles of endoplasmic reticulum (ER) (Brawer and Sonnenschein, 1975; Srebro and Lach, 1987).

3.3 Topography of the Gomori-positive Astroglia

Gomori-positive astrocytes are relatively abundant in the subependymal zone throughout the neuraxis and in blood-brain barrier-deficient regions including all of the circumventricular organs (Creswell et al., 1964; Goldgefter, 1976; Keefer and Christ, 1976; Srebro and Cichoki, 1971). In the rat, where the topography of these cells is best known, GPA are dispersed throughout the telencephalon, with relatively high concentrations of these cells in the olfactory bulb, the caudate nucleus adjacent to the lateral ventricle, and the hippocampus (Schipper and Mateescu-Cantuniari, 1991; Keefer and Christ, 1976). A highly stratified topography of GPA within the dorsal hippocampus was delineated in a study employing dual histochemical/immunohistochemical labeling to identify peroxidase-positive (DAB) granules within GFAP-positive astrocytes. In this region, numerous DAB-positive astrocytes are confined to the hilus of the dentate gyrus

and the lacunosum molecular layer and stratum oriens of regions CA1 and CA2 adjacent to the corpus callosum. Other hippocampal layers exhibit GFAP-positive astrocytes with little or no detectable DAB reactivity and include the granule cell and inner molecular layers of the dentate gyrus, and the pyramidal cell layer and stratum radiatum of CA1 and CA2 (Schipper and Mateescu-Cantuniari, 1991). In the diencephalon, GPA are prominent in the basal aspect of the hypothalamus, notably in the arcuate nucleus and ventral premammillary area, in the third ventricular subependymal zone, and the organum vasculosum of the lamina terminalis (OVLT). In the mesencephalon, GPA are frequently observed in the periaqueductal gray, dorsal to the raphe nuclear complex and in the superficial aspect of the superior colliculi. The area postrema in the rhombencephalon contains numerous Gomori-positive glia and, to a lesser extent, these cells are also present in the nucleus gracilis, dorsal motor nucleus of the vagus, locus coeruleus, olivary nuclear complex and lateral cerebellum. Scattered GPA have also been identified in Rexed's laminae 1 and 2 of the spinal cord (Keefer and Christ, 1976).

In serial sections derived from adult human autopsy material, topographically superimposable CAH-positive and DAB-positive astrocytes are found throughout the periventricular forebrain, in the optic tract and globus pallidus, and within the diencephalon (Schipper et al., 1988; Schipper, 1991). In the latter, these cells appear to be concentrated in the OVLT, infundibular region, and capsule of the mammillary body. As in other vertebrates, the endogenous peroxidase activity in human astrocytes resists extremes of pH, tissue preheating and aminotriazole, and is therefore non-enzymatic in nature (*ibid*).

### 3.4 Putative Biological Functions of Gomori-positive Astroglia

Based on the premise that abundant porphyrins and heme respectively mediate orange-red autofluorescence and non-enzymatic peroxidase activity in GPA, several laboratories have ascribed the following biological functions to these cells: (i) supply of heme to neurons for the synthesis of heme-containing proteins such as the cytochromes and catalase (De

Matteis et al., 1981; Young et al., 1990), (ii) provision of iron required for myelination (Becker and Wolfgram, 1978), (iii) photostimulation of sexual cyclicity (Fox, 1953), and (iv) stimulation of neurite outgrowth (Schipper, 1991) analogous to the effects observed in cultures of mouse neuroblastoma cells following hemin and protoporphyrin IX administration (Ishii and Maniatis, 1978). As discussed in the Conclusion of this thesis, these putative functions have been cast into doubt given recent evidence from Dr. Schipper's laboratory that (i) non-heme iron mediates non-enzymatic peroxidase activity in Gomori glia, and (ii) porphyrin biosynthesis is *suppressed* in these cells and cannot, therefore, account for their autofluorescent properties (Wang et al., 1995). In 1971, Srebro conjectured that the glial peroxidase activity may subserve an antioxidant function by degrading cytotoxic lipoperoxides and thereby confer protection to periventricular brain regions (Srebro, 1971). However, as described below (Section 5), direct experimental evidence indicates that the non-enzymatic (iron-mediated) peroxidase activity in these cells is more likely to *generate* free radicals than to quench them.

## 3.5 Modulation of the Gomori-positive Periventricular Glial System

### 3.5.1 Aging

Gomori-positive astroglia first appear at the end of the first week in the post-natal rabbit hypothalamus (Srebro and Slebodzinski, 1966) and in the fourth week in rats and mice (Koritsánszky et al., 1967; Maksymowicz and Srebro, 1972). Over time, numbers of Gomori-positive astrocytes and their cytoplasmic inclusions accumulate in the hypothalamic arcuate nucleus and in other periventricular and limbic brain regions. In rats, a significant increase in astrocytic granules occurs between 6 and 14 months of age, and between 4 and 13 months in mice (Schipper et al., 1981). Early ovariectomy of female rats arrests the senescence-dependent proliferation of Gomori granules in the arcuate nucleus, indicating that sex hormones may influence the accumulation of these astrocytic inclusions (Schipper et al., 1981; described in Sex Hormones). In humans, numbers of Gomori-positive granules increase in the basal ganglia and throughout the periventricular forebrain between the ages of 3 and 69 years (Schipper et al., 1988). As

described above, histochemical and morphological studies have demonstrated that the Gomori-positive granules are a unique form of glial inclusion constitutively different from the aging pigment, lipofuscin.

### 3.5.2 X-Irradiation

A marked increase in the numbers of Gomori-positive glia occurs within the rat arcuate nucleus following exposure to head X-irradiation (Srebro, 1971). The author of this study suggested that the accumulation of peroxidase-positive granules in glia inhabiting periventricular brain regions may contribute to the protection of blood-brain barrier-deficient regions by degrading cytotoxic, blood-borne substances (Srebro, 1971). However, recent studies have demonstrated that GPA, rather than conferring protection to the surrounding neuropil, may instead promote the generation of neurotoxic free radical species (see Section 5).

### 3.5.3 Sex Hormones

In adult female rodents, an anovulatory syndrome known as the persistent estrus (PE) state, characterized by the development of polycystic ovaries and persistent vaginal cornification, spontaneously develops with aging (Aschheim, 1976; Schipper et al., 1981). In senescent rodents exhibiting PE there is a discrete accumulation of GPA granules within the hypothalamic arcuate nucleus, a neuroendocrine locus implicated in the regulation of gonadotropin secretion (Schipper et al., 1981). The onset of PE can be hastened by continuous light exposure (Singh and Greenwald 1967) or by administration of estradiol valerate (EV) (Brawer et al., 1978, 1980). In these animals, constant light treatment (Brawer et al., 1980) and multiple (Brawer and Sonnenschein, 1975) or single (Brawer et al., 1978; Schipper et al., 1990a) intramuscular injections of 2mg EV greatly accelerate the age-related accumulation of peroxidase-positive astrocyte granules in the arcuate nucleus, a region rich in estrogen receptors (Brawer and Sonnenschein, 1975; Brawer et al., 1980, 1983; Casanueva et al., 1982; Schipper et al., 1990a). In the arcuate

nucleus of hyperestrogenized rats, there is an average 60% reduction in the total number of β-endorphin-immunoreactive neurons. In contrast, other hypothalamic neuronal populations surveyed appeared unaffected by EV treatment, indicating that the loss of β-endorphinergic neurons in this region is relatively selective (Desjardins et al., 1993). At the ultrastructural level, this estrogenization promotes the collapse of dendritic profiles, degeneration of axon terminals, formation of myelin figures and the accumulation of phagocytic microglial cells within the arcuate nucleus (Brawer and Sonnenschein, 1975; Brawer et al., 1978; Brawer et al., 1980; Olmos et al., 1989; Pérez et al., 1990; Naftolin et al., 1990). These neurodegenerative changes are often seen in close proximity to GPA exhibiting a massive proliferation of peroxidase-positive inclusions. Conversely, this EV- and light-induced astrocytic reaction and the accumulation of reactive microglia in the arcuate nucleus can be abolished by early ovariectomy. These results suggest that an ovarian derivative, possibly estrogen, and not the administration of EV or constant illumination *per se*, is directly responsible for the arcuate lesion in PE rats (Brawer et al., 1980). Repeated monthly injections of EV to male rats (Brawer et al., 1980) or chronic release of high-physiologic levels of unconjugated estradiol via Silastic implants in gonadectomized females (Brawer et al., 1983) produce identical histopathological changes in the arcuate nucleus. In normal, aging, female rats and mice exhibiting PE, the progressive increase in GPA granules within the arcuate nucleus can be blocked by early gonadectomy (Schipper et al., 1981). Taken together, these observations indicate that aging of the neuroendocrine hypothalamus may be hastened by abnormal patterns of circulating ovarian estradiol resulting from EV or constant light exposure (Schipper et al., 1981; Schipper 1993). Conceivably, the progressive estrogen-induced neurodegeneration in the arcuate nucleus affects the integrity of gonadotropin-regulating neural circuitry in this brain region (Brawer et al., 1980), and anovulatory sterility arises once the synaptic connections therein become disrupted. This is consistent with the fact that (i) surgical deafferentation of the anterior hypothalamus arrests sexual cyclicity and induces the PE state (Blake et al., 1972, 1973; Halasz, 1969), and (ii) early ovariectomy (estrogen withdrawal) permits sexual cyclicity to be maintained in extremely old rodents (at ages normally incompatible with estrous cyclicity) which have been implanted with young or

old ovarian grafts (Aschheim, 1976).

Interestingly, Landfield and co-workers have demonstrated that corticosterone administration enhances, whereas adrenalectomy diminishes, age-associated gliosis in glucocorticoid receptor-rich regions of the rodent hippocampus (Landfield, 1978; Landfield et al., 1978). Thus, during aging, several classes of steroid hormones appear to have the potential to render dysfunctional the neural circuitry subserving their own regulation.

### *3.5.3.1 The Specificity of the Gomori Astroglial Response to Estrogen*

Estrogen, but not testosterone or progesterone, stimulates peroxidase activity in a variety of sex hormone-responsive tissues including rat vagina, cervix and uterus as well as in estrogen-dependent mammary carcinoma (Anderson et al., 1975; Lyttle and DeSombre, 1977). Similarly, Schipper and co-workers demonstrated that the biogenesis of peroxidase- (DAB-) positive cytoplasmic granules in arcuate astrocytes is stimulated by the administration of estradiol (Schipper et al., 1990a), but not by androgens (Brawer et al., 1983) or progestins (Schipper et al., 1990b). Based on these observations, Schipper and co-authors suggested that Gomori-positive astroglia may represent a novel estrogen "target tissue" (Schipper et al., 1990a). This concept is supported by studies demonstrating the existence of estrogen receptor-positive astrocytes in periventricular and preoptic brain areas of humans, guinea pigs and rats (Lee 1982; Stopa et al., 1989; Langub and Watson, 1992).

As discussed earlier, Gomori astrocyte granules emit distinct autofluorescence in the porphyrin range, suggesting that heme or some other metalloporphyrin may be responsible for the peroxidase activity in these inclusions. Estrogen is known to induce δ-aminolevulinic acid synthase, the rate-limiting enzyme in heme biosynthesis, in various tissues including liver (Proud et al., 1989). Moreover, in rodents, estrogen promotes the sequestration of porphyrins within discrete cytoplasmic inclusions of the Harderian gland

(Payne et al., 1979). Accordingly, in 1990, Schipper et al. hypothesized that hyperestrogenization results in sustained induction of δ-aminolevulinic acid synthase in periventricular astrocytes, leading to the formation of peroxidase-positive, metalloporphyrin-rich cytoplasmic inclusions (Schipper et al., 1990a). However, as discussed above (Section 3.4), it is now clear that some alternative mechanism must mediate this estrogenic effect given recent, direct evidence from Schipper's lab that (i) porphyrin-heme biosynthesis is *suppressed* in Gomori-positive astrocytes and (ii) *non-heme* iron is responsible for the peroxidase activity in these cells (Wang et al., 1995).

### *3.5.3.2 The Free Radical Hypothesis of Estrogen-related Hypothalamic Damage*

The non-enzymatic, peroxidase activity characteristic of GPA inclusions may contribute, in part, to estrogen-related neurodegeneration observed in the arcuate nucleus. In various estrogen target tissues, the oxidative metabolism of estrogen incurs the formation of cytotoxic semiquinones and other free radical intermediates (Horning et al., 1978; Kalyanaraman et al., 1984, 1985). The mammalian hypothalamus contains the enzyme estrogen 2/4-hydroxylase and some poorly-characterized peroxidases which are able to convert estradiol to 2- or 4-hydroxyestradiol (catecholestrogen) (Ball and Knuppen, 1978; MacLusky et al., 1983; Mondschein et al., 1986). In turn, highly reactive semiquinone radicals are formed when catecholestrogens are substrates of peroxidase/$H_2O_2$-catalyzed reactions. Catecholestrogens may also undergo spontaneous autoxidation resulting in the generation of semiquinone radicals and reactive oxygen species including $H_2O_2$ and superoxide anion (Kalyanaraman et al., 1984, 1985). Semiquinones and reactive oxygen species may initiate peroxidation of lipid membranes and promote DNA damage and thereby mediate the known teratogenic and carcinogenic effects of estrogen in various sex steroid target tissues (Horning et al., 1978; Jellinck and Fletcher, 1970; Metzler and McLachlan, 1978). In hyperestrogenized rats, estrogen-derived cytotoxic radicals may cause lipid peroxidation and neuronal membrane disintegration resulting in the aberrant axo-dendritic profiles and myelin figures observed in the arcuate nucleus (Schipper, 1991; Schipper, 1993). Conceivably, the non-enzymatic, peroxidase activity of GPA inclusions

in the arcuate nucleus catalyzes the conversion of catecholestrogen to cytotoxic semiquinone derivatives and thereby engenders lipid peroxidation and the development of neuropathological changes in this region (Schipper et al., 1990a; Schipper, 1993). In the normal, aging, female rat, oxidation of ovary-derived estradiol by glial peroxidase activity within the basal hypothalamus may progressively disrupt the neural circuitry subserving phasic gonadotropin release culminating in senescence-related anovulation (senile PE; Schipper et al., 1990a). Evidence implicating a role for Gomori-positive astroglia in free radical generation is derived from studies on the oxidation of catechols by peroxidase-positive astrocytes in primary brain cell cultures (see Section 5).

#### *3.5.3.3 The Putative Role of Peroxidase-positive Astrocytes in Goldthioglucose Neurotoxicity*

In mice, the neurotoxic effects resulting from goldthioglucose (GTG) administration are especially severe in the GPA-rich brain regions of the mediobasal hypothalamus and circumventricular organs (De Matteis et al., 1981; Young, 1988). GTG has been shown to potentiate iron-induced lipid peroxidation (Beckman and Greene, 1988). Based on these observations, Young et al. have proposed that accumulation of iron-containing astrocytes in periventricular brain regions may be responsible for the latters' enhanced vulnerability to GTG-induced neurodegeneration (Young, 1989). In an earlier study, Young and co-workers (1978) reported that estrogen enhances the vulnerability of the arcuate nucleus to GTG in ovariectomized rats. Schipper (1991) suggested that estrogen-induced increases in GPA inclusions may account for this increased sensitivity to GTG neurotoxicity.

### 3.5.4 Other Modulators of GPA In Situ

Various experimental manipulations have been shown to alter the numbers of GPA in the rodent hypothalamus. Nephrectomy (Rzehak and Srebro, 1973) and exposure to salicylate-quinine-lithium (Togal; Srebro and Szirmai, 1971) induce significant increases

in GPA in the rat arcuate nucleus. Conversely, GPA are fewer in number in the hypothalamus of magnesium-deficient rats (Srebro and Stachura, 1972) and are virtually absent in mutant, copper-deficient "blotchy" mice (Terr and Weiner, 1983). In female and male mice, adrenalectomy increases and decreases, respectively, numbers of hypothalamic GPA (Srebro et al., 1973). The mechanisms responsible for these experimental alterations of GPA numbers are unknown.

## 4. GOMORI-POSITIVE ASTROCYTES IN PRIMARY CULTURE

### 4.1 In Vitro "Aging"

In the early 1970's, Srebro and Macinska reported that Gomori-positive cells with tinctorial and fluorescent attributes akin to those of periventricular astrocytes *in situ* are present in *in vitro* cultures of rodent embryonic and human fetal brain tissue (Srebro and Macinska, 1972, 1973). Gomori glia in hamster and mouse diencephalic explants were first observed on day 14 *in vitro* and progressively accumulated over the ensuing 2-3 weeks (Srebro and Macinska, 1972). In periventricular brain explants derived from a 6-week-old human embryo, Gomori glia appeared after 5 weeks in culture and increased in number thereafter (Srebro and Macinska, 1973). More recently, Schipper and coworkers detected Gomori/DAB-positive astrocytes in dissociated embryonic and neonatal rat brain primary cell cultures (Schipper et al., 1990c; Schipper and Mateescu-Cantuniari, 1991). As in the rat hypothalamus (Schipper et al., 1990a), peroxidase-positive inclusions were localized exclusively to GFAP-positive astrocytes in culture by combining DAB histochemistry with anti-GFAP immunohistochemistry. The numbers of peroxidase-positive astrocytes and their granule content progressively increased between days 10 and 46 *in vitro*, consistent with earlier observations in diencephalic explants and in the intact rodent hypothalamus. In contrast with 10 day old cultures where DAB-positive astrocytes represented fewer than 1% of all cells, older cultures were replete with peroxidase-positive granules deposited within the cytoplasm of isolated flat and stellate astroglia and in astrocytes forming confluent monolayers. The astrocytic inclusions appeared invisible under LM and phase-dark under phase-contrast microscopy in unstained preparations, but

exhibited Gomoriphilia with CAH staining. As *in vivo*, the astrocytic inclusions emitted an orange-red autofluorescence consistent with the presence of porphyrins or oxidized flavoproteins, and contained a non-enzymatic, peroxidase activity that was unaffected by catalase inhibition, tissue preheating, or extreme pH modification (Schipper et al., 1990c; Schipper 1991).

4.2 The Cysteamine Model

The characterization of an *in vitro* model for the age-related accumulation of GPA permitted further investigation of factors which may modulate the astrocytic peroxidase activity. Since GPA have been implicated in the oxidation of catechols to free radical intermediates (Schipper et al., 1990a), Schipper et al. hypothesized that free radicals may, in turn, regulate the peroxidase activity of GPA (Schipper et al., 1990a,c). As described below, experiments utilizing the sulfhydryl compound, cysteamine (CSH), have provided unique opportunities to explore the role of free radicals and other potential mechanisms in the biogenesis of GPA granules.

### 4.2.1 Properties of Cysteamine

CSH is an aminothiol compound naturally present in mammalian cells where it is formed by pantethinase-mediated enzymatic degradation of coenzyme A (Abiko, 1967; Dupre et al., 1970). The chemical structure of CSH, also referred to as 2-aminoethanethiol or 2-mercaptoethylamine, is $H_2N$-$CH_2$-$CH_2$-SH. In peripheral organs such as kidney and heart, CSH is considered a metabolic precursor of taurine. In the brain, however, formation of taurine, a neurotrophic compound and potential neuromodulator proceeds via the "cysteine sulfinate pathway" and does not appear to involve CSH (Tappaz et al., 1992). CSH readily forms mixed disulfide conjugates with proteins. Measurements have estimated the concentration of total (free and conjugated) CSH in rat liver and kidney to be very low, in the range of 18-20 nmol/g tissue (Duffel et al., 1987). In contrast, neither CSH, nor its disulfide form, cystamine were detectable in rat brain (Kuriyama et al., 1984). However, low levels of activity of CSH dioxygenase, the enzyme which catalyzes

oxidation of CSH to hypotaurine, have been detected in ox, dog, and mouse brain, but not in rat (Kataoka et al., 1988).

### 4.2.2 In Vitro, Systemic and CNS Effects of CSH

Schwedes et al. (1977) demonstrated that somatostatin administration prevents the development of CSH-induced duodenal ulcers reported 4 years earlier by Selye and Szabo (1973). Oral or systemic administration of CSH to rats causes a depletion of somatostatin-like immunoreactivity (SLI) in rat plasma and gastrointestinal tract, and a similar, albeit reversible, effect on SLI throughout the CNS (Ceccatelli et al., 1987; Sagar et al., 1982; Szabo and Reichlin, 1981). However, more recent studies appear to suggest that somatostatin molecules, instead of being lost, are chemically altered by CSH via disulfide bond interactions, thereby deterring their detection using conventional immunolabeling techniques (Kwok et al., 1992). In several studies, CSH causes reversible depletions of pituitary prolactin concentrations *in vivo* and *in vitro* but did not alter prolactin immunoreactivity when incubated with the purified neuropeptide (Millard et al., 1982). However, on the basis of subsequent experiments, others have suggested that the CSH-mediated prolactin depletion results from thiol-disulfide interactions (Lorensen and Jacobs, 1984) and interference with immunodetection (Scammel and Dannies, 1984). CSH reversibly suppresses hypothalamic noradrenaline levels (reviewed in Vécsei and Widerlöv, 1990), an effect which may be due, in part, to the ability of CSH to inhibit the activity of dopamine-β-hydroxylase *in vitro* (Terry and Craig, 1985). This latter effect can be blocked by addition of N-ethylmaleimide, a compound which reacts with sulfhydryl groups (*ibid*). Furthermore, CSH was found to disturb hypothalamo-hypophyseal mechanisms subserving GH, TSH, and corticosterone secretion (*ibid*).

Based on the ability of CSH to alter brain SLI, the relationship between CSH and many behavioral processes has been investigated. CSH produces deficits in active avoidance behavior, and in some studies, impairs performance in passive avoidance tests and increases or decreases locomotor and exploratory activity. CSH also exerts a biphasic

action on kindled seizures in rat cortex and hippocampus, consisting of an early excitatory phase and a subsequent anticonvulsant or inhibitory effect (reviewed in Vécsei and Widerlöv, 1990).

### 4.2.3 Therapeutic Uses of CSH

CSH is currently used as a therapeutic agent in subjects with nephropathic cystinosis. In this disorder, the amino acid cystine accumulates abnormally within renal lysosomes. Under experimental conditions, CSH appears to form a disulfide conjugate with cystine facilitating extralysosomal cystine transport and preventing irreversible intralysosomal deposition of the amino acid (Gahl et al., 1987; Pisoni et al., 1985; Thoene et al., 1976). CSH has also been used to treat sickle cell anemia (Hassan et al., 1976) and acetaminophen (paracetamol) poisoning (Prescott et al., 1974). In the latter, CSH may exert its protective role by increasing the activity of the rate-limiting enzyme of heme catabolism, heme oxygenase, with resultant decreases in cytochrome P-450, a hemoprotein involved in the bioactivation of acetaminophen (Peterson et al., 1989). CSH has been shown to be a free radical-scavenging agent (Aruoma et al., 1988) and radioprotectant in experimental oncology (von Sonntag, 1987). However in the presence of ferrous iron and other transition metals, CSH may generate several prooxidant species (thiyl, hydroxyl and superoxide radicals and $H_2O_2$) and thereby subject certain tissues to oxidative stress (Munday, 1989).

SLI is increased in the basal ganglia of subjects with HD (Aronin et al., 1983; Marshall and Landis, 1985). Based on this observation and the aforementioned SLI-reducing action of CSH, Shults et al. (1986) administered CSH to Huntington's patients but did not observe improvement in motor or cognitive functions. On account of its ability to suppress plasma prolactin levels (Millard et al., 1982), some have advocated the use of CSH in the management of prolactin-secreting pituitary adenomas and other causes of hyperprolactinemia (Vécsei and Widerlöv, 1990).

### 4.2.4 CSH Induces Peroxidase Activity In Cultured Astrocytes

As discussed above, Schipper et al. hypothesized that free radicals may provoke the formation of GPA (Schipper, 1991; Schipper et al., 1990a, c). On the basis of its free radical-scavenging capability (Aruoma et al., 1988), dissociated rat brain cell cultures were exposed to CSH to determine whether the sulfhydryl would retard the time-dependent accumulation of peroxidase-positive astrocyte granules (Schipper et al., 1990c). Contrary to hypothesis, 6-day-old cultures treated with 88-880 μM of CSH (twice-weekly with each change of culture media) exhibit a robust accumulation of peroxidase-positive inclusions by day 18 *in vitro*, a time when few such inclusions are present in unexposed controls (Schipper et al., 1990c; Schipper and Mateescu-Cantuniari, 1991). Exposure to cystamine, the disulfide dimer of CSH, at lower concentrations in the range of 8.8-88 μM elicits similar effects, but causes marked cytotoxicity at 880 μM. As in untreated, older cultures and in GPA of the aging periventricular brain, the CSH-induced astrocytic inclusions are CAH-positive, emit an orange-red autofluorescence consistent with the presence of porphyrin or oxidized flavoproteins, and exhibit non-enzymatic peroxidase activity (Schipper et al., 1990c).

### 4.2.5 Fine Structural Features of CSH-induced Astrocyte Inclusions

At the ultrastructural level, 18-day-old CSH-treated astrocytes contain numerous membrane-bound cytoplasmic inclusions which are variable in size (0.3-1.5 μm in diameter) and round or ovoid in shape. The inclusions consistently exhibit an intensely electron-dense granular matrix reminiscent of periventricular astrocyte granules *in situ* (McLaren et al., 1992; Brawer and Sonnenschein, 1975; Brawer et al., 1978). Around the perimeter of many of the larger granules, concentric stacks of membrane appear to form myelin-like figures or fingerprint bodies. As in the periventricular rodent brain, clusters of the dense inclusions occasionally appear to make contact with cisternal elements (possibly profiles of ER) filled with a similar electron-opaque substance (Brawer and Sonnenschein, 1975; Brawer et al., 1978; Srebro and Lach, 1987; McLaren et al., 1992).

Conversely, and in accordance with previous *in situ* studies, the electron-lucent Golgi complexes do not appear to be associated with the electron-dense cisternal networks and granule clusters. In non-osmicated material, the DAB reaction product is visualized as a moderately dense, granular precipitate deposited within some, but not all, of the inclusions. Within strongly-labeled inclusions, the DAB reaction product is either dispersed throughout the granule matrix or restricted to discrete internal compartments. Using electron microprobe X-ray analysis, elemental iron is detected in many of the CSH-induced astrocytic inclusions. Moreover, the concentration of iron in these inclusions correlates strongly with the intensity of DAB staining, whereas the metal is undetectable in inclusions devoid of DAB reactivity (McLaren et al., 1992). These astrocyte granules are not stained with Prussian blue, a marker of ferric and hemosiderin iron, further supporting the contention that ferrous iron is responsible for the non-enzymatic peroxidase activity in these cells (Schipper et al., 1990c).

## 5. THE PUTATIVE ROLE OF PEROXIDASE-POSITIVE ASTROCYTES IN BRAIN AGING AND NEURODEGENERATIVE DISEASE

In 1990, Schipper et al. conjectured that neurodegenerative changes occurring in the aging and estrogenized rodent hypothalamus may result from exposure of the neuropil to cytotoxic semiquinone radicals formed by GPA-mediated oxidation of catecholestrogen. To test this hypothesis, Schipper and coworkers used electron spin resonance spectroscopy (ESR) to assay the oxidation of 2-hydroxyestradiol (catecholestrogen; 0.1-10 mM) to its *ortho*-(*o*)-semiquinone derivative in suspensions containing cellular homogenates derived from CSH-pretreated and control (untreated) 18-day-old primary astrocyte cultures (Schipper et al., 1991). NADPH and $H_2O_2$, cofactors required to sustain peroxidase-mediated catechol oxidations in acellular systems, were added to the suspensions (Kalyanaraman et al., 1986). Control cultures manifested a weak pro-oxidant capacity possibly attributable to (i) the small amount of pseudoperoxidase activity present in these cells at this age (Schipper et al., 1990c), (ii) constituent cellular enzymatic oxidases and peroxidases, and (iii) the autoxidation of catecholestrogen which may occur, to a limited extent, at neutral pH (Graham, 1978; Graham et al., 1978; Rosenberg, 1988). Homogenates of CSH-pretreated cultures, however, exhibited a marked oxidation of catecholestrogen to its *o*-semiquinone moiety as detected by ESR. Removal of $H_2O_2$ from the incubation medium resulted in a sustained reduction in the levels of *o*-semiquinone produced, underscoring the role of astroglial peroxidase activity in the accelerated oxidation of catecholestrogen. In contrast, removal of NADPH from the reaction suspension elicited only a slight reduction in the amplitude of the *o*-semiquinone signal. This may be due to the high content of sulfhydryls in GPA which may serve as alternate reducing agents during pseudoperoxidase-catalyzed reactions. Intracellular thiols may confer cytoprotection to the astroglia by complexing with, and reducing, estrogen-derived free radicals (Abdul-Hajj and Cisek, 1986, 1988; Jellinck and Fletcher, 1970; Maggs et al., 1983). However, leakage of free radical intermediates into the intercellular space may promote oxidative damage to surrounding neural elements (Kontos et al., 1985)

Since inclusion-containing astrocytes in CSH-treated primary brain cell cultures are tinctorially, histochemically and morphologically identical to GPA in the hypothalamic arcuate nucleus, the results of the ESR study support the notion that GPA are capable of generating oxidative stress within the medial basal hypothalamus *in situ*. The latter, in turn, may promote peroxidation of neuronal membranes resulting in failure of gonadotropin-regulating circuitry characteristic of the senile PE state (see Section 3.5.3.2). Further evidence supporting a role for GPA-derived free radicals in estrogen-related neurotoxicity and the development of PE has recently been obtained in studies employing antioxidants. Dietary supplementation with α-tocopherol (vitamin E; Desjardins et al., 1992) or administration of 21-aminosteroids (Schipper et al., 1994), blocks EV-induced depletion of hypothalamic β-endorphin, a consistent feature of EV-related hypothalamic damage, and prevents the development of anovulation and acyclicity in these rats.

In addition to catecholestrogen, the catechol moieties of the neurotransmitters, norepinephrine and dopamine may also serve as oxidizable substrates for the peroxidase activity of GPA *in situ*. The semiquinones derived from the oxidation of norepinephrine and dopamine are known neurotoxins and are readily produced by peroxidase-mediated reactions *in vitro* (Metodiewa et al., 1989). Barbeau (1984) has suggested that dopaquinones, semiquinone species generated by the autoxidation or peroxidase-catalyzed oxidation of dopamine, may play an important role in the pathogenesis of Parkinson's disease. In the same ESR study described above, upon addition of dopamine (1 mM) in place of catecholestrogen to reaction medium containing $H_2O_2$ and NADPH, homogenates of CSH-pretreated astroglia generated considerably higher levels of dopamine-*o*-semiquinone relative to untreated controls (Schipper et al., 1991). Thus, GPA may aggravate the neuronal damage observed in Parkinson's disease and other free radical-related neurodegenerations by enhancing the conversion of catechols (endogenous or administered) and environmentally-derived xenobiotics to neurotoxic intermediates. The progressive, age-related accumulation of these redox-active glial inclusions in rodent and human brain may predispose the senescent nervous system to parkinsonism and other free radical-related neurological disorders (Schipper et al., 1991).

## 6. CSH INDUCTION OF ASTROCYTE GRANULES: MECHANISM(S) OF ACTION

Due to their homology to GPA which naturally accumulate in the aging periventricular brain, CSH-treated astrocyte cultures provided Schipper and co-workers with a unique model to study the formation of the peroxidase-positive astroglial inclusions. In 1990, these authors suggested three possible mechanisms by which CSH or its metabolite(s) may stimulate the formation of GPA granules (Schipper et al., 1990c; Schipper, 1991). 1. Excessive intracellular sulfur resulting from sustained CSH exposure may be stored in discrete cytoplasmic inclusions which thereby acquire an affinity for Gomori stains (Srebro, 1969; Wagner and Pilgrim, 1978). To test this hypothesis, equimolar concentrations of the sulfur-containing amino acid, L-cysteine, which differs from CSH by the presence of an additional carboxyl group on the α-carbon, was administered to dissociated rat brain cell monolayers. In contradistinction to CSH, L-cysteine treatment did not induce the Gomori phenotype in cultured astroglia. In addition, mere sulfur loading would not explain the induction of autofluorescence or peroxidase activity in the CSH-treated cells. 2. CSH may derange porphyrin-heme metabolism in cultured astroglia resulting in the sequestration of metalloporphyrins within discrete cytoplasmic inclusions. An excess of porphyrins and heme could account for the autofluorescence and peroxidase activity in these cells, respectively (Askarov et al., 1984; Goldfischer et al., 1966; Goldgefter et al., 1980; Van Steveninck et al., 1987). CSH upregulates the activity of heme oxygenase and decreases total heme content in rat liver (Peterson et al., 1989). Various inducers of heme oxygenase elicit a prolonged rebound induction of δ-aminolevulinic acid synthase, the rate-limiting enzyme subserving porphyrin/heme biosynthesis (Kappas and Drummond, 1984; Rosenberg et al., 1982). Based on these observations, Schipper et al. (1990c) hypothesized that sustained activation of this enzyme may be responsible for the CSH-induced accumulation of metalloporphyrins in cultured astroglia. However, as discussed above (Section 3.4) and in the Conclusion of this thesis, recent evidence indicates that porphyrin/heme biosynthesis is *depressed* in CSH-pretreated cells, suggesting that the autofluorescence and peroxidase activity in the latter are not due

to accumulation of metalloporphyrins (Wang et al., 1995). 3. Although CSH has been used clinically as a free radical scavenging radioprotectant (von Sonntag, 1987), in the presence of transition metals and under certain redox conditions, CSH may revert to a generator of free radical intermediates (Munday, 1989; Svensson and Lindvall, 1988). As such, CSH may promote oxidative stress and stimulate the formation of GPA akin to the effects of ionizing radiation, a free radical generator, on rodent periventricular astroglia (Srebro, 1971; see Section 3.5.2). Much of the later work performed in Dr. Schipper's laboratory, including the data presented in this thesis, support this third mechanism of action.

Cells subjected to a variety of insults, including free radicals, commonly undergo a "cellular stress response" characterized by the concerted upregulation of a superfamily of polypeptides termed stress or heat shock proteins (HSPs). Based on the fact that CSH induces a stress protein, heme oxygenase-1, in rat liver (Peterson et al., 1989), we set out to elucidate the role of the cellular stress (heat shock) response in the biogenesis of peroxidase-positive astrocyte granules in CSH-treated glial cultures and in astrocytes of the aging periventricular brain.

## 7. THE CELLULAR STRESS RESPONSE

Various developmental stages in the fruit fly, *Drosophila melanogaster*, correspond to a pattern of ballooning or "puffing" of different regions along the polytene chromosomes of their salivary gland cells. These puffs represent chromosomal stretches undergoing enhanced gene expression. In 1962, Ritossa discovered a novel and rapidly-induced pattern of chromosomal puffing while studying the effects of elevated temperatures on gene activity in *Drosophila* salivary gland cells (Ritossa, 1962). More than a decade later, Tissières and colleagues (1974) reported that the chromosomal regions exhibiting heat-induced puffing contain the genetic code for a select set of proteins which are preferentially expressed in response to heat shock. It is now clear that the enhanced expression of these so-called "heat shock proteins" (HSPs) is a ubiquitous response of cells in virtually all organisms to sudden increases in their optimal growth temperatures. HSPs from diverse eukaryotes typically share a very high level of amino acid sequence identity. Furthermore, the sequence of the major 70 kDa HSP in eukaryotes is approximately 50% identical to its homologue in *E. coli*, and various other eukaryotic HSPs exhibit close similarities to the polypeptide sequences of prokaryotic HSPs (Bardwell and Craig, 1984; Craig, 1985; Lindquist, 1986). This significant degree of evolutionary conservation indicates that HSPs are an important group of proteins which may play crucial roles in maintaining long-term cellular viability under adverse conditions.

### 7.1 HSPs: Role In The Cellular Defense Against Heat Shock

Knockout of the genes coding for certain bacterial and yeast HSPs abolishes the ability of these organisms to survive hyperthermic challenge (Craig et al., 1987; Georgopoulos, 1977). In mammalian cells, microinjection of antibodies against a major HSP or competitive inhibition of its gene expression augments heat-induced cell killing (Johnston and Kucey, 1988; Riabowol et al., 1988). Conversely, transfection of the gene encoding a low-molecular weight human HSP confers thermoresistance to rodent cells confronted with otherwise lethal heat shock (Landry et al., 1989). Additionally, enhanced cellular

resistance to ordinarily lethal heat shock treatment can be induced in cells previously exposed to a mild, sublethal hyperthermic stimulus and allowed to recover at their normal growth temperature (Gerner and Scheider, 1975; Henle and Leeper, 1976). This "induced" or "acquired thermotolerance" correlates with the enhanced synthesis of HSPs occurring in response to a host of non-lethal stressors (Henle and Leeper, 1982; Lee et al., 1987; Li and Hahn, 1978; Li and Werb, 1982; Oesterreich et al., 1991). Moreover, a gradual loss of thermotolerance is associated with a return of HSPs to their prestressed basal levels, further underscoring the importance of these proteins in conferring cytoprotection against hyperthermic stress (Landry et al., 1982).

## 7.2 Induction Of Heat Shock-Related Proteins Under Various Environmental Stress Conditions

In addition to heat shock, the upregulation of HSP synthesis may be stimulated by a variety of metabolic stressors including: ionizing radiation, transition heavy metals, ethanol, amino acid analogues, arsenite, sulfhydryl reactive agents, nutrient deprivation, and various oxidative agents (Compton and McCarthy, 1978; Hightower, 1980; Levinson et al., 1979; Morimoto et al., 1990; Welch, 1990; Schlesinger, 1990). Several proteins which are strongly induced by glucose starvation, exposure to calcium ionophores (Lee, 1987; Lee, 1992; Welch et al., 1983) and ß-mercaptoethanol (Subjeck and Shyy, 1986) are termed glucose-regulated proteins (GRPs). Although somewhat less responsive to heat shock treatment, the GRPs are nevertheless structurally very similar to the HSPs and are classified as members of the mammalian HSP superfamily. In view of the large repertoire of insults capable of eliciting heat shock gene expression, the heat shock response came to be regarded as a general defense mechanism against hostile environmental agents and is often referred to as the cellular stress response, with the various HSPs and GRPs being collectively known as stress proteins (Morimoto et al., 1990; Welch, 1990). Throughout this thesis, the terms stress proteins and HSPs are interchangeable, unless the text warrants specific description of a GRP.

## 7.3 Abnormal Proteins Induce HSP Expression

The incorporation of amino acid analogues into nascent peptides disrupts proper protein folding and provokes the synthesis of a group of proteins identical to those induced by heat shock (Hightower, 1980). Similarly, the oxidation of protein thiol constituents resulting in the alteration of native polypeptide configuration activates HSP expression (Freeman et al., 1995; Lee and Hahn, 1988). Because many other inducers of stress proteins also cause protein denaturation (Hightower, 1980), it has been suggested that the intracellular accumulation of improperly folded or aberrant proteins represents a universal activator of the cellular stress response (Goff and Goldberg, 1985; Hightower, 1980). Strong support for this hypothesis derives from the demonstration, in 1986, that injection of denatured, but not intact, proteins into frog oocytes activates heat shock gene expression (Ananthan et al., 1986). Conversely, pretreatment of chicken embryo cells with deuterium oxide or glycerol, agents which enhance protein stability, blocked the induction of HSPs after heat shock (Edington et al., 1989).

## 7.4 Induction Of HSPs In Certain Pathophysiological States

Following tissue ischemia, free radicals, generated upon reperfusion of the affected area (Fridovitch, 1983; McCord, 1985), may oxidize and damage proteins and thereby induce a stress response (reviewed in Ananthan et al., 1986). Furthermore, the febrile reaction to various infections commonly elicits a heat shock response in mammalian hosts. Additionally, the stress associated with intracellular growth of a microbial pathogen may induce HSP synthesis in host cells independent of fever (Schlesinger, 1990; Young, 1990). Stress proteins are also expressed subsequent to lytic viral infection, and may be related to the accelerated synthesis of viral proteins within host cells (Nevins, 1982).

## 7.5 Expression Of HSPs Under Normal Physiological Conditions

Most HSPs are constitutively expressed in normally growing, unstressed cells and their levels may be increased (i) during the early S phase of the cellular mitotic cycle, (ii) at various stages of development and differentiation, and (iii) by hormones and growth factors (Schlesinger, 1990; Bond and Schlesinger, 1987). These results suggest that upregulation of HSP gene expression may occur not only in cells containing aberrant proteins but in normal cells exhibiting a high rate of protein synthesis under diverse physiological conditions (Welch, 92). In support of this, there is preliminary evidence that cells which actively synthesize and release proteins undergo enhanced HSP expression (*ibid*). Furthermore, HSPs may subserve essential housekeeping functions within cells by directly participating in the biogenesis, folding and maturation of proteins (see Section 7.7).

## 7.6 Stress-Induced Changes in Cellular Function and Morphology

Heat shock induces the preferential synthesis of HSPs and the concomitant cessation of other cellular activities. Under hostile conditions, stress-inducible gene expression occurs at the expense of the transcription of many genes which regulate cell proliferation and a host of other homeostatic functions in unstressed cells. Although poikilothermic organisms may eventually adapt to higher temperatures and resume proliferation following an initial growth arrest, warm-blooded organisms appear unable to recommence normal anabolic activities under continued heat stress and instead maintain HSP expression until the stress resolves or cell death intervenes. Biochemical changes occurring in stressed mammalian cells shortly after heat shock include (i) a sudden decrease in intracellular pH, (ii) a rapid increase in intracellular calcium, and (iii) lowered ATP levels (Findly et al., 1983; Stevenson et al., 1981; Weitzel et al., 1985). Mitochondrial function appears perturbed in heat-stressed cells (Leenders et al., 1974), presumably causing a necessary shift of energy production from aerobic respiration to anaerobic glycolysis. To facilitate this conversion, certain key enzymes in the glycolytic pathway, such as glyceraldehyde-3-

phosphate dehydrogenase and enolase, undergo enhanced synthesis following hyperthermia (Iida and Yahara, 1985; Mondovi et al., 1969; Nickells and Browder, 1988) and are considered members of the heat-inducible family of proteins.

Heat shock characteristically induces a series of morphological changes in eukaryotic cells. Shortly after heat shock, components of the pervasive cytoskeletal framework, the intermediate filaments, collapse and become re-organized around the nucleus, forming a so-called "cage" (Falkner et al., 1981; Thomas et al., 1982; Welch and Suhan, 1985). This process incurs an aggregation of mitochondria and polyribosomal elements in the perinuclear cytoplasm. Under transmission electron microscopy, the newly distributed mitochondria appear distended and the normally tightly-packed cristae undergo conspicuous swelling and disorganization (Welch and Suhan, 1985). This alteration in mitochondrial morphology may contribute to the functional disruption in cellular respiration and the decreased ATP levels observed after cell stress (Findly et al., 1983; Leenders et al., 1974; Welch, 1992). The Golgi apparatus, which typically consists of a complex of parallel, flattened cisternae and vesicular elements, often appears disrupted and fragmented in heat-shocked cells. However, it is unclear whether the heat-induced structural changes of the Golgi complex translate into any functional impairments in polypeptide glycosylation or derangements in overall cellular secretory activity (Welch, 1992; Welch and Suhan, 1985).

Additional morphologic abnormalities become apparent within the nucleus and nucleolus of cells exposed to supraphysiological temperatures. An accretion of perichromatin granules occurs in the nucleus, perhaps resulting from the condensation of unprocessed mRNA transcripts (Welch, 1992; Welch and Suhan, 1985; Heine et al., 1971). The intranuclear accumulation of mRNA precursors is in accordance with evidence demonstrating heat-induced interruption of normal RNA splicing (Mayrand and Pederson, 1983; Yost and Lindquist, 1986). It is noteworthy that in all organisms examined, most of the genes encoding the stress-responsive, but not necessarily the constitutive, forms of HSPs lack intervening sequences, or introns. Thus, mRNAs coding for HSPs may remain

unaffected by the block in splicing activity, resulting in the preferential processing of heat-inducible genes under stressful conditions (Lindquist, 1986; Yost and Lindquist, 1986). In some reports, hyperthermic treatment resulted in the appearance of unique, rod-shaped, actin-containing nuclear inclusions (Pekkala et al., 1984; Welch and Suhan, 1985). The role played by these actin-containing bodies in the cellular response to stress is unclear. The nucleolus, the site responsible for the assembly of ribosomal elements, is thermal-sensitive and exhibits heat-induced swelling, attendant decreases in its ribonucleoprotein granular components, and aggregation of remaining preribosomal granules (Welch and Suhan, 1985; Amalric et al., 1969; Simard and Bernhard, 1967; Simard et al., 1969). These observations are in keeping with other studies in which suppression of nucleolar rRNA synthesis and ribosome formation were demonstrable following hyperthermic stress (Ellgaard and Clever, 1971; Rubin and Hogness, 1975).

Among the various changes in cellular morphology associated with heat shock, alterations in nucleolar structure (and function) and in the organization of cytoplasmic intermediate filaments appear most reversible pending a return of mildly stressed cells to ambient temperatures and optimal growth conditions (Welch, 1992; Welch and Suhan, 1986). However, the putative role(s) of HSPs in this recovery process remain(s) poorly understood.

### 7.7 Structural and Functional Aspects of Stress Proteins

Individual members of the stress protein superfamily are commonly classified into the following six families on the basis of their apparent molecular weights when resolved on SDS gels: (i) high molecular weight HSPs, (ii) HSP90, (iii) HSP70, (iv) HSP60, (v) small molecular weight HSPs and (vi) ubiquitin (Healy et al., 1992; Kochevar et al., 1991). As previously mentioned, GRPs are members of the HSP superfamily, and the HSP70, HSP90 and large HSP families each contain at least one GRP constituent (Becker and Craig, 1994; Cai et al., 1993; Kochevar et al., 1991; Pouyssegur et al., 1977). The HSPs described in the following section are those germane to the work presented in this thesis.

### 7.7.1 HSP70 Family

HSP70 is one of the most thoroughly characterized HSPs and considerably more is known about the diverse functions of this HSP than virtually any other stress protein. Accordingly, the cellular and molecular biology of HSP70 is presented in greatest detail. HSP70 is a highly conserved HSP, exhibiting approximately 50% overall sequence identity, and up to 96% homology for certain domains, between *E. coli* and eukaryotic forms (Bardwell and Craig, 1984; Lindquist and Craig, 1988). In 1984, Pelham demonstrated that HSP70 participates in the protection of heat-stressed cells from sustained, deleterious nucleolar changes. In this study, overexpression of HSP70 in transfected mammalian cells enhanced nucleolar recovery after heat shock relative to non-transfected controls. Conversely, heat-induced cytotoxicity was markedly enhanced in cells microinjected with antibodies against HSP70 (Riabowol et al., 1988) and after competitive inhibition of its gene expression (Johnston and Kucey, 1988). Pelham proposed that HSP70 confers protection by catalyzing the refolding and reassembly of denatured ribonuclear proteins into biologically competent forms (Pelham, 1984, 1986). Evidence implicating HSP70 in protein folding was initially obtained when GRP78, an HSP70-like protein located within the ER, was shown to be identical to a protein previously described as the immunoglobulin heavy chain binding protein (BiP; Bole et al., 1986; Haas and Wabl, 1983; Munro and Pelham, 1986). BiP plays a transitory role in the process leading to the folding and assembly of the heavy and light chain components of immunoglobulins by binding to the newly synthesized heavy chains and shielding exposed hydrophobic regions before they become occupied by the light chains (Bole et al., 1986; Haas and Wabl, 1983). In addition, BiP transiently associates with other secretory proteins prior to their assembly into larger multimeric complexes (Copeland et al., 1986; Gething et al., 1986). Whereas the interaction of BiP with its target secretory proteins is relatively short-lived and requires ATP hydrolysis for disengagement, improperly assembled or misfolded secretory proteins within the ER tend to remain complexed to the stress protein and may trigger enhanced BiP (GRP78) expression (Kassenbrock et al., 1988; Kozutsumi et al., 1988; Normington et al., 1989). Thus, in addition to its putative

involvement in protein assembly, some have suggested that GRP78 may play a supervisory role over proteins destined for secretion, delaying the release of incompletely formed or aberrant proteins from the ER until proper assembly or, alternatively, degradation occurs (Munro and Pelham, 1986; Pelham, 1986). However, others have argued that the processes mediating retention of unfolded proteins in the ER and their subsequent translocation are not contingent upon BiP binding (Gething and Sambrook, 1992).

It is now clear that in eukaryotes, the HSP70 genes encode a number of structurally and functionally similar HSPs in the 70kDa range, collectively known as the HSP70 family. Under adverse conditions, cells upregulate the expression of a highly stress-inducible HSP70 commonly referred to as HSP72. Except for primate cells and certain human cell lines which exhibit significant basal levels of an HSP72 homologue (Welch, 1990), most eukaryotic organisms express little or no HSP72 in unstressed cells. However, all healthy mammalian cells constitutively express a closely related, but distinct, gene product known as the HSP70 cognate (HSP70c or HSP73). Both HSP73 and HSP72 appear functionally analogous, possess a high degree (>90%) of sequence homology, and transiently redistribute to the nucleus and nucleolus shortly after heat shock (Brown et al., 1993; Welch, 1992; Welch and Feramisco, 1984; Welch and Suhan, 1986). In unstressed eukaryotic cells, most of the members of the HSP70 family are constitutively expressed and have been detected in virtually all major intracellular compartments including (i) the nucleus and cytosol (HSP73; Brown et al., 1993; Welch, 1990; Welch and Feramisco, 1984), (ii) the lumen of the ER (GRP78; Munro and Pelham, 1986), (iii) the mitochondrial matrix (mt-HSP70 (GRP75); Kang et al., 1990; Scherer et al., 1990; Engman et al., 1989; Leustek et al., 1989; Mizzen et al., 1989), and (iv) plant chloroplasts (GRP75; Welch, 1992). Whereas HSP73 requires mRNA splicing for processing (Craig, 1985) and is preferentially expressed under physiologic conditions, the genes coding for the constitutive GRPs 75 and 78 may be further upregulated in response to certain metabolic challenges, including glucose withdrawal.

As in the case of BiP/GRP78, all HSP70s exhibit a strong affinity for unfolded proteins and avidly bind exposed, hydrophobic polypeptide sequences, although there is evidence that HSP70 may bind to hydrophilic stretches as well (Flynn et al., 1989). HSP70s appear to interact with folded and unfolded proteins, including those destined for intraorganellar transport, by virtue of variable C-terminal substrate recognition domains (reviewed in Gething and Sambrook, 1992). Release of the HSP70-bound peptides requires ATP hydrolysis. HSP70s contain ADP- and ATP-binding domains (Chappell et al., 1987; Welch and Feramisco, 1985) and, at the N-terminal domain, exhibit a weak ATPase activity upon conjugating with unfolded proteins (Flaherty et al., 1990; Flynn et al., 1989; Rothman, 1989), which, in E. coli, is potentiated by 2 cohort proteins, DnaJ and GrpE (Liberek et al., 1991).

#### *7.7.1.1 Role in Protein Biogenesis, Maturation, Renaturation and Degradation*

Studies on the renaturation of fully denatured proteins led to Anfinsen's demonstration, in 1973, that the amino acid sequence of polypeptide chains entirely governs the correct folding of proteins *in vitro*. Anfinsen suggested that the biologically active, native form of a protein is attained when the polypeptide chain achieves its most thermodynamically stable conformation (Anfinsen, 1973). *In vivo* however, as incomplete, nascent polypeptides progressively elongate off the ribosome into a complex cytosolic or reticular environment, diverse conditions may affect protein folding. For example, nearby proteins may interact with the unfolded preproteins, interfering with proper protein assembly and resulting in potentially deleterious protein aggregates. Based on the prevalence of HSP70s in unstressed cells and their capacity to transiently bind unfolded peptides and, to some extent, hydrolyze ATP, it is now believed that the HSP70 family may participate in the biogenesis and maturation of proteins by the following mechanisms: (i) Binding to the exposed regions of nascent polypeptides released from ribosomes. (ii) Maintaining cytosolic proteins destined for future transport into ER and mitochondria in semifolded, "translocation-competent" forms. (iii) Shielding the unfolded stretches of proteins protruding through transmembranal "channels" during their transport into the ER and

mitochondria. (iv) Indirectly facilitating protein folding and assembly into multimeric complexes by the orderly release of HSP70s from certain peptide domains of precursor proteins (Becker and Craig, 1994; Beckmann et al., 1990; Rothman, 1989). In this manner, members of the HSP70 family may preclude premature, potentially inaccurate self-folding until translation or translocation has been achieved and prevent the aberrant formation of protein aggregates. HSP70 binds directly to actin microfilaments (Margulis and Welsh, 1991a,b), possibly at its N-terminal domain (Tsang, 1993). It has, therefore, been proposed that HSP70 may transiently anchor nascent, unfolded polypeptides to the cytoskeletal framework, precluding their complexation with other proteins (*ibid*). In addition to promoting protein biogenesis and indirectly facilitating protein translocation, certain members of the HSP70 family may play a primary role in the targeting of polypeptides to specific intracellular compartments. For example, members of the HSP70 family have been shown to associate with proteins containing nuclear localization sequences and mediate the import of nucleoplasmin and other karyophilic proteins into the nuclei of mammalian cells (Imamoto et al., 1992; Shi and Thomas, 1992). In yeast, the mitochondrial HSP70 homologue, Ssc1p, has been shown to mediate the transfer of cytosolic proteins into the mitochondrial matrix (Kang et al., 1990). Similarly, proper functioning of the ER-localized BiP/GRP78 yeast homologue, Kar2p, is required to sustain transport of proteins into the ER lumen (Vogel et al., 1990b). It has been suggested that HSP70s located within mitochondria and ER bind to incoming polypeptide chains with higher affinity than their cytosolic counterparts, thereby averting a reversal in movement toward the cytosol and effectively "pulling" the peptide across the organellar membranes (Brodsky et al., 1993; Kang et al., 1990; Pfanner et al., 1990; reviewed in Martinus et al., 1995). Cytosolic HSP70s may also promote the proteolytic processing of proteins by facilitating their transport into lysosomes. For example, HSP73 binds to the signal sequence of certain intracellular proteins targeted for lysosomal degradation (KFERQ), inducing a conformational change in the polypeptide and rendering it conducive to lysosomal import (Chiang et al., 1989; Terlecky et al., 1992). HSP70s disassemble protein complexes including the lambda bacteriophage DNA replication complex (Hoffmann et al., 1992) and exhibit ATP-ase uncoating of clathrin-caged vesicles

(Rothman and Schmid, 1986; Chappell et al., 1986). The unravelling of partially folded polypeptides for transport into ER, mitochondria and lysosomes likely occurs in a similar ATP-dependent manner. HSP70 has been detected, in conjunction with other stress proteins (see HSP90, Section 7.7.2.1), in heterocomplexes with progesterone and glucocorticoid receptors (Smith et al., 1992; Hutchinson et al., 1992). The ATP-dependent unfoldase activity of HSP70 has been suggested to maintain these receptors in a state conducive to hormone-binding (Hutchinson et al., 1992). Due to this preoccupation with protein assembly, configuration and transport, the HSP70 family and other HSPs with related functions are collectively referred to as "molecular chaperones" or "chaperonins" (Ellis and van der Vies, 1991; Ellis et al., 1989). Pelham (1986) further proposed that the molecular chaperones may serve to renature aggregates of damaged proteins and thereby restore their biological activity. Evidence in support of this proposal has been obtained in *E. coli* (Hwang et al., 1990; Skowyra et al., 1990) but it remains unclear whether chaperonins function in this capacity in eukaryotic cells. However, based on the actin-binding properties of HSP70, a role in the regeneration and reconstitution of the filamentous cytoskeleton, which collapses after heat shock, has been surmised (Tsang, 1993).

#### *7.7.1.2 Role in Transcriptional Regulation of Heat Shock Genes*

Promoters located in the 5'-flanking regions of DNA coding for HSPs contain heat shock elements (HSE) consisting of inverted repeats of the sequence nGAAn (Perisic et al., 1989). In Drosophila, humans and most eukaryotic cells growing at ambient temperatures, the HSE of the HSP70 promoter is unoccupied by protein. After hyperthermic challenge, however, a protein referred to as the heat shock factor (HSF) occupies the HSE, inducing transcription of the heat shock gene (Rabindran et al., 1993). In contrast, in yeast *Saccaromyces cerevisiae*, HSF exhibits constitutive HSE-binding in the absence of heat shock, and temperature-dependent phosphorylation of the HSF appears to mediate its ability to activate transcription (Jakobsen and Pelham, 1988; Nieto-Sotelo et al., 1990; Sorger and Nelson, 1989; Sorger and Pelham, 1988). Three vertebrate HSFs have been

sequenced (HSF1, HSF2, HSF3) which range in size from 50-70 kDa. Although the N-terminal DNA-binding domain and various hydrophobic coiled-coil (leucine zipper) regions permitting HSF oligomerization are well conserved among the various HSFs, the remainder of their polypeptide sequences is highly variable (Lis and Wu, 1993; Rabindran et al., 1993). HSF1 appears to play a primary role in the induction of the cellular stress response and is activated following heat shock and exposure to heavy metals, amino acid analogues and oxidative stress (Baler et al., 1993). HSF2 is not heat responsive, but is activated during hemin-induced differentiation of erythroid K562 cells and may be implicated in embryogenesis (Mezger et al., 1987; Sistonen et al., 1992). Avian-derived HSF3, unlike HSF1, does not exhibit a rapid response to heat shock and appears unresponsive to various conditions which activate HSFs 1 and 2, but is co-expressed with the other HSFs in a cell-type-specific manner during development (Nakai and Morimoto, 1993).

HSP70 may play a negative, self-regulatory role in the induction of the heat shock response (DiDomenico et al., 1982; Morimoto, 1993). In support of this hypothesis, exogenous HSP70 prevents the *in vitro* activation of HSF1 (Abravaya et al., 1992). Furthermore, human cell transfectants that constitutively express high levels of HSP70 exhibit marked reductions in HSF activation subsequent to hyperthermic challenge (Mosser et al., 1993). In higher eukaryotic organisms, stress induces an oligomerization of the monomeric non-DNA-binding form of HSF1 present in the cytoplasm and nucleus to an activated trimeric form which accumulates in the nucleus (Sarge et al., 1993). It has been hypothesized that members of the HSP70 family transiently associate with the monomeric form of HSF1, ensuring its deactivation during normal periods of growth (Craig and Gross, 1991; Morimoto, 1993). Conceivably, under conditions of stress resulting in protein denaturation, the number of substrates competing for association with HSP73/HSP70c increases, thereby exhausting the pool of free HSP70c, leading to the dissociation of inactive HSF1-HSP70c complexes. Upon release from HSP70, the HSF1 monomers oligomerize into a trimeric state which attains DNA-binding capacity and translocates to the nucleus where transcription of the heat shock genes is activated. The

resultant enhanced expression of HSPs, including HSP70, would serve to reestablish normal protein balance, enabling a free pool of HSP70c to reform as the latter is released from newly resolved or degraded proteins. HSP70 has been found in complexes associated with the activated trimeric form of HSF1 (Abravaya et al., 1992). This observation suggests that HSP70, when expressed in sufficiently high levels in stressed cells or, alternatively, newly released HSP70c, may be responsible for the down-regulation of the heat shock response by causing dissociation of HSF1 from the HSE and its reconfiguration into the deactivated monomeric form (Abravaya et al., 1992; Baler et al., 1992).

### 7.7.2 HSP90 Family

#### *7.7.2.1 HSP90*

The genes coding for HSP90 are approximately 40% homologous between prokaryotes and eukaryotes (Lindquist and Craig, 1988). In humans, at least 2 genes, designated $\alpha$ and $\beta$, code for HSP90. Their gene products correspond to HSPs 86 and 84 in mice (Hickey et al., 1986b, 1989; Moore et al., 1989; Rebbe et al., 1987; Ullrich et al., 1986). Both HSP90 $\alpha$ and $\beta$ are constitutively present in unstressed eukaryotic cells and, in some instances, may account for 1-2% of the total cellular protein (Hickey et al., 1986b, 1989; Lai et al., 1984). Eukaryotic HSP90 is a phosphoprotein, exists as a dimer when purified, and *in vivo*, is found predominantly in the cytoplasm (and to a much lesser extent, in the nucleus) of cells growing under normal conditions (Akner et al., 1992; Schlesinger, 1990; van Bergen en Henegouwen et al., 1987; Welch, 1992). Both HSP90 isoforms are upregulated in human cells in response to heat shock (Hickey et al., 1986b; Simon et al., 1987). Heat shock induces a translocation of HSP90 from the cytoplasm to the nucleus in cultured human and murine cells. This effect is reversible pending removal of the heat stress and recovery of the cells (Akner et al., 1992; van Bergen en Henegouwen et al., 1987). The role of HSP90 in the development of tolerance to extreme temperatures is unclear. In mammalian cells, reduction of HSP90 content by expression of its antisense RNA enhances their sensitivity to heat-induced killing relative to wild-type controls

(Bansal et al., 1991). Furthermore, HSP90 is constitutively over-expressed in a line of CHO cells resistant to heat shock (Yahara et al., 1986).

*In vitro*, HSP90 exhibits chaperone-type functions, as determined by its ability to prevent the aggregation of chemically-denatured proteins including the enzyme citrate synthase and the Fab fragment of a monoclonal antibody (Wiech et al., 1992). In addition, $pp60^{src}$, the retroviral transforming gene product with tyrosine kinase activity, is maintained in an inactive state in association with HSP90 until reaching its final destination in the cell membrane (reviewed in Brugge, 1986). Similarly, HSP90 exhibits transient associations with other retrovirus-encoded tyrosine kinases including yes, fps, ros, and fes (*ibid*). HSP90 *induces* the kinase activity of heme-regulated eIF-2$\alpha$ kinase (Rose et al., 1989), which is thought to lead to widespread suppression of protein synthesis (Hardesty and Kramer, 1989; Rose et al., 1987). Indeed, some have speculated that HSP90 may be responsible, in part, for the generalized down-regulation of gene transcription observed in cells undergoing a stress response (Akner et al., 1992). Much of the interest concerning the function of HSP90 has centered on its interaction with steroid hormone receptors (reviewed in Lindquist and Craig, 1988). The receptors for estrogen, progesterone, and glucocorticoids all appear to be maintained in an inactive state when associated with HSP90, HSP70, and a 56kDa HSP in an "aporeceptor complex" (Bohen and Yamamoto, 1994; Lindquist and Craig, 1988; Maytin, 1995). It is thought that in the absence of steroid hormone, the aporeceptor complex remains folded and inactive by virtue of HSP-binding (Hutchinson et al., 1994). With increasing steroid concentrations, an ATP-dependent conformational shift of the receptor occurs which is mediated by the unfolding and stabilizing properties of HSP70 and HSP90, respectively, and the hormone (ligand) binds to its receptor. In support of this, heat-induced dissociation of HSP90 from the glucocorticoid receptor (GR) promoted loss of its steroid-binding capacity (Bresnick et al., 1989). Once steroid is bound, the activated receptor-ligand complex is translocated from the cytoplasm to the nucleus to stimulate transcription. Binding of hormone to the receptor and dissociation of HSP90 and other cohort proteins from the complex was shown to correlate with the receptor's acquisition of DNA-binding capacity (Renoir et al., 1986;

Sanchez et al., 1986). Some have suggested that HSP90 associates with the hormone-binding domain of the steroid receptor and stereochemically interferes with DNA-binding until ligand displaces the HSP (Dalman et al., 1991). In contrast, others have argued that HSP90 directly associates with the receptor's DNA-binding domain, or, alternatively, interferes with the dimerization of receptors (Demarco et al., 1991; Pratt et al., 1989). Receptor dimerization is a prerequisite for transcriptional activation of various steroid-responsive genes (Demarco et al., 1991). In summary, HSP90 has been associated with (i) the development of thermotolerance in certain mammalian cells, (ii) the prevention of aggregation of denatured polypeptides, *in vitro*, (iii) the refolding and reconferral of function to denatured enzymes, *in vitro*, (iv) the chaperoning and intracellular transport of nascent kinases and (v) the fostering of appropriate steroid-receptor form and function.

### *7.7.2.2 GRP94*

GRP94, also referred to as ERp99 or endoplasmin, exhibits strong sequence similarity (50%) to, and is considered a member of, the HSP90 family of stress proteins (Kulomao et al., 1986; Mazzarella and Green, 1987). In contrast to the predominately cytoplasmic-localization of HSP90, GRP94 is most abundant in the ER, and has also been identified in the Golgi and in association with the plasma membrane (Lewis et al., 1985; Lindquist and Craig, 1988; Olden et al., 1978). GRP94 is a glycoprotein possessing low affinity but high capacity calcium-binding sites (Koch et al., 1986), and has been implicated in the sorting of proteins destined for transport (Gething and Sambrook, 1992; Little and Lee, 1995) and the regulation of intracellular calcium levels (Brostrom et al., 1990). In analogy to other stress proteins, chaperoning functions have also been ascribed to GRP94 based on the following observations: (i) GRP94 synthesis is upregulated in response to the accumulation of misfolded proteins in the ER (Kozutsumi et al., 1988); (ii) Immature or mutant, but not the native, forms of various polypeptides are complexed to GRP94 and other ER-derived GRPs (Navarro et al., 1992; Ramakrishnan et al., 1995; Schaiff et al., 1992); (iii) GRP94 appears to bind ATP (Clairmont et al., 1992) and possess ATPase

activity (Li and Srivastava, 1993); and (iv) GRP94, in conjunction with GRP78, facilitates the folding of nascent immunoglobulin chains in the ER (Melnick et al., 1994). GRP94 is constitutively synthesized during normal growth conditions from a single copy gene and exists as a dimer (Kang and Welch, 1991; Lee et al., 1983). Its expression is markedly enhanced (approximately 10-fold) in response to glucose starvation, anoxia, and particularly treatments which perturb normal ER function including ionophore-mediated depletion of intracellular $Ca^{2+}$, inhibitors of ER $Ca^{2+}$-ATPase, blockers of protein glycosylation (Gomer et al., 1991; Lee, 1992; Little and Lee, 1995), but not by heat shock or oxidative stress (Lee, 1987; Subjeck and Shyy, 1986).

### 7.7.3 Small Molecular Weight HSPs

#### *7.7.3.1 HSP27 and αB-crystallin*

Relative to their higher molecular weight counterparts, the small molecular weight HSPs (weighing between 15 and 40 kDa) exhibit less cross-species homology. However, a characteristic feature of many small HSPs is the conserved "α-crystallin domain" consisting of 90-100 residues in the C-terminal halves of the proteins (reviewed in de Jong et al., 1993). The two isoforms of α-crystallin, αA and αB, are closely related and expressed in abundance in the lens. αB-, and to a much lesser extent, αA-crystallin have also been found in various non-lenticular tissues (*ibid*). αB-crystallin is a 23 kDa protein which, unlike αA-crystallin, is inducible in mouse 3T3 cells with heat shock (Klemenz et al., 1991). Analogous to other HSPs, the promoter region of the αB-crystallin gene contains a HSE sequence (*ibid*). In mammals, an additional low molecular weight protein exhibiting 70% sequence similarity to the α-crystallins has been identified as HSP27 (Hickey et al., 1986a; Ingolia and Craig, 1982). In humans, three genes appear to code for HSP27 (McGuire et al., 1989) and may be evolutionary descendants of a common α-crystallin/small HSP gene (de Jong et al., 1993). Both αB-crystallin and HSP27 exhibit development-associated regulation of their expression in rat tissues and in various lower eukaryotic organisms (*ibid*). HSP27 was initially identified as an estrogen-inducible protein in human breast cancer cells (Edwards et al., 1980). Analysis of the gene

encoding Drosophila HSP27 suggests that the promoter region contains both steroid responsive and heat shock responsive elements (Cohen and Meselson, 1985; Hoffman et al., 1987). In addition, estradiol and heat treatment both regulate the induction of HSP27 in human breast cancer cells *in vitro* (Fuqua et al., 1989). In the normal adult, HSP27 expression is observed in diverse tissues but appears to be preferentially expressed in estrogen target tissues of the female reproductive system (reviewed in Ciocca et al., 1993). HSP27, akin to HSPs 72 and 90, may chaperone steroid receptors, and appears identical to the estrogen receptor-associated protein, p29 (Ciocca and Luque, 1991).

In unstressed eukaryotic cells examined under LM, HSP27 exhibits a primarily perinuclear distribution proximal to the Golgi complex. However, following hyperthermia, HSP27 expression may increase by 10-20 fold, and the protein appears to accumulate within the nucleus as determined by immunoelectron microscopic analysis of heat-shocked cells (Arrigo et al., 1988a). In contrast, Nover et al. (1989) have argued that hyperthermia does not result in an intranuclear localization of small HSPs, but rather induces the formation of large, cytoplasmic "heat shock granules" which may be associated with the newly collapsed cytoskeleton in the immediate nuclear vicinity. These authors have speculated that the heat shock granules form to sequester and protect proteins and untranslated cellular mRNAs from heat-induced degradation (Nover et al., 1989). Nevertheless, upon recovery of the cells from heat shock, HSP27 appears to "exit" the nucleus and redistribute into the cytoplasm (Arrigo et al., 1988a). Similarly, αB-crystallin exhibits cytoplasmic localization in normally growing murine cells and, in heat shocked cells, is found in association with the nucleus (Klemenz et al., 1991). Heat treatment also induces the formation of heat shock granule-like aggregates containing αB-crystallin (*ibid*). HSP27 and αB-crystallin have been identified in large, 700 kDa complexes associated with newly synthesized proteins (Zantema et al., 1992). Furthermore, the supramolecular aggregates dissociate with heat treatment, suggesting that the two stress proteins subsume different functions when separated during heat shock than when complexed in the unstressed state (*ibid*). Recently, it was shown that enhanced expression of human HSP27 or αB-crystallin in mouse L929 transfectants correlates with increased resistance to

TNFα-, menadione- and $H_2O_2$-mediated oxidative stress (Mehlen et al., 1995). However, at similar levels of expression, HSP27 appeared twice as effective as αB-crystallin in protecting transfectants against TNFα cytotoxicity. In earlier studies, selective overexpression of human HSP27 appeared sufficient to confer thermoresistance to rodent cell transfectants (Landry et al., 1989) and enhanced their tolerance to oxyradical-generating anticancer agents and $H_2O_2$ (Huot et al., 1991). αB-crystallin and HSP27 also appear to be molecular chaperones. For example, αB-crystallin has been shown to protect lens-specific proteins from thermal aggregation *in vitro* (Horwitz, 1992). In another study, both αB-crystallin and HSP27 prevented heat-induced aggregation of enzymes *in vitro*, and promoted their refolding after urea denaturation in an ATP-independent fashion (Jakob et al., 1993). Protection from heat-induced derangements of the microfilament network was apparent in cells overexpressing only HSP27 (reviewed in Arrigo and Landry, 1994) and may be attributable to its ability to modulate actin polymerization *in vitro* (Miron et al., 1991). Based on the observation that HSP27 copurifies with large proteolytic enzyme complexes ("prosomes"), a role in the degradation of polypeptides has also been proposed (Arrigo et al., 1988b).

HSP27 is phosphorylated at multiple serine residues as an early response to heat shock (Zhou et al., 1993) and can be separated into at least four isoelectric variants representing altered phosphorylation states (reviewed in Arrigo and Landry, 1994). Whether phosphorylation of HSP27 is a prerequisite for its chaperoning and protective properties remains controversial (Knauf et al., 1994; Lavoie et al., 1993). Many agents, including TNFα and mitogenic stimulants, increase HSP27 phosphorylation (conceivably via induction of mitogen-activated protein (MAP) kinases) without eliciting a concomitant upregulation of HSP27 gene expression (Arrigo and Landry, 1994). MAP kinase is known to modulate the cellular reaction to many external stimuli (reviewed in Davis, 1993). Accordingly, it has been suggested that HSP27, a substrate of MAP kinase-activated protein 2, may additionally play a role in the cellular signal transduction pathway (Arrigo and Landry, 1994).

### *7.7.3.2 Heme Oxygenase-1*

Although heme oxygenase (HO) does not exhibit sequence homology to the HSP27/αB-crystallin family, it exhibits a molecular mass of 32 kDa and is considered a small HSP. HO, which exhibits immunolocalization to the ER (Muller-Eberhard and Nikkilä, 1989), has long been known to catalyze the rate-limiting step in heme degradation. In this step, the heme ring is cleaved and converted to biliverdin, ferric iron is released, and carbon monoxide (CO) is produced (Maines, 1988). In mammalian cells, the bile pigment, biliverdin, is subsequently converted to bilirubin via the action of biliverdin reductase. Two isoforms of HO have been characterized and appear to be different gene products (Cruse and Maines, 1988). The activity of the first, HO-1, was initially shown to be induced by transition metals, oxidative stressors (Maines, 1982; Maines and Kappas, 1974, 1977), and its substrate, hemin (Shibahara et al.,1978). More recently, it was demonstrated that the gene encoding rat (Müller et al., 1987) and human (Shibahara et al., 1989) HO-1 contains a HSE sequence in its promoter region. Furthermore, in rodent cells, HO-1 gene transcription and protein expression are inducible by heat shock (Dwyer et al., 1992; Raju and Maines, 1994). The message for HO-1 is also enhanced in some, but not all, human cells faced with hyperthermic challenge (reviewed in Mitani et al., 1989). Thus, HO-1 exhibits many features typical of HSPs, and is often referred to as HSP32 (Caltabiano et al., 1986). However, the upregulation of HO-1 in response to various stressors seems to occur independently of other major HSPs (Dwyer et al., 1992; Mitani et al., 1992; Raju and Maines, 1994), suggesting that HO-1 expression may be regulated by its own transcription factor(s) (Raju and Maines, 1994). For example, the promoter regions of HO-1 genes contain metal- and IL-6-responsive element sequences and binding sites for various transcription factors including AP-1, AP-2, and NF-κB (Alam and Zhining, 1992; reviewed in Lavrovsky et al., 1994). It is noteworthy that the HO-1 message, unlike those of other HSPs, contains 4 introns and requires RNA splicing (Müller et al., 1987). As discussed above (Section 7.6), heat stress perturbs this cellular process (Mayrand and Pederson, 1983; Welch et al., 1983). Raju and Maines (1994) have suggested that the block in HO-1 expression may be circumvented by the presence of

diverse copies of genes encoding HO-1. Conceivably, some of the resultant transcripts may not require processing.

The inducibility of HO-1 in response to heat and a variety of other agents which promote oxidative stress has led to the prevailing belief that its expression is a fundamental component of the cellular anti-oxidant defense system (Applegate et al., 1991; Keyse and Tyrrell, 1989; Stocker, 1990). Stocker (1990) suggested that enhanced HO activity serves to catabolize prooxidant heme and generate bilirubin, a potent oxyradical scavenger *in vitro* (Stocker et al., 1987). However, recent studies suggest that a reevaluation of the assumed anti-oxidant role of HO-1 may be necessary (Balla et al., 1992; Nutter et al., 1994). For example, whereas HO-1 was shown to be strongly induced in human cells in response to menadione-derived oxidative stress, inhibition of its activity did not enhance oxyradical-mediated damage to DNA and, in fact, afforded protection from cytotoxicity relative to controls (Nutter et al., 1994). The HO-mediated release of CO may exacerbate oxidative stress in keeping with the ability of CO to promote mitochondria-derived oxyradical generation (Zhang and Piantadosi, 1992).

HO-2, the second isozyme, is constitutively expressed in various tissues but, in contrast to HO-1, is not inducible under any stressful conditions examined (Maines et al., 1986; Trakshel et al., 1986). The work presented in this thesis deals exclusively with the inducible form of HO, HSP32/HO-1.

### 7.7.4 Ubiquitin

Heat shock of mammalian cells typically provokes a robust increase in protein degradation of ordinarily long-lived polypeptides (Parag et al., 1987). In eukaryotes, most of the ATP-dependent proteolytic processing of peptides is performed by an extremely well conserved ubiquitin system (reviewed in Jentsch, 1992). Ubiquitin (Ub), a central component of the system, is a 76-amino acid-long polypeptide weighing 8.5 kDa which exhibits greater than 82% sequence homology amongst diverse eukaryotic organisms (Wostmann et al., 1992).

The formation of an isopeptide bond between the C-terminal glycine of Ub and side-chain amino groups of lysine on substrate proteins has been shown to influence various cellular processes including DNA repair, cell cycle control and proteolysis (Finley and Chau, 1991; Jentsch, 1992). Attachment of Ub to a protein substrate occurs in a stepwise manner and is dependent on the action of diverse enzymes which are members of the Ub system (reviewed in Wilkinson, 1994): (i) Ub is first activated by a Ub-activating enzyme, E1. In this ATP-dependent step, Ub becomes linked to E1 through a high energy thiol-ester bond. (ii) Activated Ub is then transferred via transthiolation to one of many Ub-conjugating enzymes, collectively referred to as E2. (iii) Covalent attachment of Ub to the substrate protein may now occur by the action of E2. In certain, but not all cases, a third enzyme termed Ub-protein ligase, recognin, or E3, has been shown to facilitate the E2-mediated transfer of Ub to the target protein. Ub-attachment and subsequent assembly of a multi-Ub chain on a protein appears essential to target the substrate for degradation. The multi-Ub chain is formed by another Ub-conjugating enzyme which joins individual Ub monomers by isopeptide linkage. Multi-ubiquitinated proteins are recognized as proteolytic substrates by a 1500 kDa cytosolic proteolytic complex referred to as the 26S protease. The Ub-dependent 26S protease requires ATP both for its assembly, and for its proteolytic activity (reviewed in Hershko and Ciechanover, 1992). The recovery of Ub from the substrate and its recycling into a free form capable of becoming activated is achieved by Ub-isopeptidases. Ubiquitination of targeted proteolytic substrates imparts a specificity to the Ub system, and is thought to account for the selective degradation of short-lived as well as stress-damaged proteins (reviewed in Hershko and Ciechanover 1992 and Jentsch, 1992).

In all species examined, Ub is encoded by three different classes of genes (reviewed in Wilkinson, 1994). The gene products of classes I and II appear fused to ribosomal zinc finger proteins and may modulate protein synthesis as well as Ub-dependent proteolysis. In *S. cerevisiae* there are four Ub genes, UBI1 through UBI4, which encode individual Ub polypeptides. Expression of the UBI1, UBI2, and UBI3 gene products, members of classes I and II, occurs at ambient temperatures under normal growth parameters but is

rapidly downregulated following heat shock. Constitutive expression of Ub genes suggests that the protein may play important roles in the regulation of cellular processes. In this respect, Ub-dependent degradation of various short-lived regulatory proteins has been reported. In yeast, the MAT $\alpha$2 transcriptional repressor requires multi-ubiquitination prior to its degradation (Hochstrasser et al., 1991). Similarly, the rapid proteolysis of cyclin, a regulator of the mitotic cell cycle, appears to be Ub-dependent (Glotzer et al., 1991). Ub is found covalently associated to histone molecules in the nucleus and may regulate chromatin structure and function (Yarden et al., 1986). Ub has also been reported to bind various cell surface molecules including the receptors for: platelet-derived growth factor, growth hormone, T-cell antigen, and TNF (reviewed in Wilkinson, 1994). Conceivably, ubiquitination of these receptors would serve to downregulate their activities and modulate signal transduction pathways. Ub has also been associated with cellular reorganization during embryogenesis, as well as development-related programmed cell death (reviewed in Mayer et al., 1991a). In the hawkmoth *Manduca sexta*, hormone-induced apoptosis of intersegmental muscles is contingent upon enhanced polyubiquitin gene expression (Schwartz et al., 1990). Induction of Ub under these circumstances, however, is not accompanied by similar upregulation of other heat shock genes.

In contrast to the constitutive and stress-refractory properties of class I and II genes, expression of the gene product encoded by UBI4, a class III gene, is markedly enhanced in humans and other organisms under stressful conditions including heat shock and nutritional deprivation (Choi et al., 1988; Finley et al., 1987). The class III gene encodes a Ub precursor arranged as pentameric head-to-tail Ub sequence repeats (Özkaynak et al., 1987). Furthermore, in yeast and chicken, the class III gene for Ub contains a consensus heat shock promoter (Bond and Schlesinger, 1986; Finley et al., 1987). Thus, class III-encoded Ub is a member of the HSP superfamily and represents the transcript implicated in the cellular response to adverse conditions (Bond and Schlesinger, 1985, 1986). Furthermore, two members of the E2 family of Ub-conjugating enzymes, UBC4 and UBC5, associated with the selective proteolysis of short-lived and aberrant proteins, have

also been shown to be heat-inducible (Seufert and Jentsch, 1990).

In 1988 Schwartz et al. employed immunoelectron microscopy to demonstrate the presence of free Ub in lysosomes of hepatoma cells. In 1990, an accumulation of intralysosomal Ub-protein conjugates was also demonstrated in fibroblasts (Laszlo et al., 1990). This latter finding suggested that large-scale incorporation of ubiquitinated substrates within lysosomes may occur independent of a non-specific, autophagic process (*ibid*). Furthermore, these observations contested the earlier belief, largely based on observations of Ub in association with non-membrane-bound inclusions, that the Ub system acts independently of the lysosome system (Ciechanover et al., 1984). Indeed, Ub exhibits high resistance to acidic conditions and proteolytic digestion and is likely capable of withstanding the harsh intralysosomal environment (reviewed in Wilkinson, 1988). It has been suggested that ubiquitinated proteins undergo lysosomal degradation when they are insoluble, or associated with the cytoskeleton or cellular membranes (Mayer et al., 1991b). Curiously, however, Ub-protein conjugates do not appear to increase in various disorders of lysosomal storage (Zhan et al., 1992). Consequently, some authors continue to assert that Ub incorporation into lysosomes is the result of normal autophagy (reviewed in Wilkinson, 1994). Other studies have demonstrated that the native, but not the mutant thermolabile, form of E1 Ub-activating enzyme promotes stress-induced lysosomal breakdown of cellular, long-lived proteins (Gropper et al., 1991), possibly by facilitating lysosomal maturation (Gropper et al., 1991; Lenk et al., 1992). Notwithstanding the above, today there is a consensus that Ub is implicated in both non-lysosomal as well as lysosomal proteolysis (Mayer et al., 1991b; Wilkinson, 1994).

The mechanism whereby Ub detects proteins destined for degradation remains to be defined. It has been suggested that Ub exhibits "semi-random" conjugation to most proteins, whether or not they are denatured (reviewed in Wilkinson, 1994). The hypothesis states that transient binding of Ub to stable, native proteins occurs because "proofreading" proteases continually remove Ub from normal proteins. In contrast, binding of Ub to the exposed hydrophobic surfaces of aberrant or stress-damaged proteins

may, conceivably, promote a conformational change in the stress protein rendering the Ub-protein complex conducive to polyubiquitination and subsequent degradation.

The cellular stress response can be elicited by many agents which share the ability to cause intracellular accumulation of aberrant proteins. Upregulation of Ub with a concomitant increase in degradation of abnormal proteins is considered an adaptive response to stress. It has been suggested that activation of the Ub system may be contingent upon prior failure of the other HSP superfamily members to adequately chaperone and catalyze the refolding and renaturation of damaged proteins (Wilkinson, 1994). In contrast, an early model suggested that Ub maintains the HSF in an inactive state (Munro and Pelham, 1985). In this model, a rapid, initial increase in Ub substrates (i.e. denatured proteins), as might occur following sudden exposure to heat shock, would promote de-ubiquitination of the HSF in favor of conjugation with the damaged proteins. HSF would then be free to activate transcription of the HSP genes. However, evidence in support of this model is scarce. Furthermore, a recent study demonstrated that the immediate effects of $H_2O_2$-mediated oxidative challenge consisted of a *decrease* in the amount of Ub-protein conjugates formed, with a coincident reduction in proteolytic capability (Shang and Taylor, 1995). Reactivation of Ub-dependent protein conjugation and proteolysis to levels above non-stressed controls occurred following removal of the stress and only after significant recovery of the cells. Thus, in spite of the uncertainty regarding the temporal involvement of Ub in the cellular response to stress, the Ub system does appear to be essential for the reestablishment of protein homeostasis following exposure to stress.

## 8. HSPs IN THE BRAIN: ISCHEMIA, AGING AND NEURODEGENERATION

In brain, the elevated demands for oxygen and energy accompany an enhanced sensitivity to metabolic stress compared with other tissues. HSP70c is constitutively expressed to a high degree in the CNS, and represents approximately 1% of total axonal protein (de Waegh and Brady, 1989). Furthermore, numerous studies have demonstrated that the CNS responds to a diverse array of insults including hyperthermia, ischemia, and physical and chemical stressors by upregulating the message and expression of various HSPs, especially members of the HSP70 family (Brown, 1990; Dienel et al., 1986; Tytell et al., 1993a). The enhanced resistance to forebrain ischemic cytotoxicity observed in rats preconditioned with mild, transient heat shock has been attributed to the heat-induced upregulation of stress protein expression (Chopp et al., 1989). Additionally, widespread induction of neural HSP72 following mild ischemia in gerbils correlated with tolerance to subsequent severe ischemic challenge (Kitagawa et al., 1991). Interestingly, following hyperthermia, HSP72 immunoreactivity is prominent in astrocytes and vascular endothelia *in situ*, and to a lesser extent, in neurons (Marini et al., 1990). Similarly, relative to cultured astrocytes, neurons exhibit an attenuated heat shock response *in vitro* (*ibid*; Nishimura et al., 1991). Large numbers of astroglia, ependyma, and to a lesser extent, select neuronal populations, upregulate HO-1 expression, *in situ*, following thermal stress (Ewing and Maines, 1991). Furthermore, treatment of rats with chemical depletors of the antioxidant, glutathione, results in robust induction of HO-1 in astrocytes, ependymal cells, Bergmann glia and leptomeninges, but not in neurons (Ewing and Maines, 1993). Cultured astrocytes, but not neurons, strongly overexpress HO-1 following exposure to oxidative stress (Dwyer et al., 1995). As discussed above (Section 7.7.3.2), induction of HO-1 may promote the accumulation of free radical-scavenging bile pigments (Stocker, 1990), and thereby fortify the brain's antioxidant potential. A stronger induction of the stress response in astrocytes relative to neurons may underlie the enhanced astroglial resistance to various challenges including heat shock (Nishimura et al., 1991), exposure to oxidative stress (Dwyer et al., 1995) and ischemia (see below; reviewed in Sharp and Sagar, 1994). Nevertheless, neuronal HSP expression also appears to confer

cytoprotection to neurons exposed to excitotoxins (Lowenstein et al., 1991; Rordorf et al., 1991). Pretreatment of cultured neurons with mild heat shock protected them from subsequent glutamate-induced excitotoxicity. Furthermore, inhibition of heat shock gene transcription or HSP expression prevented this cytoprotective effect. It has been difficult, however, to ascribe a well-defined, protective role to HSP72 upregulation. For example, in a rat model of global ischemia, although strong immunoreactivity for HSP72 was evidenced in surviving CA3 and CA4 pyramidal neurons, the stress protein was also markedly expressed in CA1 neurons which exhibit enhanced cytotoxicity under hypoxic conditions (Chopp et al., 1991; Simon et al., 1991). Ischemic insults of shorter duration similarly resulted in overexpression of HSP72 in preferentially affected CA1 and dentate hilus neurons (Simon et al., 1991). Taken together, it appears that concerted upregulation of HSP72 cannot, in all cases, be considered conclusive evidence of impending neuronal survival in the face of adverse conditions. It is clear that upregulation of stress protein expression occurs in cells experiencing, or that have undergone, stress of a potentially cytotoxic nature (Gonzalez et al., 1989; Nowak, 1993). In the postischemic gerbil brain, ischemia-sensitive CA1 neurons of the hippocampus exhibit prolonged induction of HSP72 mRNA but little expression of the HSP72 protein (Nowak, 1991). However, under the same conditions, resistant CA3 hippocampal neurons immunoreactive for HSP72 only transiently express HSP72 mRNA as detected by *in situ* hybridization (*ibid*). Based on these studies, it has been suggested that the duration of HSP72 mRNA transcription may be used to indicate the neuronal populations at risk for ischemia-induced cytotoxicity. Alternatively, enhanced accumulation of Ub-protein conjugates in the ischemia-sensitive CA1 region was shown to occur with increasing severity of ischemic insult (Hayashi et al., 1992). The authors conjectured that accumulation of Ub-protein conjugates may precede, and play a role in, the development of ischemia-related neurotoxicity. Others have cited the role of Ub in developmental apoptosis (Section 7.7.4) and suggested that during pathogenic processes, this function of Ub is reactivated (Mayer et al., 1991a). Hayashi et al. (1992) nonetheless conceded that it was not clear whether the observed ubiquitination of proteins promoted or resulted from neuronal cell death.

Various studies have demonstrated an apparent "hierarchy of vulnerability" of cells in an ischemic territory (reviewed in Sharp and Sagar, 1994). Brief focal ischemia results in enhanced transcription and expression of the HSP70 gene product restricted to hippocampal pyramidal neurons residing in the ischemic region. In contrast, ischemia of longer duration causes upregulation of HSP70 within a rim of astrocytes surrounding the damaged neurons, and apparently results in translational inhibition of the latter. Finally, severe ischemia sufficient to cause hippocampal infarction results in HSP70 overexpression in capillary endothelial cells, but not in neurons or astroglia within the ischemic core. Based on these observations, it has been suggested that translational blockade as a result of ischemic damage occurs first in neurons, and then in astroglia. Capillary endothelial cells, on the other hand, appear relatively resistant to similar levels of hypoxia, and may be the last cell type in an infarcted area to cease expression of stress proteins. The induction of HSP70 in astrocytes and neurons within the CNS has also been documented in various studies following stereotaxic injection of kainic acid (Uney et al., 1988), flurothyl-induced status epilepticus (Lowenstein et al., 1990), cortical stab wounds (Brown et al., 1989), and spinal cord trauma (Gower et al., 1989).

Certain studies, though few in number, have examined the relationship between aging and the ability to mount a cellular stress response in the brain. For example, relative to younger rats, older animals exhibit an age-related deficiency in HSP70 mRNA induction in brain (and other tissues) following heat stress (Blake et al., 1991). However, the heat-induced increase in colonic temperature of the aged rats in this study was lower than that observed in younger controls. Based on this observation, the authors concluded that impaired upregulation of HSP70 in heat-stressed, older animals resulted from an age-associated decline in heat-generating capacity rather than an age-related failure to mount a heat shock response. Subsequently, Pardue et al. (1992) performed a comparable study with young and old rats exhibiting similar body temperature changes following heat shock. Relative to younger thermal-stressed animals, induction of HSP70 mRNA was, nonetheless, blunted in dentate gyrus granule cells and pyramidal cells of the hippocampus in heat-stressed older rats. Few studies have examined the normal distribution of HSP

expression in the CNS of young versus old, unstressed animals. In one such study, Tytell et al. (1993b) assayed retinal HSP70 expression in young and old rats and could not detect an age-related difference between young and old animals. However, following hyperthermic treatment, levels of inducible HSP70 in older animals were significantly attenuated relative to younger animals (*ibid*; reviewed in Tytell, 1994).

There is a well documented reduction in the ability of older organisms to cope with stress and maintain vital homeostatic mechanisms under adverse conditions (Shock, 1977). Age-related impairments in the heat shock response system may contribute to the decreased ability of senescent organisms to mount adaptive responses under stressful conditions (Heydari et al., 1994). It has been suggested that inability of older cells to adequately promote the post-translational conversion of inactive HSF to its oligomeric DNA-binding form underlies this functional deficit (*ibid*). Specifically, a reduced capacity of senescent neural tissues to mount a cytoprotective heat shock response may be a factor predisposing the aging CNS to neurodegeneration.

A number of studies have examined the distribution of Ub in the normal and diseased human brain. Pappolla et al. (1989) demonstrated intensely Ub-positive deposits distributed throughout the white matter of normal, aged ($\geq$70 yrs), but not normal, young ($\leq$33 yrs) human brain. Although ultrastructural detection of the Ub deposits was not performed, under LM, Ub immunoreactivity appeared to localize to axonal elements. In another study, immunoelectron microscopy detected granular Ub-immunoreactivity within glia and myelin lamellae of white matter (Dickson et al., 1990). Ub-positive dystrophic neurites in cortical areas, and axonal spheroids in the substantia nigra and striatum were also found to accumulate with advancing age in normal subjects (*ibid*). Recently, an age-dependent accumulation of vacuole-laden, Ub-immunopositive astrocytes within the globus pallidus of normal human brain was demonstrated (Abe et al., 1994). The authors suggested that this was a normal age-related effect because numbers of these astrocytes were not further increased in basal ganglia derived from subjects with AD, PD, multiple system atrophy, or MS.

Corpora amylacea (CA) are glycoprotein-rich inclusions that accumulate in subpial and subependymal regions of the human brain as a consequence of aging, and are found in greater numbers in AD and other neurodegenerative conditions (reviewed in Cissé et al., 1993). Typically, CA have been localized to astrocytes or the extracellular matrix of affected brain regions (Anzil et al., 1974; Palmucci et al., 1982; Ramsay, 1965). They have also been observed, albeit to a lesser extent, within neuritic processes (Anzil et al., 1974; Yagashita and Itoh, 1977). CA from senescent and AD brain contain Ub-protein conjugates, and exhibit immunoreactivity for HSP28 (HSP27), HSP70, and HO-1 (Cissé et al., 1993; Schipper et al., 1995). Based on these observations, the authors suggest that CA in the aging and degenerating brain may derive from the accumulation of abnormal, glycosylated proteins resulting from sustained physiological stress.

The expression of Ub and various HSPs has been associated with a large number of pathologic structures found in neurodegenerative states. Of these, the neurofibrillary pathology of AD has been most extensively studied. Ub levels in the AD brain are noticeably higher than in non-demented age-matched controls (Wang et al., 1991). Initial studies revealed that Ub is a component of the neurofibrillary tangles (NFT) and senile plaques characteristic of AD (Mori et al., 1987; Perry et al., 1987). Relative to age-matched controls, HSP72 is dramatically overexpressed in AD brain, and, like Ub, exhibits colocalization to neuritic plaques and NFT (Hamos et al., 1991). HSP27 is similarly overexpressed in AD relative to non-demented controls (Renkawek et al., 1994a). Reactive atrogliosis is often illustrative of underlying neuropathology, and occurs in the AD brain. In AD, HSP27 was immunolocalized to degenerating reactive astrocytes, especially in areas rich in senile plaques, and exhibited occasional colocalization to NFT (*ibid*). In another study, HSP28 (HSP27) was increased over controls in the temporal, frontal and parietal lobes of AD and staining appeared over damaged neuronal elements and senile plaques (Shinohara et al., 1993). αB-crystallin-immunopositive hypertrophic astrocytes (*ibid*), as well as microglia, appeared more numerous in AD, relative to controls, and were concentrated in areas containing plaques and NFT (Renkawek et al., 1994b). In the AD brain, HO-1 immunostaining is dramatically increased in cortical and

hippocampal neurons and astrocytes relative to age-matched, non-demented controls and is found in association with the neuritic plaques and NFT (Smith et al., 1994; Schipper et al., 1995).

Several other neuropathological states are characterized by the accumulation of aberrant, cytoplasmic inclusions in neural cells. Many of these inclusions, including Lewy bodies in PD, Pick bodies in Pick's disease, and Rosenthal fibres in Alexander's disease, are ubiquitinated and may be associated with other stress proteins. A summary of stress protein-containing, neural inclusion bodies is presented in Table 1. However, much of the neuropathology associated with HSP expression may represent end-stage or "graveyard" pathology. In such cases, there is considerable difficulty resolving the cause and effect relationship between HSP induction and development of neuropathologic features. Regarding the ubiquitination of abnormal structures in neurodegenerative states, Wilkinson (1994) recently stated that "It is not known whether ubiquitin is present at the time of deposition or only detected late in the process as a reflection of the cells attempt to deal with this pathological situation." Due to the inherent difficulties in studying inclusion biogenesis using human tissue, there is considerable value in the generation of tissue culture and animal models in which the development of stress-related inclusions can be thoroughly investigated. The work described in this thesis focuses on the biogenesis of Gomori-positive astrocyte inclusions (Sections 3 and 4) in both primary culture and intact animal models. The primary objective of this thesis is to delineate the temporal features of astroglial inclusion biogenesis and the relationship of the latter to the cellular stress (heat shock) response.

**Table 1 Stress Protein-containing Neural Inclusion Bodies**

| Cell Type | Inclusion Body | Characteristic Clinical Condition | Location | Structural Protein | Stress Protein |
|---|---|---|---|---|---|
| ASTROCYTES | Corpora amylacea | Aging; AD | Subpial and periventricular regions | Unknown | Ubiquitin[a]; HSP27[a],72[a]; HO-1[b] |
| | Rosenthal fibres | Alexander's disease | Subpial and perivascular regions; white matter | GFAP | Ubiquitin; αB-crystallin; HSP27 |
| | Gliofibrillary inclusion | Infantile hypoxic encephalopathies | White matter | GFAP | Ubiquitin; αB-crystallin |
| | Gliofibrillary tangles | CBG; PSP | White matter | PHF-tau | Ubiquitin |
| NEURONS | Granulovacuolar bodies | Aging; AD | Hippocampus | Tau, NF | Ubiquitin; HO-1[c] |
| | Marinesco bodies | Aging | Substantia nigra | Unknown | Ubiquitin |
| | Neuroaxonal dystrophy | Aging | Dorsal column nuclei; substantia nigra; globus pallidus | Unknown | Ubiquitin |
| | Dystrophic neurites | Aging; AD & prion diseases | Associated with senile plaques | Unknown | Ubiquitin; HO-1[b] |
| | Dystrophic neurites | DLBD | CA2/3; basal forebrain; brainstem nuclei | NF | Ubiquitin |
| | Cortical Lewy bodies | DLBD | Limbic & para-limbic cortices; amygdala | NF | Ubiquitin; αB-crystallin; |
| | Ballooned neurons | CBG; Pick's disease | Limbic & para-limbic cortices; amygdala | NF | Ubiquitin; αB-crystallin; HSP27[d] |
| | Bunina bodies | ALS | Anterior horn cells | occasionally NF | Ubiquitin[e] |

Table 1 continued

| Cell Type | Inclusion Body | Characteristic Clinical Condition | Location | Structural Protein | Stress Protein |
|---|---|---|---|---|---|
| NEURONS | Lewy bodies | PD; DLBD | Substantia nigra; brainstem monoaminergic; basal forebrain | NF | Ubiquitin; αB-crystallin |
| | Lewy body-like inclusions in ALS | ALS-sporadic and familial | Anterior horn cells | NF | Ubiquitin; αB-crystallin |
| | Neurofibrillary tangles & neuropil threads | AD; PSP | Cortex; hippocampus; basal forebrain; brainstem nuclei | PHF-tau | Ubiquitin; HO-1[b,e] |
| | Pick bodies | Pick's disease | Hippocampal dentate fascia; cerebral cortex | PHF-tau | Ubiquitin |
| | Neuropil grains | CBG; Dementia with grains | Cortex; hippocampus | PHF-tau | Ubiquitin |
| | Ubiquitin reactive inclusions | Dementia lacking distinctive histopathology | Cortex (small neurons in layer 2); dentate fascia | Unknown | Ubiquitin |
| OLIGODEN-DROGLIA | Granular degeneration of myelin | Aging | Brain and spinal cord myelin | Unknown | Ubiquitin |
| | Glial cytoplasmic inclusions | Multisystem atrophy | White matter | PHF-tau | Ubiquitin; αB-crystallin[f] |

**TABLE 1 is modified after Dickson and Yen, 1994. Additional data are referenced. Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CBG, corticobasal ganglionic degeneration; DLBD, diffuse Lewy body disease; GFAP, glial fibrillary acidic protein; NF, neurofilament; PD, Parkinson's disease; PHF, paired helical filaments; PSP, progressive supranuclear palsy**

References: a: Cissé et al, 1993
b: Schipper et al, 1995
c: Smith et al, 1994
d: Kato et al, 1992
e: Migheli et al, 1994
f: Tamaoka et al, 1995

## CHAPTER 2

**Role of the cellular stress response in the biogenesis of cysteamine-induced astrocytic inclusions in primary culture.**

Authors: Mydlarski MB, Liang J-J, and Schipper HM (1993)

Published in: J Neurochem (1993) 61:1755-1765.

Key words: Astrocyte, Cysteamine, Heme oxygenase, Heat shock proteins, Glucose regulated protein, Peroxidase activity.

## ABSTRACT

Cysteamine (CSH; 2-mercaptoethylamine) stimulates the accumulation of peroxidase-positive inclusions in cultured astroglia akin to those observed in the aging periventricular brain. Because CSH induces the synthesis of a stress protein (heme oxygenase) in rat liver, we hypothesized that aspects of the cellular stress response may play a role in the biogenesis of CSH-induced astrocyte granules. In the present study, we performed indirect immunofluorescent staining and immunoblotting for various stress proteins in rat neuroglial cultures. Exposure of astrocyte cultures to CSH enhanced immunostaining for heme oxygenase-1 (HO-1) and heat shock proteins 27, 72, and 90, but not glucose-regulated protein 94, relative to untreated cultures. CSH-pretreated astrocytes exhibited enhanced tolerance to $H_2O_2$ toxicity relative to untreated cells, providing physiological evidence of an antecedent stress response in the former. In addition, exposure for 12 days exposure to $H_2O_2$, a known inducer of the stress response, elicited astrocyte granulation similar to that observed with CSH. Chronic induction of HO-1 and other stress proteins may participate in the biogenesis of metalloporphyrin-rich inclusions in CSH-treated astroglial cultures and in astrocytes of the aging periventricular brain.


**Abbreviations used:** CSH, cysteamine (2-mercaptoethylamine); DAB, 3,3'-diaminobenzidine; DIV, days *in-vitro*; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; GRP, glucose-regulated protein; HO-1, heme oxygenase-1; HSP, heat shock protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

# INTRODUCTION

A unique subpopulation of granule-laden neuroglia has been described in hippocampus, striatum and periventricular brain regions of many terrestrial vertebrates including humans (Schipper, 1991). The cytoplasmic granules are histochemically distinct from lipofuscin, exhibit an affinity for Gomori stains (chrome alum hematoxylin, aldehyde fuchsin), emit an orange-red autofluorescence consistent with the presence of porphyrin, and stain intensely with the peroxidase marker, 3,3'-diaminobenzidine (DAB) (Srebro and Cichocki, 1971; Keefer and Christ, 1976; Goldgefter et al., 1980). The endogenous peroxidase activity is non-enzymatic in nature and is felt to be mediated by heme (ferrous) iron or by a related metalloporphyrin (Schipper et al., 1990a). In rodent brain, the astrocytic inclusions accumulate with aging (Schipper et al., 1981) and in response to X-irradiation (Srebro 1971) and chronic estrogen exposure (Brawer et al., 1980, 1983; Schipper et al., 1990a).

In dissociated rat fetal and neonatal brain cell cultures, astroglial granules with identical tinctorial and histochemical properties progressively accumulate with time *in vitro* and can be massively induced by exposure to the sulfhydryl agent, cysteamine (CSH; 2-mercaptoethylamine) (Schipper et al., 1990b). The presence of iron-mediated DAB oxidation has been confirmed in CSH-induced astroglial inclusions at the ultrastructural level using energy-dispersive microprobe analysis in conjunction with peroxidase cytochemistry (McLaren et al., 1992). We demonstrated that the pseudoperoxidase activity is capable of oxidizing dopamine and other catechols to their respective orthosemiquinone radicals suggesting that Gomori-positive astrocytes may promote free radical-related neurodegeneration in the aging nervous system (Schipper et al., 1991). In the present study, we attempted to delineate the mechanism(s) responsible for the accumulation of these iron-rich astrocytic organelles. Because CSH induces a stress protein (heme oxygenase EC 1.14.99.3) in rat liver (Peterson et al., 1989), we hypothesized that chronic or repeated activation of the cellular stress response may participate in the biogenesis of CSH-induced astrocyte granules. To test this hypothesis,

we determined whether (a) CSH induces the expression of various stress proteins in cultured astroglia, (b) CSH-treated astroglia exhibit relative resistance to $H_2O_2$ toxicity consistent with an antecedent stress response, and (c) chronic $H_2O_2$ exposure, a known inducer of the cellular stress response, stimulates the accumulation of peroxidase-positive astroglial inclusions.

## MATERIALS AND METHODS

### Materials

Sprague Dawley neonatal rats were obtained from Charles River. Ham's F12, high glucose Dulbecco's modified Eagle's medium (DMEM), heat-inactivated horse serum, and fetal calf serum were purchased from Gibco (Grand Island, N.Y). Poly-D-lysine, penicillin-streptomycin, CSH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DAB were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies for heat shock proteins (HSPs) 27, 72, and 90 and glucose-regulated protein (GRP) 94 were obtained from StressGen Biotechnologies Co. (Victoria, B.C.). Monoclonal and polyclonal anti-glial fibrillary acidic protein (anti-GFAP) antisera were kindly provided by Dr. E. Wang of our institution. Fluorescein isothiocyanate (FITC)-conjugated goat-derived monoclonal and polyclonal IgG antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA). Rhodamine-conjugated, goat-derived monoclonal and polyclonal IgG antibodies were purchased from Organon Teknika Cappel (West Chester, PA). Rabbit-derived polyclonal antibody raised against rat liver heme oxygenase-1 (HO-1) was kindly provided by Dr. T. Yoshida (Yamagata University, Yamagata, Japan).

### Brain Cell Cultures

Primary neuroglial cultures were prepared by mechanoenzymatic dissociation of cerebral tissue derived from 2-day-old Sprague Dawley rats as previously described (Schipper and Mateescu-Cantuniari, 1991). Cells ($3.5 \times 10^5$/mL) were plated on eight-chamber (LabTek) culture slides precoated with 0.01% poly-D-lysine, on Primaria 24 multiwell plates, and on 100 mm Primaria dishes. Cells were grown in Ham's F12 and DMEM (50:50 v/v) supplemented with 10 mM HEPES, 5% heat-inactivated horse serum, 5% fetal calf serum, and penicillin-streptomycin (50 U/mL and 50 µg/mL, respectively). The cultures were incubated at 37°C in humidified 95% and 5% $CO_2$, and culture medium was changed twice weekly. Cultures were either left untreated (control), or received 880 µM CSH or

0.1 mM $H_2O_2$ with each change of culture medium between 6 and 18 days *in vitro* (DIV).

Immunofluorescence

Indirect immunofluorescence for HSPs 27, 72, and 90, GRP94 and HO-1 was performed on DIV 6 following exposure for 6 h to CSH. Cell monolayers were washed in 0.1 M phosphate-buffered saline (PBS; pH 7.2) before fixation. Cultures fixed in methanol at -20°C for 5 min were subsequently incubated for 90 min at room temperature with anti-HSPs 27, 72 (inducible), and 90 or anti-GRP94 monoclonal antibody diluted 1:200 in 0.15% bovine serum albumin-phosphate-buffered saline (pH 7.2). The secondary antibody consisted of FITC-conjugated goat anti-mouse IgG (1:100 dilution) applied for 30 min at room temperature. Astroglia were identified by subsequent immunolabeling with rabbit-derived anti-GFAP antiserum (1:100 dilution) for 30 min at room temperature followed by rhodamine-conjugated, goat anti-rabbit IgG (1:200 dilution) for 30 min at room temperature. Other astroglial cultures fixed in methanol/acetone (50:50, v/v) at -20°C for 5 min were permeabilized in 2% Triton prior to incubation at room temperature for 1 h with anti-HO-1 polyclonal antibody diluted 1:50 in PBS containing 0.15% bovine serum albumin. The secondary antibody consisted of FITC-conjugated goat anti-rabbit IgG (1:200 dilution) applied for 30 min at room temperature. Colocalization of HO-1 immunoreactivity to astrocytes was performed using a mouse-derived anti-GFAP monoclonal antibody and laser scanning confocal microscopy (BioRad). Nonspecific mouse ascites fluid and normal rabbit sera served as controls for monoclonal and polyclonal antibodies, respectively. The monolayers were rinsed in PBS, mounted in PBS/glycerol (50:50, v/v), and examined and photographed under epifluorescence using a Leitz Diaplan microscope equipped with appropriate filters.

Gel Electrophoresis and Immunoblotting

Following 6 h exposure to CSH or $H_2O_2$, astrocyte monolayers were washed with ice cold PBS and gently scraped with a rubber policeman. The cell suspensions were spun at 1000

rpm (112 g) for 5 min and the cell pellets were solubilized in 5 volumes of 4% SDS sample buffer by passage through 21- and 25-gauge needles. The samples were heated to 100°C for 5 min, cooled at room temperature and centrifuged for 10 min at 10,000 rpm (6720 g). SDS/PAGE was carried out according to the method of Laemmli (1970) using 7-12.5% polyacrylamide separating gels and 4% stacking gels. Quantitation of protein was performed by the method of Lowry et al. (1951). One hundred µg of total cell protein was loaded onto each lane. The proteins were stained with Coomassie blue (0.1%) or were electrophoretically transferred to nitrocellulose membranes according to the technique of Towbin et al. (1979). The nitrocellulose membranes were blocked with 0.5% Tween-20 in Tris-buffered saline (0.02 M Tris/HCl, pH 7.2, 0.5 M NaCl) at room temperature for 1 h and incubated overnight at 4°C with anti-HSPs 27, 72, and 90, or GRP 94 (1:1000 dilution) or anti-HO-1 (1:100 dilution). For HO-1 staining, the nitrocellulose paper was washed several times and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. For staining of the other stress proteins, washed nitrocellulose membranes were incubated in rabbit anti-mouse IgG solution and then incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1-h incubations at room temperature). The visualization medium consisted of 50 mg/100 ml of 4-chloro-1-naphthol and 5.3 mM $H_2O_2$ in Tris-buffered saline.

Cytotoxicity

Phase contrast microscopy

On DIV 18, cell monolayers were rinsed with PBS, incubated for 24 h in serum-free culture medium to eliminate exogenous CSH and serum-derived antioxidants, and subsequently exposed for an additional 24 h to serum-free media containing 0, 0.1, 1.0 or 10.0 mM $H_2O_2$. The cultures were examined for cytotoxicity using phase contrast microscopy and photographed (Khera and Whalen, 1988).

## MTT cytotoxicity assay

The tetrazolium salt (MTT) assay, based on the ability of living cells to convert MTT into an insoluble blue colored formazan (Mosmann, 1983), was used as a quantitative index of cell viability following exposure to $H_2O_2$ (modified after the method of Manthorpe et al., 1986). The assay was performed on astrocytes pretreated with CSH for either 6 h (on DIV 6; short-term CSH) or 12 days (DIV 5-18; long-term CSH) and on untreated controls. $H_2O_2$ in serum-free media was serially diluted in 96-well microplates. Following a 24 h wash in serum-free media, the cells were trypsinized and 8 x $10^5$ cells/mL was added to 96-well microplates containing varying concentrations of $H_2O_2$ (0-6.25 mM). Blanks consisted of equimolar concentrations of $H_2O_2$ without cells. Cells were incubated at 37°C for 22 h prior to addition of tetrazolium (final concentration, 1 mg/mL) and maintained at 37°C for an additional 2 h. Formazan solubilization was performed as described by Hansen et al. (1989). Spectral absorbance at 570 nm was determined using an automated microwell multiscanning spectrophotometer (THERMO-MAX, Molecular Devices Corp). Statistical analysis of the MTT results was performed using the Statistical Package for the Social Sciences (SPSS 4.1). Multifactorial two-way ANOVA, followed by Student-Newman-Keuls post hoc test, was used to determine statistical significance at the 0.050 level.

## HPLC analysis

Concentrations of intracellular CSH and its oxidized disulfide form, cystamine, were assayed in the treated cultures immediately before and following the 24-h washout period using a Hewlett Packard (HP1090) liquid chromatograph with a Bio Analytical System LC-4B dual amperometric detector. The upstream (reductive) and downstream (oxidative) Hg/Au electrodes were operated at potentials of -1.1 and 0.24 V, respectively, with a range setting of 100 nA. The column was a reverse-phase (200 x 2.1 mm) Adsorbosphere HS C-18, (particle size, 5 µm; home-lab-packed). Mobile phase composition was 0.05 M $KH_2PO_4$, 0.4 M l-octane sulfonic acid, 0.1 mM EDTA, pH 2.93 (with $H_3PO_4$). The flow

rate was 0.2 mL/min with column temperature maintained at 48°C. Standard peaks were determined for CSH- and cystamine-$dH_2O$ with 10 mM HCl. Cultured monolayers were scraped off the Petri dish surface in PBS and centrifuged for 3 min at 1000 RPM (112 g). Cell pellets were homogenized in 2.7 mM $Na_2$ EDTA. After addition of 0.2 M $HClO_4$ and centrifugation at 10,000 RPM (6720 g) for 10 min, supernatants were collected, passed through BAS MF-1 microfiltration system with a NYLON 66 filter (pore size, 0.2 µm), and injected (5-µL volumes) into the chromatograph. Cell pellets were used for protein quantification (Lowry, 1951).

DAB Histochemistry

On DIV 18, cell cultures treated with 0.1 mM $H_2O_2$ and control monolayers were rinsed in PBS and fixed in 4% paraformaldehyde for 30 minutes at room temperature. Staining of the cultures for endogenous peroxidase activity was performed using modified Karnovsky medium consisting of 0.05% DAB and 0.09% $H_2O_2$ in 0.1 M PBS, as previously described (Schipper et al., 1990b).

## RESULTS

### Immunofluorescence

Untreated (control) astroglial cultures exhibited faint or no staining for HSPs 27, 72, and 90, and moderate cytoplasmic staining for GRP 94 and HO-1. In contrast, GFAP-positive astroglia exposed to CSH for 6 h showed strong immunostaining for HO-1 and HSPs 27, 72, and 90, whereas staining for GRP 94 remained unchanged from controls (Fig. 1). The foamy cytoplasmic appearance in HO-1 immunostained cultures is consistent with immunolocalization of the enzyme to the endoplasmic reticulum (Muller-Eberhard and Nikkilä, 1989). Colocalization of HO-1 immunoreactivity to GFAP-expressing astrocytes was confirmed using laser scanning confocal microscopy (data not shown). Immunofluorescent labeling of HO-1, and HSPs 27 and 72 was characterized by heterogeneous cytoplasmic staining with prominent nuclear rim intensification. Cells expressing HSP 90 exhibited both diffuse and punctate cytoplasmic fluorescence with occasional perinuclear enhancement.

### Gel electrophoresis and immunoblotting

In the Coomassie Brilliant Blue-stained gels, exposure for 6 h to CSH consistently induced novel protein bands at approximately 27 and 90 kDa (Fig. 2). In contrast, a 94-kDa band was present in control preparations and did not appear to change with CSH treatment. Distinct 32- (HO-1) and 72-kDa bands could not be clearly discerned in the treated and control preparations. Many additional bands normally seen in control gels were either diminished or completely eliminated by CSH treatment. A broad 55-kDa band was inconsistently observed in the CSH-treated gels, which may represent induction or accumulation of cytoskeletal proteins.

In western blots of untreated astrocyte cultures, HSPs 27 and 90 were consistently undetectable and there was occasional faint staining of HSP 72 and moderate levels of HO-1 (Fig. 2). In contrast, the CSH-treated cells exhibited prominent staining for HSPs

27 and 90, and increased staining of HSP 72 (Fig. 2). Treatment of cultures with either CSH or $H_2O_2$ significantly increased the HO-1 protein band intensity relative to controls. CSH induction of band triplets of HSP 27 probably represents various phosphorylation states of the activated protein in this system (Crete and Landry, 1990; Zhou et al., 1993). In accord with the immunofluorescence data, moderate expression of GRP 94 was noted in immunoblots of control cultures and did not appear to be influenced by CSH exposure (Fig. 2). We observed a progressive increase in HSP 27, 72, and 90 expression in untreated cultures from DIV 6 to 18, although HSP immunostaining was generally more robust in CSH-exposed cells at any given time point (data not shown).

Cytotoxicity studies

Phase microscopy

On DIV 20, in the absence of $H_2O_2$ exposure, both control and CSH-treated cultures exhibited healthy confluent monolayers composed of phase-dark, flat, and process-bearing astrocytes and moderate numbers of rounded cells (macrophages and oligodendroglia) overlying the astroglial substratum (Fig. 3) (Khera and Whalen, 1988). Control cultures exposed to concentrations of $H_2O_2$ between 0.1 mM and 10.0 mM showed progressive cytotoxicity characterized by retraction of processes, cytoplasmic shrinkage, formation of phase-bright plaques, and detachment of cells from the monolayer surface (Fig. 3). In contrast, cytotoxicity was significant in the CSH-pretreated cultures only with the 10 mM $H_2O_2$ dose and was less severe than that observed in control cultures at 1.0 mM $H_2O_2$ (Fig. 3).

MTT assay

To control for variations in numbers of CSH-exposed and control cells and potential density-dependent cytotoxicity (Spitz et al., 1987), an MTT cytotoxicity assay was performed using equivalent numbers of control and CSH-treated astroglia. In the long-term CSH experiment (see Materials and Methods), even in the absence of $H_2O_2$, CSH-

pretreated cells exhibited significantly greater viability after trypsinization and reseeding into serum-free medium relative to control cells. Moreover, in the control tissue, a significant decline in cell viability first occurred at 0.4 mM $H_2O_2$, whereas significant cytotoxicity in CSH-treated cells only became apparent with the 0.8 mM dose (Fig. 4). The MTT cytotoxicity assay was repeated using the short-term CSH protocol (see Materials and Methods) to correlate $H_2O_2$ resistance with the initial induction of HSPs. In the absence of $H_2O_2$, CSH-pretreated astroglial cultures again displayed significantly greater viability than the control cultures following trypsinization and reseeding into serum-free medium. However in contrast to the long-term CSH experiment, the first significant decline in cell viability occurred in both control and CSH-pretreated cultures at 0.05 mM $H_2O_2$ and further increases in $H_2O_2$ concentration yielded similar declines in cell viability for both groups (Fig. 4).

HPLC analysis

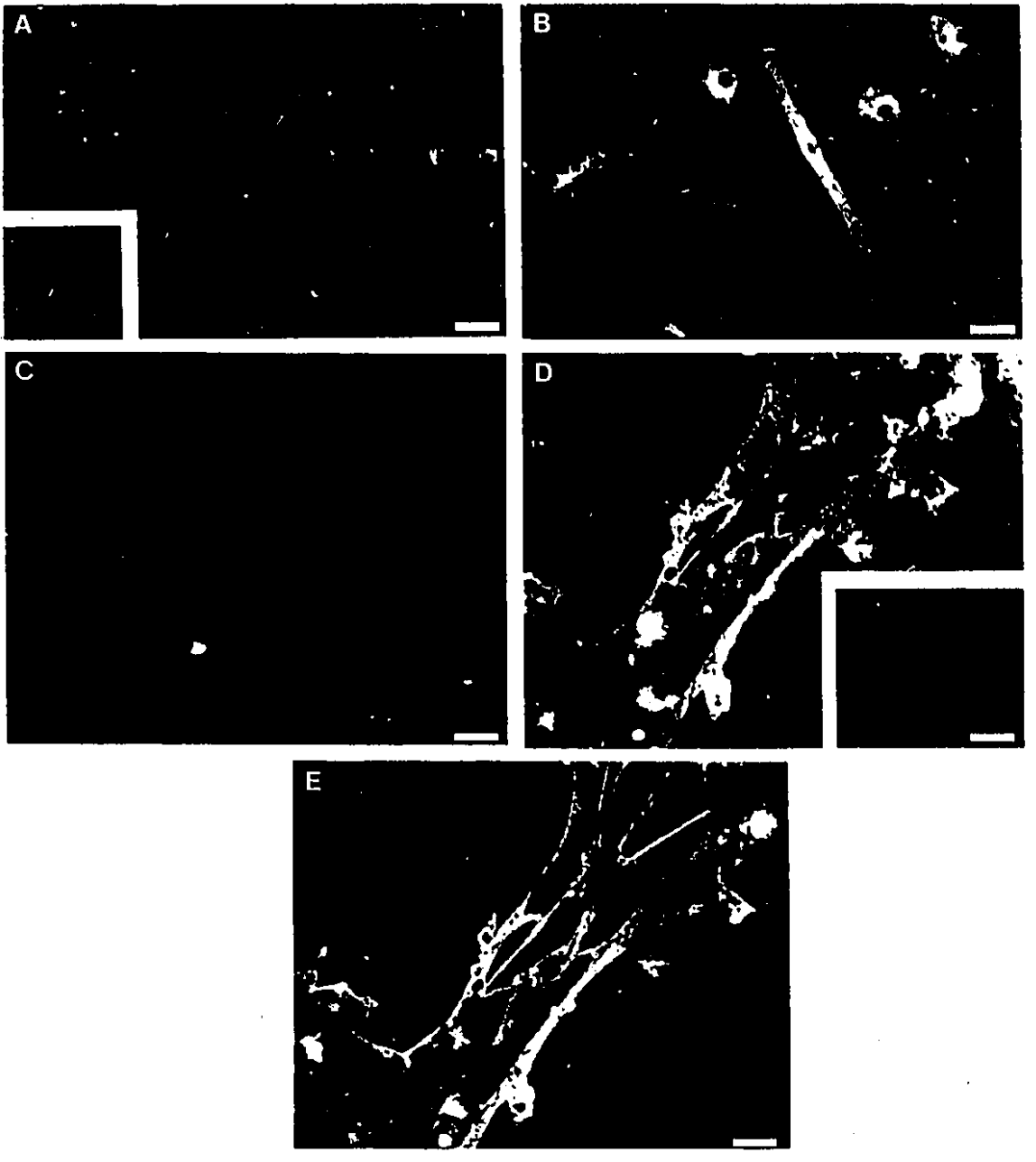
In standard solutions, peaks for CSH and its oxidized form, cystamine, were readily detected by HPLC at retention times of 3.85 and 11.64 min, respectively. In the absence of a washout period, CSH was detected in cell homogenates derived from CSH-treated (0.43 ng/μg total cell protein, mean ± SEM of 3 samplings; range = 0.29-0.5 ng/μg), but not control, cultures on DIV 18. However, following a 24-h washout period, intracellular CSH could no longer be detected in the CSH-pretreated preparations. Cystamine, the disulfide form of CSH, was not detectable in any cell homogenates either before or after washout. Although the potential formation of CSH-derived mixed disulfides was not monitored, the HPLC results strongly argue against the possibility that CSH or cystamine serves as a direct cytoprotectant in the $H_2O_2$ toxicity assays.

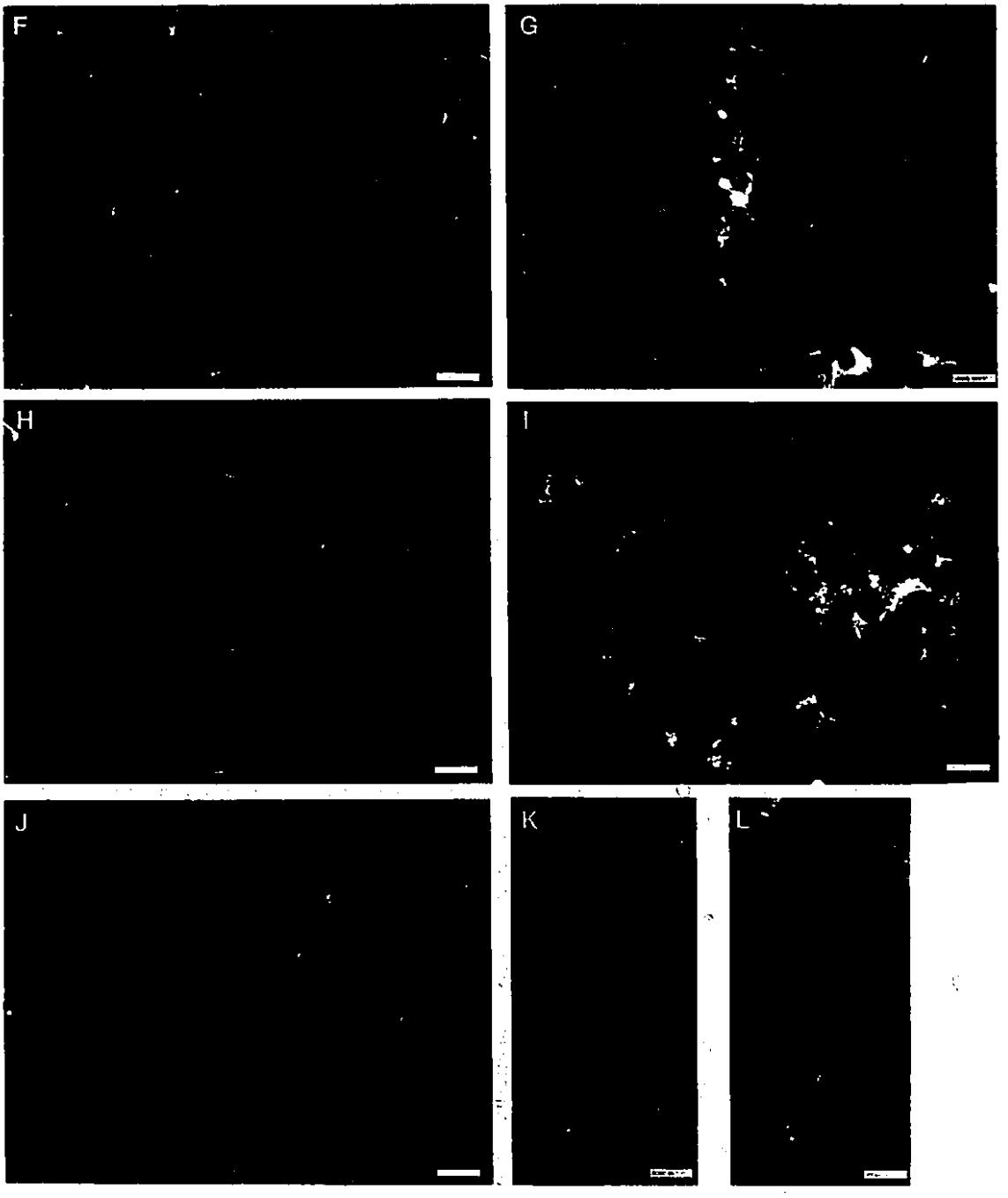
DAB histochemistry

Long-term exposure of astroglial cultures to $H_2O_2$ elicited a robust accumulation of DAB/peroxidase-positive cytoplasmic inclusions in comparison with untreated controls

(Fig. 5). DAB staining in these cells is similar to that observed in cultured astroglia following prolonged CSH treatment (Schipper et al., 1990b).

**Fig. 1** Immunofluorescent labeling of stress proteins and GFAP in CSH-treated (880 μM x 6 h) and control glial cultures. Shown are control and CSH-treated cultures stained for HO-1 (**A** and **B**, respectively), HSP27 (**C** and **D**, respectively), HSP72 (**F** and **G**, respectively), HSP90 (**H** and **I**, respectively) and GRP94 (**J** and **K**, respectively). With the exception of GRP94 (which is constitutively present), CSH induces the expression of stress proteins in these cultures. Cells immunoreactive for HSP27 (**D**) and GRP94 (**K**) are shown to be GFAP-positive astrocytes in (**E**) and (**L**), respectively. No immunostaining is observed when normal rabbit serum is substituted for anti-HO-1 (**A**; inset) or non-specific mouse ascites fluid for anti-HSPs 27,72,90 and anti-GRP94 (**D**, inset). Bar = 25 μm.


A
B
C
D
E


F
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L

**Fig. 2** Protein electrophoresis and immunoblotting for stress proteins in control, CSH-treated (880 µM x 6 h) and $H_2O_2$-treated (0.1 mM x 6 h) astroglial cultures. **A:** Coomassie Blue stained-SDS-PAGE of control (lane 2) and CSH-treated (lane 3) preparations. After CSH treatment, certain protein bands disappear, new bands are visible at 27 and 90 kDa, and a band at 94 kDa remains unchanged. Molecular weight markers (lane 1). **B:** Western blots for HO-1 reveal that CSH (lane 2) and $H_2O_2$ (lane 3) induce the 32 kDa HO-1 protein relative to control levels (lane 1). CSH also induces HSP27 (**C**, lane 2), HSP72 (**D**, lane 2) and HSP90 (**E**, lane 2) relative to controls (lane 1 in c, d, and e, respectively). GRP94 staining is seen in untreated material and remains unchanged following CSH exposure (**F**, lane 1 and 2, respectively). Primary antibody was also replaced with non-specific mouse ascites fluid (D-F, lane 3). M denotes molecular mass markers (C and F).

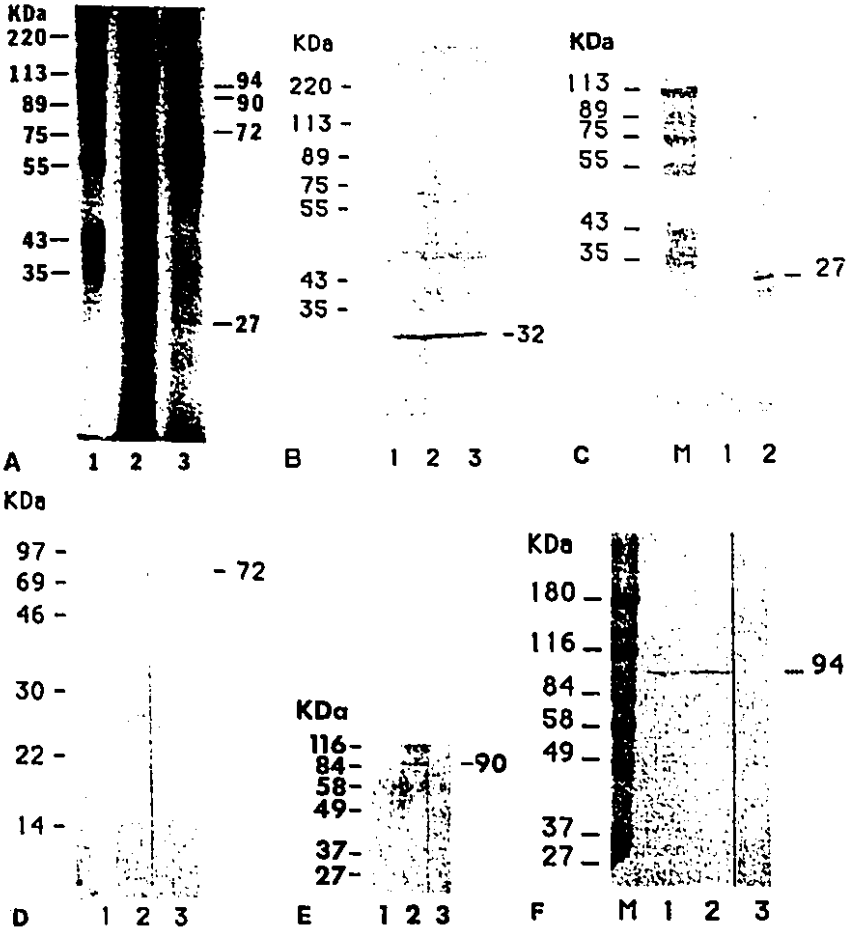
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**Fig. 3** Phase contrast micrographs depict cytotoxic effects of $H_2O_2$ on CSH-pretreated (880 µM, DIV 6-18) and control astrocyte cultures. Control and CSH-pretreated monolayers were exposed for 24 h to 0 (**A** and **B**, respectively), 0.1 mM (**C** and **D**, respectively), 1.0 mM (**E** and **F**, respectively) or 10 mM (**G** and **H**, respectively) $H_2O_2$. Progressive monolayer disruption is observed in the controls between 0.1 and 10.0 mM $H_2O_2$. In contrast, CSH-pretreated cultures appear relatively healthy at 0.1 and 1.0 mM $H_2O_2$, and toxicity at 10.0 mM (H) is less severe than that observed in controls at 1.0 mM $H_2O_2$ (E). Bar = 100 µM.

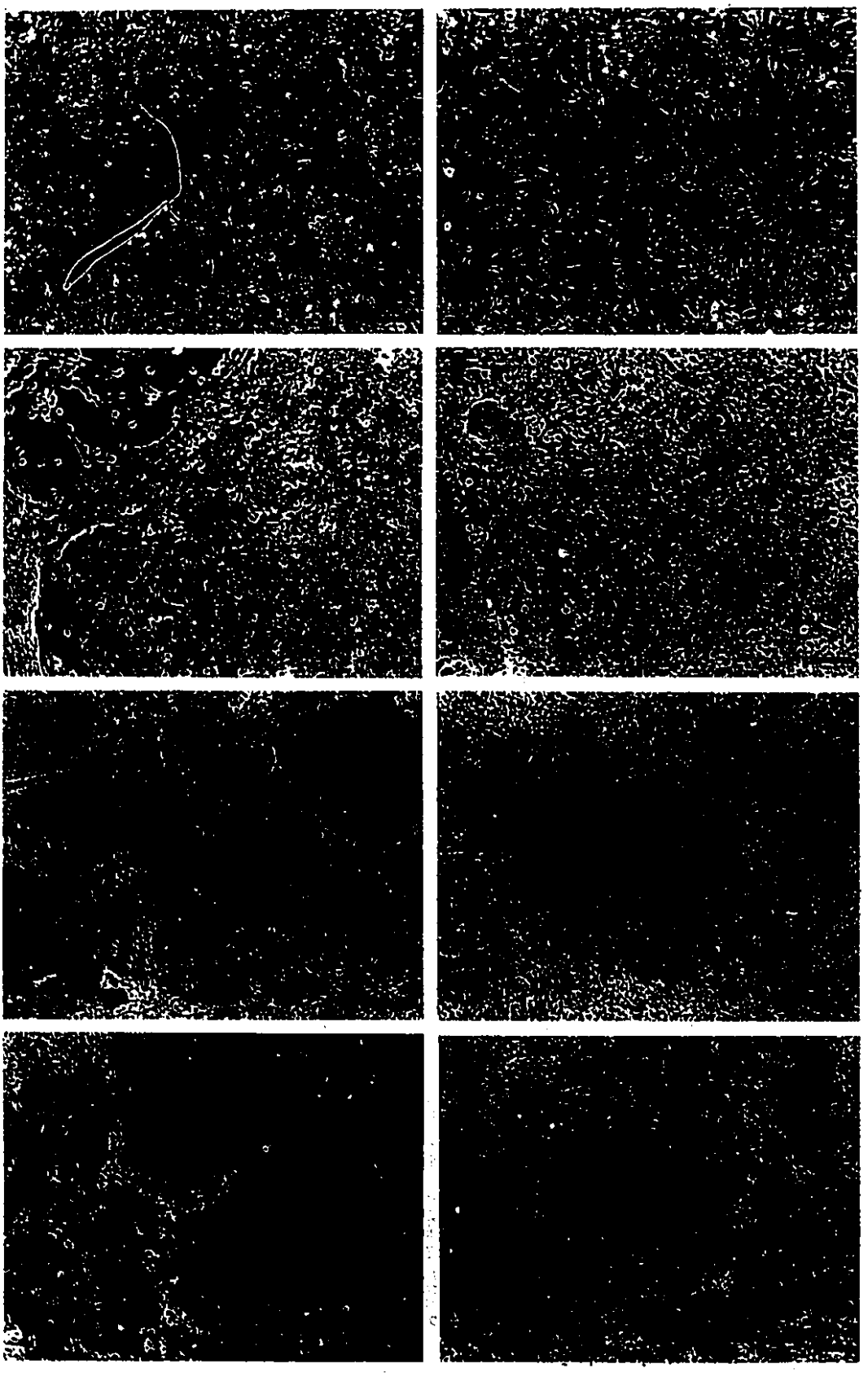
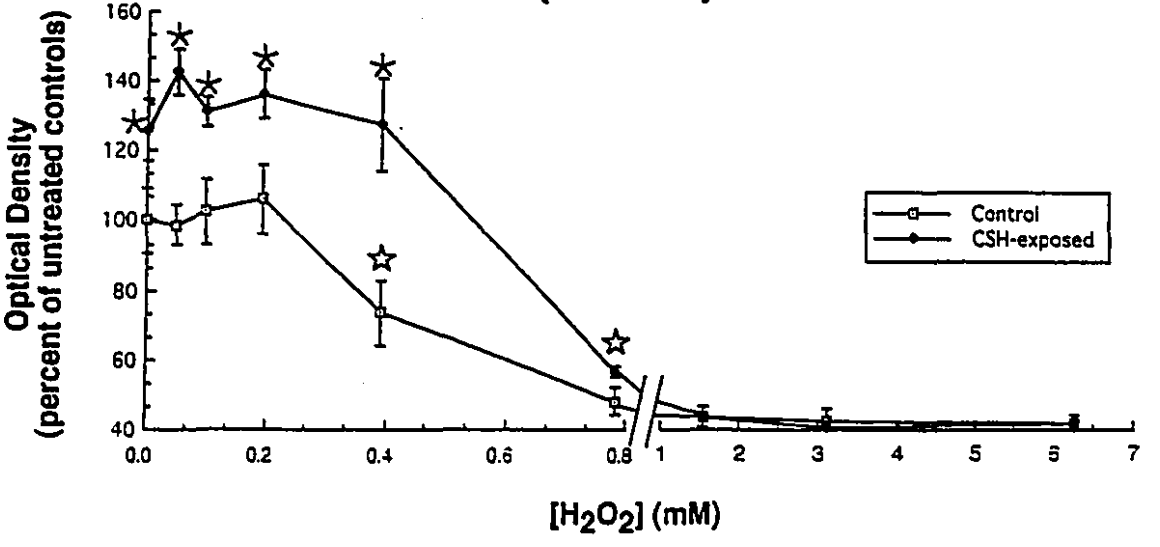


**Fig. 4** Automated MTT cell viability assay depicts cytotoxic effects of trypsinization and $H_2O_2$ exposure on fixed numbers (40,000 cells/well) of control and CSH-pretreated astrocytes. Optical density correlates directly with cell viability. **A:** Long-term CSH exposure (880 µM, DIV 6-18). CSH-pretreated cells (diamonds) exhibit increased resistance to mechanical trauma and $H_2O_2$ exposure relative to controls (squares). **B:** Short-term CSH exposure (880 µM x 6 h). As in A, CSH-pretreated cells (diamonds) exhibit robust resistance to mechanical trauma relative to controls (squares). However, normalizing for the effects of mechanical stress, both groups show similar declines in cell viability with increasing $H_2O_2$ concentrations. An asterisk denotes a significant difference from control values ($p \leq 0.050$ by Student-Newman-Keuls post hoc test). An open star denotes first significant decline in cell viability relative to respective conditions at 0 mM $H_2O_2$ ($p \leq 0.050$, Student-Newman-Keuls).

A

# MTT Cell Viability Assay (DIV 18)

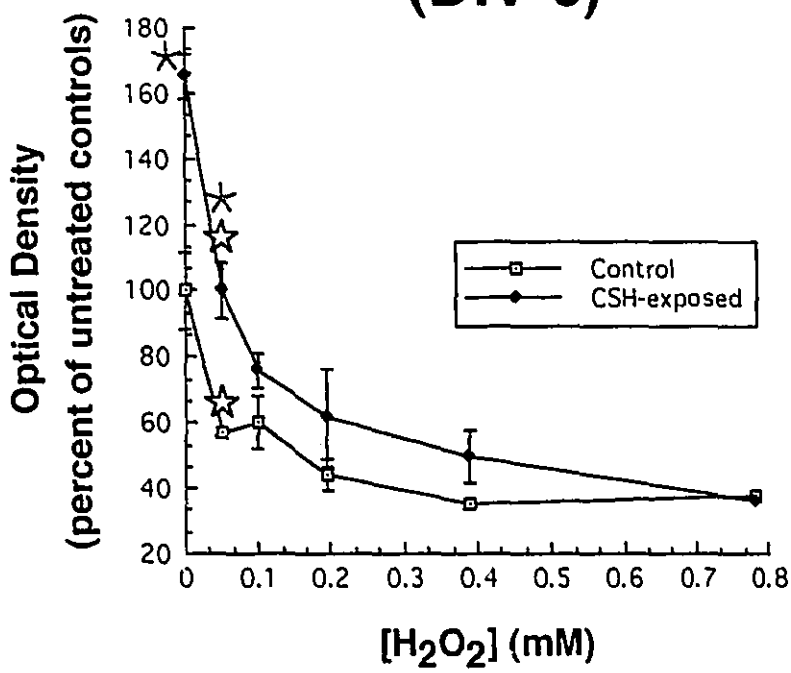



☆ indicates significant difference from controls at same dose ($p \leq 0.050$)

☆ indicates significant decline in cell viability relative to respective conditions at 0 mM $H_2O_2$ ($p \leq 0.050$)

B

# MTT Cell Viability Assay (DIV 6)




✳ indicates significant difference from controls at same dose ($p \leq 0.050$)

☆ indicates significant decline in cell viability relative to respective conditions at 0 mM $H_2O_2$ ($p \leq 0.050$)

**Fig. 5** Effects of repeated $H_2O_2$ exposure on peroxidase activity in cultured astroglia. $H_2O_2$ treatment (0.1 mM, DIV 6-18) results in massive accumulation of peroxidase-positive cytoplasmic inclusions in comparison with untreated controls (**inset**). DAB stain. Bar = 25 μM.

## DISCUSSION

In dissociated fetal or neonatal astroglial cultures, administration of CSH between 6 and 18 DIV induces the accumulation of cytoplasmic granules with histochemical and morphological features identical to those which characterize a subpopulation of glia in the aging periventricular brain. Thus, this sulfhydryl compound appears to accelerate aging-related phenotypic changes in these cells (Schipper et al., 1990b). As *in situ*, the accumulation of porphyrins and heme is thought to be responsible for the orange-red autofluorescence and non-enzymatic (ferrous iron-mediated) peroxidase activity in the CSH-induced inclusions, respectively (Schipper et al., 1990b; McLaren et al., 1992). In the intact aging brain and in the CSH-treated cultures, the origin of the astrocytic granules and mechanisms responsible for their biogenesis remain obscure. The results of the present study indicate that CSH induces HO-1 and various HSPs in cultured astroglia before granulation occurs. These observations suggest that the development of the astrocytic organelles may be dependent on an antecedent cellular stress response.

CSH induces derangements in porphyrin-heme metabolism in liver (Peterson et al., 1989) and may influence metalloporphyrin biosynthesis and sequestration in astrocytes (Schipper et al., 1990b). In rat liver, CSH induces HO-1 (Peterson et al., 1989) resulting in a rapid conversion of heme to biliverdin (Tenhunen et al., 1969; Maines and Kappas, 1974). In response to acute cellular stress, induction of HO-1 may protect cells by catabolizing pro-oxidant metalloporphyrins such as heme (Misra and Fridovich, 1972; Gutteridge, 1987) to bile pigments with free radical-scavenging capabilities (Stocker, 1990; Applegate et al., 1991). Conversely, the results of the present study suggest that chronic or repeated stress may result in complex dysregulation of the genes subserving porphyrin-heme biosynthesis leading to the progressive accumulation of redox-active metalloporphyrins (Maines and Kappas, 1975; Schipper et al., 1990b).

The HO-1 gene has a heat-shock element in its promoter region (Pelham, 1985; Müller et al., 1987) and is up-regulated by metal ions, ionizing radiation, sulfhydryl reactive

agents and $H_2O_2$ as part of a concerted cellular stress response (Keyse and Tyrell., 1987, 1989; Applegate et al., 1991; Dwyer et al., 1992). In the present study, we observed that exposure of astrocyte cultures to CSH for 6 h induced HSPs 27, 72, and 90 in addition to HO-1. The intensification of perinuclear staining for HSPs 27, 72 and 90 noted in CSH-exposed astrocytes is consistent with reports of translocation of these stress proteins from cytoplasmic to nuclear compartments in response to heat shock (Welch and Feramisco, 1984; Welch and Suhan, 1986; Arrigo and Welch, 1987; Kochevar et al., 1991).

CSH-induced HSPs in cultured astroglia may be responsible for the relative cytoprotection from mechanical and $H_2O_2$ damage as determined by phase contrast microscopy and a sensitive MTT cell viability assay. In many tissues, the increased expression of HSPs associated with a sublethal insult confers enhanced resistance to a host of subsequent stressors (Li and Hahn, 1978; Li and Werb, 1982; Spitz et al., 1987; Morimoto et al., 1990; Oesterreich et al., 1991). HSPs may protect cells undergoing stress by prevention of damage to the translational apparatus (Liu et al., 1992), maintenance of lipid membrane integrity (Burdon et al., 1987), accelerating degradation of denatured and abnormal proteins (Ananthan et al., 1986) and prevention of deleterious protein aggregation by prior binding to exposed hydrophobic surfaces (Finley et al., 1984).

The specific CSH-induced stress protein(s) directly responsible for subsequent $H_2O_2$ resistance in astrocytes remain(s) to be determined. Huot et al. (1991) demonstrated that transfected fibroblasts over-expressing HSP27 were more resistant to treatments with $H_2O_2$, sodium arsenite, heat and certain anti-cancer agents relative to nonover-expressing transfected controls. Astrocytes assessed for cytotoxicity at 24 h following the initiation of CSH exposure, long before granulation occurs, exhibited robust resistance to mechanical stress but similar vulnerability to $H_2O_2$ relative to controls. In contrast, cells pretreated with CSH for 12 days, a regimen that induces the accumulation of peroxidase-positive granules (Schipper et al., 1990b), displayed significant resistance to both mechanical trauma and $H_2O_2$. These observations raise the interesting possibility that, in

addition to the classic heat shock response, the non-enzymatic peroxidase activity induced by CSH in these cells confers some cytoprotection against $H_2O_2$. The decreased vulnerability of control DIV 18 cells as compared to DIV 6 cells at the lower range of $H_2O_2$ concentrations (Fig. 4) may be due to the gradual increase in HSP expression noted in untreated cultures with time *in vitro* (Nishimura et al., 1991; present study).

The CSH-induced astrocytic inclusions may represent "heat-shock granules" akin to those observed in other cells following sustained stress. In some cases, heat-shock granules consisting of polymerized small HSPs, such as HSP27 and HSP-protein complexes, may protect various mRNAs and critical proteins from stress-related damage and degradation (Pelham, 1986; Nover et al., 1989). Using laser scanning confocal microscopy, we have recently observed that HSP27 colocalizes to the orange-red autofluorescent astrocyte inclusions in CSH-treated astroglial cultures and in subependymal regions of the adult rat brain (Mydlarski and Schipper, 1993). Since CSH-induced astrocyte inclusions are histochemically and morphologically identical to those that accumulate in periventricular brain regions as a function of aging, our results indicate that chronic or repeated induction of the cellular stress response may be responsible for the biogenesis of peroxidase-positive "stress granules" in astrocytes of the aging periventricular brain. It is of interest that in all species surveyed, the glial inclusions predominate in blood-brain barrier-deficient regions of the CNS (reviewed by Schipper, 1991). Astrocytes inhabiting these areas may be particularly susceptible to HSP induction (and subsequent granulation) by unidentified blood-borne stressors.

The astroglial stress proteins induced by CSH, namely HO-1, and HSPs 27, 72 and 90, are typically up-regulated by heat shock and oxidative stressors (Applegate et al., 1991; Dwyer et al., 1992; for reviews see Schlesinger et al., 1982; Donati et al., 1990). In contrast, CSH had little or no effect on the expression of GRP94, a stress protein responsive to glucose deprivation and calcium ionophores but not to heat shock or oxidative stress (Welch et al., 1983; Gomer et al., 1991). The oxidation of CSH in the presence of transition metals generates several pro-oxidant species including the thiyl

radical, superoxide anion, $H_2O_2$ and the hydroxyl radical (Munday, 1989). Furthermore, $H_2O_2$, a potent oxidant and inducer of HO-1 in rat astrocytes (Dwyer et al., 1992; present study) and other mammalian cells (Keyse and Tyrell, 1987), stimulated the accumulation of peroxidase-positive astrocyte granules following prolonged treatment (Fig. 5), analogous to the effects of CSH (Schipper et al., 1990b). Taken together, our results suggest that sustained intracellular oxidative stress may be the "final common pathway" responsible for the concerted up-regulation of HO-1 and other heat shock proteins that participate in the biogenesis of metalloporphyrin-rich astrocytic inclusions *in vitro* and in the intact aging brain. In support of this hypothesis, ionizing radiation, a known generator of intracellular prooxidant intermediates, increases numbers of peroxidase-positive glial granules in the rat hypothalamus in a dose-dependent manner (Srebro, 1971).

Peroxidase-positive astrocytes accumulate in aging human forebrain (Schipper, 1991) and identical non-enzymatic (heme-mediated) peroxidase activity induced in rat astroglia by CSH exposure promotes the robust oxidation of dopamine to potentially neurotoxic semiquinone intermediates (Schipper et al., 1991). These observations raise the possibility that specific stress-related derangements in glial porphyrin-heme metabolism may play an active role in the development of parkinsonism and other free-radical-related neurodegenerations.

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## Connecting Text #1

The results of the first study (Chapter 2) indicated that CSH evokes the elaboration of a host of cellular stress proteins in primary astrocyte cultures prior to the accumulation of peroxidase-positive cytoplasmic inclusions in these cells. The following study (Chapter 3) was undertaken to determine whether multiple subcutaneous injections of CSH would accelerate the aging-dependent accretion of DAB-positive astrocyte granules in the intact rat brain, and, if so, whether this would also occur in the context of a generalized cellular stress response.

## CHAPTER 3

**Cysteamine gliopathy *in situ*: a cellular stress model for the biogenesis of astrocytic inclusions.**

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Key words: astrocyte, cysteamine, glucose regulated protein, heat shock protein, metalloporphyrin, peroxidase activity, stress response

## ABSTRACT

In cultured astroglia, cysteamine induces the accumulation of peroxidase-positive cytoplasmic inclusions in the context of a generalized cellular stress response. In the present study, systemic cysteamine administration over a 3 week period induced HSP27, 72, 90, and GRP94 (stress proteins) in astrocytes and significantly increased numbers of peroxidase-positive astrocytic inclusions in various brain regions relative to controls. Similar patterns of HSP expression were also observed at 24 hr following cysteamine treatment indicating that cellular stress may be a very proximal event in the biogenesis of the astrocytic inclusions. The topography of glial peroxidase activity may provide a "map" of CNS regions particularly prone to oxidative stress during normal aging and under pathologic conditions.

## INTRODUCTION

Astrocytes classically respond to neural injury by undergoing "reactive gliosis". The latter is characterized by cellular hypertrophy, induction of GFAP mRNA and protein, elaboration of cytoskeletal intermediate filaments, and, in some cases, cell proliferation (1-3). Less commonly, as occurs in hepatic encephalopathy and certain rare neurodegenerative conditions, astrocytes exhibit specific cytopathological changes suggesting that they may be primary targets of the disease process (4,5). In the course of normal aging (6) and in response to X-irradiation (7) and chronic estrogenization (8,9), a sub-population of astrocytes residing in periventricular and limbic regions of the vertebrate brain accumulate unique cytoplasmic inclusions that are morphologically and histochemically distinct from lipofuscin. These cytoplasmic granules exhibit an affinity for the Gomori stains, chrome alum hematoxylin and aldehyde fuchsin (7,10,11), emit an orange-red autofluorescence consistent with the presence of porphyrin (12) and stain intensely with the peroxidase marker, diaminobenzidine (DAB; 9,13). The endogenous peroxidase activity is non-enzymatic in nature (9,14) and appears to be mediated by heme (ferrous) iron or by a related metalloporphyrin (9,15). This peroxidase activity has been shown to oxidize neutral catechols to potentially neurotoxic semiquinone radicals implicating these cells in the pathogenesis of parkinsonism and other free radical-related neurodegenerations (16).

In cultured fetal or neonatal rat astroglia, the sulfhydryl agent, cysteamine (CSH) stimulates the accumulation of cytoplasmic inclusions that are structurally and histochemically identical to those observed in astrocytes of the aging periventricular brain (15,17). Within 6 hr of CSH exposure, the astroglia express heat shock protein (HSP)27, 72, and 90 suggesting that chronic or repeated induction of the cellular stress response participates in the biogenesis of peroxidase-positive astrocyte inclusions *in vitro* and *in situ* (18; Mydlarski, Liang and Schipper, submitted for publication). In addition, CSH rapidly up-regulates the expression of the stress protein, heme oxygenase-1 (HO-1) resulting in derangement of porphyrin-heme metabolism that may promote the

sequestration of metalloporphyrins within the developing inclusions (9, Mydlarski, Liang and Schipper, submitted for publication). The present study was undertaken to determine whether systemic CSH administration to young adult rats stimulates the accumulation of peroxidase-positive astrocytic granules in the context of a generalized glial cellular stress response. We describe a novel experimental "gliopathy" which may serve as a model for the role of chronic oxidative stress in the biogenesis of aging- and disease-related CNS inclusions.

## MATERIALS AND METHODS

Treatment of animals

Six week old male Sprague Dawley rats were obtained from the Canadian Breeding Farms, maintained under a 12-hr light: 12-hr dark cycle and allowed free access to standard rat chow and water.

*Long-term CSH study:* At eight weeks of age, animals were injected subcutaneously twice weekly for 3 weeks (total of 6 injections) with either a) 300 mg/kg CSH HCl (Sigma Chemical CO., St. Louis, MO; neutralized to pH 7.2 with NaOH in 0.5 ml of 0.9% NaCl), b) 150 mg/kg CSH, or c) 0.9% NaCl only (control). Each treatment group contained 4-6 animals. At the time of each injection, total body weights were recorded and animals were assessed for gross systemic and CNS toxicity up until the time of perfusion. The animals were perfused four days after the final injection.

*Short-term CSH studies*: additional groups of animals were perfused 24 hr following a single injection of either 300 mg/kg CSH, 150 mg/kg CSH or saline vehicle. Each treatment group contained 4-6 animals. Following treatment, the rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 250-300 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). The brains were immediately removed and immersed in cold fixative for 6 hr. Forty micron thick coronal sections were cut on a vibratome (Lancer series 1000) in preparation for histochemistry and immunolabeling.

General neuropathology

Coronal brain sections derived from the long-term CSH study were stained with either hematoxylin and eosin, Luxol fast blue/cresyl violet, modified Bielschowsky's silver method, or Perl's ferric ferrocyanide method.

Peroxidase histochemistry-GFAP immunohistochemistry

As previously described (19), free-floating sections were washed in Tris-buffered saline (TBS; pH 7.6, 4°C) and incubated in modified Karnovsky medium for the demonstration of (brown) endogenous peroxidase activity (20). The incubation medium consisted of 0.05% 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO) and 0.45% $H_2O_2$ in 0.1 M PBS (final pH 6.5) and was applied for 30 min. The sections were subsequently treated for 30 min with 0.2% Triton-X diluted in TBS, exposed to 10% goat serum in PBS for 30 min, and incubated overnight at 4°C with a rabbit-derived polyclonal antibody directed against GFAP (1:40 dilution; kindly provided by Dr. E. Wang of our institution). The tissue was subsequently processed by the Vectastain avidin-biotin-peroxidase complex (ABC; Vector, Burlingame, CA) technique based on the method of Hsu and Raine (21). The second reaction product was visualized as a pink precipitate using α-naphthol as chromogen with pyronin B intensification as previously described (19). Although the entire brain was sectioned and examined for DAB histochemistry and HSP staining (see below), data analysis was largely restricted to the hypothalamic arcuate nucleus, the third ventricular ependyma and subependymal zone, the dorsal hippocampus and ventral hippocampal commissure, the striatum (caudate-putamen), the parietal cortex, and the corpus callosum. Peroxidase-positive astrocytes in these brain regions were examined and photographed using a Leitz Diaplan photomicroscope. Control sections were processed as above with omission of either a) DAB (with pre-incubation in 1% $H_2O_2$ in TBS for 30 min to eliminate endogenous peroxidase activity), b) the anti-GFAP antibody, or c) $H_2O_2$ in the α-naphthol reaction.

*Quantitation of peroxidase-positive astrocyte granules*: For each region surveyed, numbers of peroxidase-positive astrocyte granules were quantitated in 400x fields with the aid of an ocular grid by a single investigator unaware of the tissue source. For each animal, a minimum area of 0.625 $mm^2$ per region was evaluated and the data expressed as granules/0.1 $mm^2$. For each region, the results of the various treatments were compared using Student's two-tailed t-test.

HSP and somatostatin immunohistochemistry

Free-floating rat brain sections were pre-incubated in 3% $H_2O_2$ for 30 min to eliminate endogenous peroxidase activity and washed in PBS. Sections were then stained with a mouse monoclonal antibody directed against either HSP27, HSP72 (inducible), HSP90, or glucose regulated protein (GRP)94 (StressGen, Victoria, B.C.). Antibody dilutions were 1:200 for anti-HSP27 and 1:100 for the other stress proteins. Immunohistochemistry was performed using peroxidase-conjugated goat anti-mouse IgG (secondary antibody) and the ABC kit. Following incubation in DAB-$H_2O_2$, the stress proteins were visualized as brown precipitates. These sections were subsequently incubated with anti-GFAP polyclonal antibody (1:40 dilution, overnight at 4°C). GFAP was visualized as a pink precipitate using the ABC kit with goat anti-rabbit IgG (secondary antibody) and α-naphthol-pyronin as chromogen. To control for possible background HSP staining, some sections were processed with non-specific ascites fluid (1:100-1:200 dilutions) in place of the stress protein monoclonal antibody. Qualitative and semiquantitative analyses of each stress protein were performed on a minimum of two animals per experimental group. In addition, some sections derived from the long-term CSH experiment were immunostained for somatostatin using a rabbit-derived polyclonal antibody against the native peptide (1:500 dilution; kindly provided by Dr. A. Beaudet, Montreal Neurological Institute) and the ABC kit. In control preparations, bovine serum albumin-PBS was used in place of the anti-somatostatin antiserum.

## RESULTS

Cysteamine Toxicity

Toxicity profiles were identical for the 150 mg/kg and 300 mg/kg doses. A) Skin: local skin ecchymoses and ulceration were observed after the first CSH injections but not in saline-treated controls. B) body weight: total body weights progressively declined after the second CSH injections relative to controls. C) CNS: CSH-treated animals exhibited lethargy between the 2nd and 4th injections. Between the 4th and 6th treatments, lethargy resolved and the animals appeared irritable with some exhibiting repetitive head-shaking when lifted.

General Neuropathology

There was no evidence of CSH-induced neuronal damage or demyelination in Nissl-, myelin-, and silver-stained sections. In the latter, many glial cells exhibited argyrophilic cytoplasmic granules (Fig. 1) which have been shown to correspond to the DAB-positive astrocytic inclusions described below (22). Perl's method for ferric iron revealed infrequent, small iron deposits in striatum and corpus callosum in both CSH-treated and control specimens. The staining pattern was most consistent with oligodendroglia and myelin iron (Fig. 2). The vastly more abundant DAB-positive astrocyte granules (see below) containing heme *ferrous* iron remained unstained by Perl's method. Sections stained with anti-GFAP revealed robust astrocytic hypertrophy (gliosis) in the corpus callosum, ventral hippocampal commissure and striatum of the chronically CSH-treated animals (Fig. 3). GFAP staining in the acutely-treated animals did not differ substantially from controls.

**Peroxidase-positive astrocyte granules**

As previously reported (19), peroxidase-positive cytoplasmic granules were visualized as brown (oxidized DAB) deposits surrounded by pink (oxidized α-naphthol-pyronin) GFAP-

positive cytoplasm (Fig. 4). In all regions surveyed, the majority of astroglia were devoid of endogenous peroxidase activity. In control animals, numbers of DAB-positive astrocytes exhibited marked, but consistent, variability among the regions surveyed (Fig. 5). For example, these cells were very abundant in the dorsal hippocampus and various white matter tracts but extremely scarce in cerebral and cerebellar cortices. As depicted in Fig. 6, there was no consistent relationship between DAB-positive astrocytes and cerebral capillaries. In the absence of anti-GFAP antiserum or $H_2O_2$ in the α-naphthol medium, brown DAB-positive granules were readily visualized but astrocytes remained unlabeled. With the elimination of DAB and following pre-incubation in $H_2O_2$, all GFAP-positive astrocytes appeared devoid of endogenous peroxidase activity. Chronic CSH treatment produced significant increases in DAB-positive astrocyte granule densities in sub-ependymal regions of the third ventricle, corpus callosum, striatum, and the dorsal hippocampus (Figs. 5,7) and in the ventral third ventricular ependymal cells (Figs. 5,8). In the arcuate nucleus, a trend toward CSH-induced granulation did not reach statistical significance. There were no major differences between the effects of the 150 mg/kg and 300 mg/kg doses with respect to astrocyte granulation.

Stress protein immunohistochemistry

A) HSP27 (Fig. 9): GFAP-positive (pink) astrocytes and ependymal cells immunoreactive for HSP27 exhibited diffuse or punctate brown cytoplasmic staining. For all HSPs, brown precipitates were not observed when control ascites fluid was used in place of the specific monoclonal antibody. In saline-treated control animals, few HSP27-positive astrocytes were consistently encountered in the arcuate nucleus, adjacent to the third ventricle, hippocampus, striatum, corpus callosum, but not in cerebral cortex. In both acutely and chronically CSH-treated animals, there was a conspicuous increase in numbers of HSP27-positive astrocytes in all regions surveyed and immunopositive cells were now occasionally seen in superficial layers of the cerebral cortex. There were no discernable differences in any brain region between the low-dose and high-dose CSH treatments for any stress protein immunoreactivity profile. In control animals, third ventricular

ependymal cells were weakly immunoreactive for HSP27. Expression of the latter was strongly enhanced by chronic, and to a lesser extent by short-term, CSH exposure. No neurons immunoreactive for HSP27 were seen in any of the preparations.

B) HSP72 (Fig. 10): cells immunoreactive for HSP72 exhibited diffuse brown cytoplasmic staining. In control animals, small numbers of HSP72-positive astrocytes were observed in the arcuate nucleus and hippocampus but not in the other brain regions surveyed. In rats chronically treated with CSH, numerous HSP72-positive astrocytes were observed in the arcuate nucleus, hippocampus, striatum, and corpus callosum and small numbers of immunopositive cells appeared adjacent to the third ventricle and in the cerebral cortex. Acute CSH treatment induced similar patterns of HSP72 expression although the latter was less robust than in the chronically treated groups. In no groups was HSP72 expression detected in ependymal cells or neurons.

C) HSP90 (Fig. 11): cells expressing HSP90 exhibited diffuse brown cytoplasmic staining. In untreated control rats, virtually all GFAP-positive astrocytes were HSP90-negative. In contrast, there were abundant HSP90-positive astrocytes in the corpus callosum and lesser numbers of these cells were consistently observed in the other brain regions examined following both short-term and chronic CSH treatment. Constitutive HSP90 expression was observed in control ependymal cells and was clearly enhanced by acute and chronic CSH exposure. In the latter, tufts of ependymal cilia were often observed to be intensely immunoreactive for HSP90. Unlike the other stress proteins examined, there was prominent HSP90 immunoreactivity in neurons throughout the control brains that did not appear to change as a result of CSH treatment.

D) GRP94 (Fig. 12): as in the case of HSP27, cells expressing GRP94 exhibited both diffuse and punctate brown cytoplasmic staining. In control brains, GRP94-positive astrocytes were rarely encountered in any of the brain regions examined save for the third ventricular subependymal zone where small numbers of these cells occurred. Following chronic CSH exposure, however, there were numerous GRP94-positive astrocytes in the

arcuate nucleus, periventricular region, and corpus callosum, small numbers of these cells in hippocampus and striatum, and virtually none in the cerebral cortex. A similar pattern was observed following short-term CSH treatment except that GRP94 immunoreactivity in the hypothalamus was less pronounced. There was low-level GRP94 expression in control ependymal cells that intensified following acute and chronic CSH exposure. In all preparations, neurons were consistently GRP94-negative. The results of chronic CSH treatment on HSP expression are summarized in Table 1.

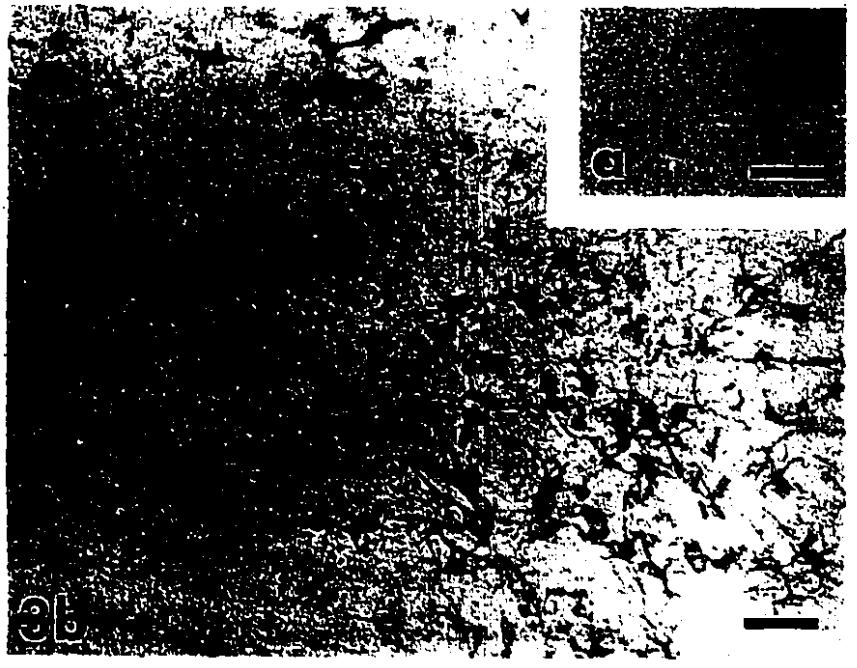
Somatostatin immunoreactivity

Prominent somatostatin immunoreactivity was observed in neuronal perikarya and fiber projections in striatum, hypothalamus and other brain regions of control animals. In accord with previous short-term CSH studies (23), chronic CSH exposure significantly diminished somatostatin immunoreactivity in fibre tracts of the ventral striatum (Fig. 13) and other brain regions.

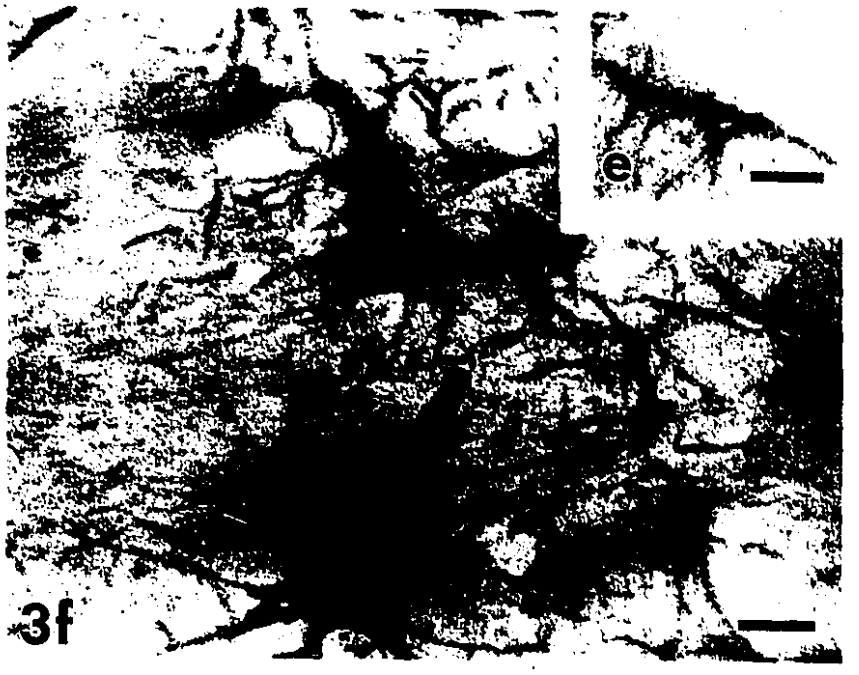
Fig. 1. Striatum of rat chronically treated with CSH (150 mg/kg). Modified Bielschowsky's silver stain. Argyrophilic inclusions depicted (arrow) are likely astrocytic in origin (see text). Bar = 10 µm.

Fig. 2. Striatum of CSH-treated animal stained with Perl's ferric ferrocyanide method. Deposits of ferric iron are far less common than DAB (heme ferrous iron)-positive astrocytic inclusions in this brain region. An iron-laden neuroglial cell (likely an oligodendrocyte) is depicted (arrow). Bar = 10 µm.

Fig. 3. Effects of long-term CSH treatment (150 mg/kg) on GFAP staining (gliosis) in corpus callosum (a-d), hippocampus (e-f) and striatum (g-h). Control levels of GFAP staining are depicted in a, c, e and g. CSH-induced astrocyte hypertrophy is shown in panels b, d, f and h. Bar = 50 μm in a, b, and 10 μm in c-h.


a
b


c
d


e
3f


g
3h

Fig. 4. DAB-GFAP labeling for peroxidase-positive astrocytes in the corpus callosum of control (a) and long-term CSH-treated (300 mg/kg; b) animals. GFAP-positive astrocytes and endogenous peroxidase activity are stained pink and brown, respectively. Peroxidase-positive (arrows) and peroxidase-negative (arrowhead) astrocytes are depicted. Bar = 10 μm.


4a

Fig. 5. Effects of long-term CSH exposure on numbers of DAB-positive astrocyte and ependymal granules in various brain regions. Columns and vertical bars represent means and standard errors of the means, respectively. Asterisks denote statistically significant increases in granule numbers relative to untreated controls ($p < 0.05$).

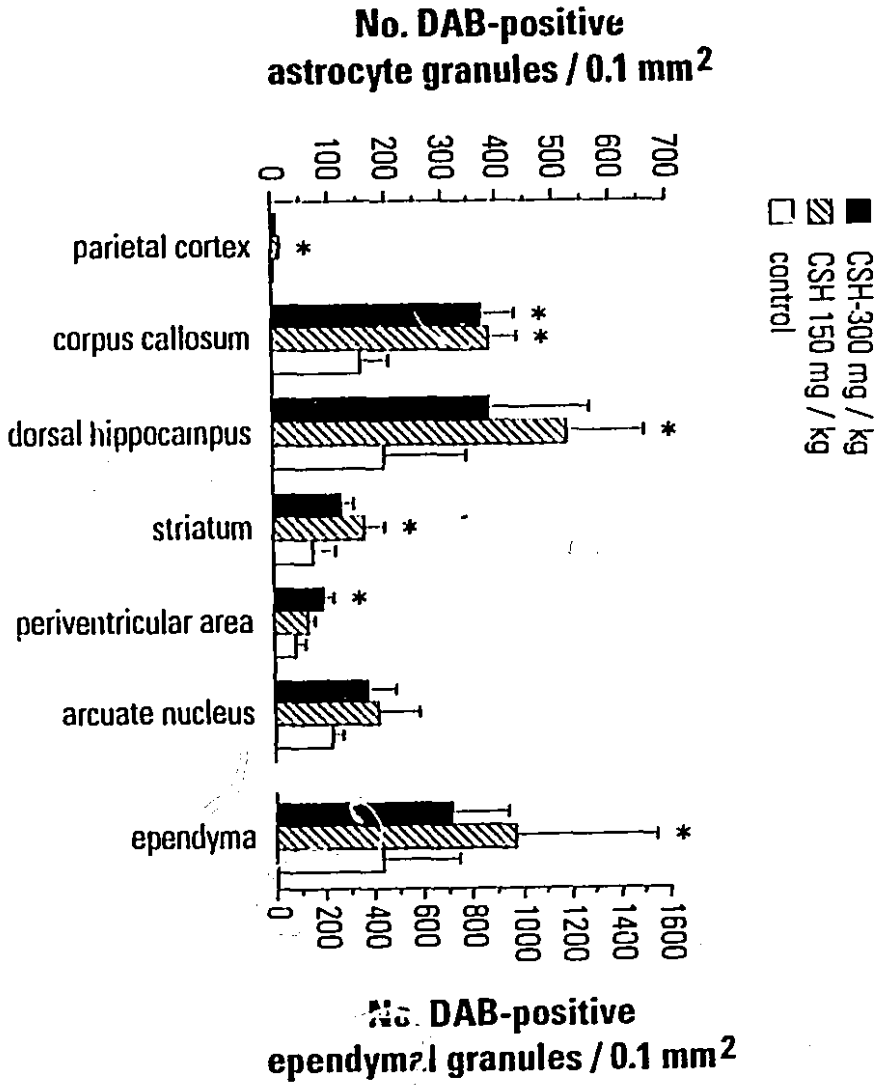
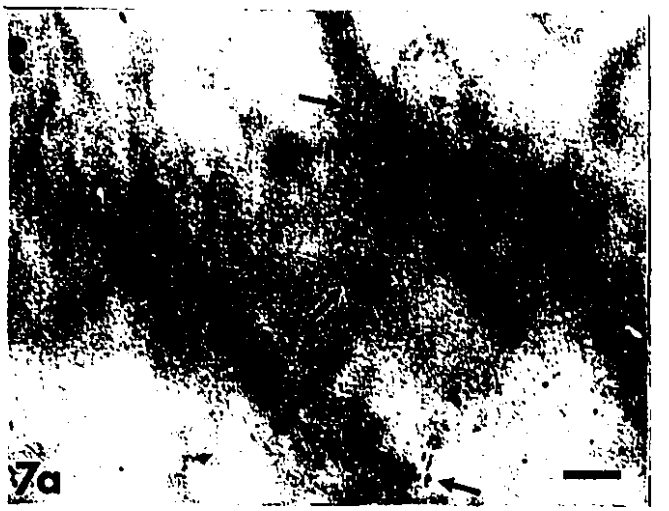

No. DAB-positive
astrocyte granules / 0.1 mm2
0
100
200
300
400
500
600
700
parietal cortex
corpus callosum
dorsal hippocampus
striatum
periventricular area
arcuate nucleus
ependyma
CSH-300 mg / kg
CSH 150 mg / kg
control
0
200
400
600
800
1000
1200
1400
1600
No. DAB-positive
ependymal granules / 0.1 mm2

Fig. 6. Relationship of DAB-positive astrocyte granules to capillaries in the ventral hippocampal commissure in CSH-treated animals. DAB-positive astrocyte granules (solid arrows) are shown at variable distances from (a) or in close proximity to (b) cerebral capillaries (open arrow). Redox-active hemoglobin (ferrous iron) renders capillary erythrocytes intensely DAB-positive as illustrated here. Bars = 10 μm.

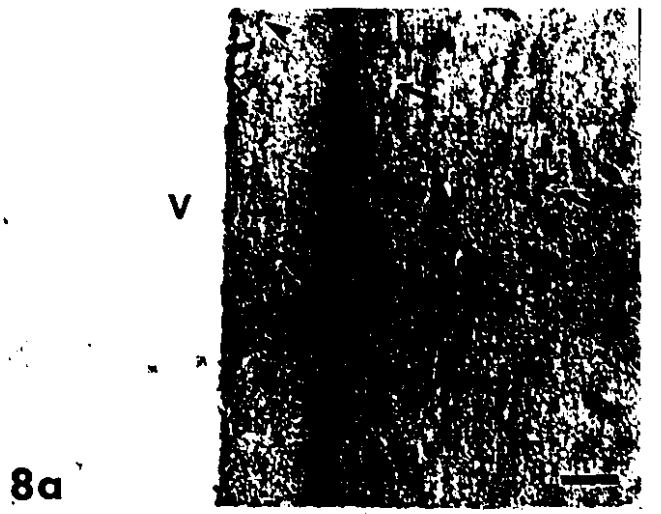

6
b

Fig. 7. Effects of long-term CSH exposure (150 mg/kg) on DAB-positive astrocyte granules in the ventral hippocampal commissure. Note the increased numbers of DAB-positive inclusions (arrows) in the treated animal (b) relative to the untreated control (a). Bar = 10 μm.


7a


7b

Fig. 8. Effects of long-term CSH treatment (150 mg/kg) on the concentration of DAB-positive ependymal granules in the ventral half of the third ventricular wall. Note the intense granulation in the treated animal (b) relative to the untreated control (a). Arrowhead = DAB-positive ependymal granules, arrows = DAB-positive astrocyte granules, V = third ventricle. Bar = 25 μm in (a) and 10 μm in (b).




8a


8b

Fig. 9. Effects of CSH exposure on HSP27 expression in GFAP-positive astrocytes and third ventricular ependymal cells. In figures 9-12, stress proteins appear brown and GFAP-positive astrocytes are labeled pink. Long-term CSH exposure (150 mg/kg) induces HSP27 in callosal astrocytes (b) and ependymal cells (d) relative to untreated controls (a, c, respectively). HSP27 is also induced in callosal astrocytes following short-term CSH treatment (300 mg/kg, f). Little or no HSP27 is observed in the corpus callosum of untreated animals (e). Arrows = HSP27-positive astrocytes; arrowheads denote HSP27-negative ependymal cells in (c) and HSP27-positive ependymal cells in (d); V = third ventricle; bar = 10 μm in (a, b) and 25 μm in (c-f).

Fig. 10. Effects of long-term CSH treatment (150 mg/kg) on HSP72 expression in GFAP-positive astrocytes. Double labeled cells (arrows) in the striatum (b) and corpus callosum (c) of treated animals are shown. In control animals (a), GFAP-positive astrocytes are typically HSP72-negative. Arrowheads = HSP72-negative astrocytes; bars = 10 μm.

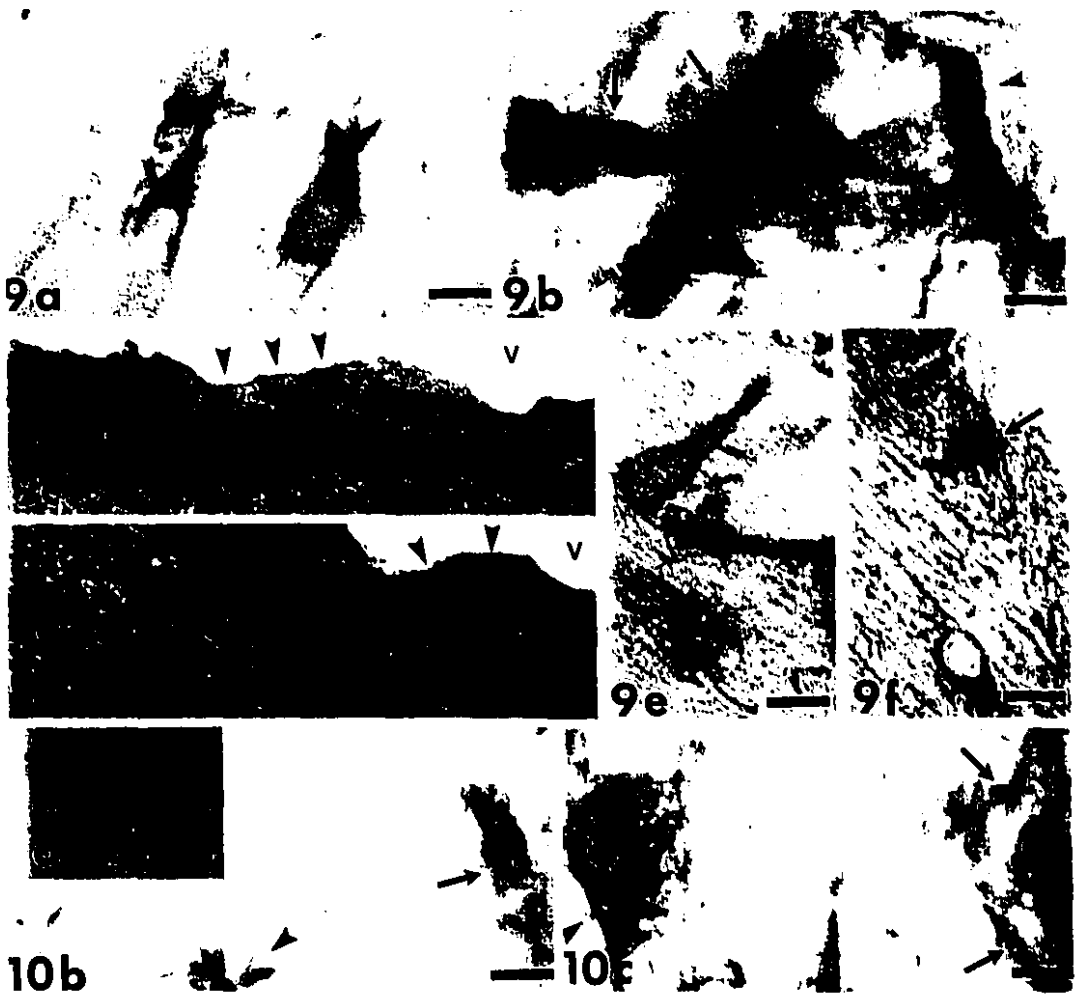

9a
9b
v
v
9e
9f
10b
10c

Fig. 11. Effects of long-term CSH (150 mg/kg) exposure on HSP90 expression in the striatum (a, b) and dorsal hippocampus (c, d). In control striatum (a), there is considerable constitutive HSP90 expression in neurons (arrows) while virtually all GFAP-positive astrocytes (arrowheads) remain HSP90-negative. Following CSH treatment (b), many GFAP-positive astrocytes express HSP90 (arrow). As in the striatum, GFAP-positive astrocytes in control hippocampus are almost invariably HSP90-negative (c) whereas significant HSP90 expression is observed in these cells (d; arrow) following CSH treatment. No HSP90 expression is seen in GFAP-positive astrocytes of treated animals when control ascites fluid is used in place of the specific monoclonal antibody (e). Arrowheads = HSP90-negative astrocytes; bars = 10 μm.

Fig. 12. Long-term CSH treatment (150 mg/kg) induces punctate GRP94 expression in GFAP-positive astrocytes of the arcuate nucleus (a, arrow) and third ventricular wall (b, arrow). With the exception of the third ventricular subependymal zone, GRP94-positive astrocytes were rarely encountered in untreated animals (not illustrated). V = third ventricle; bars = 10 μm.

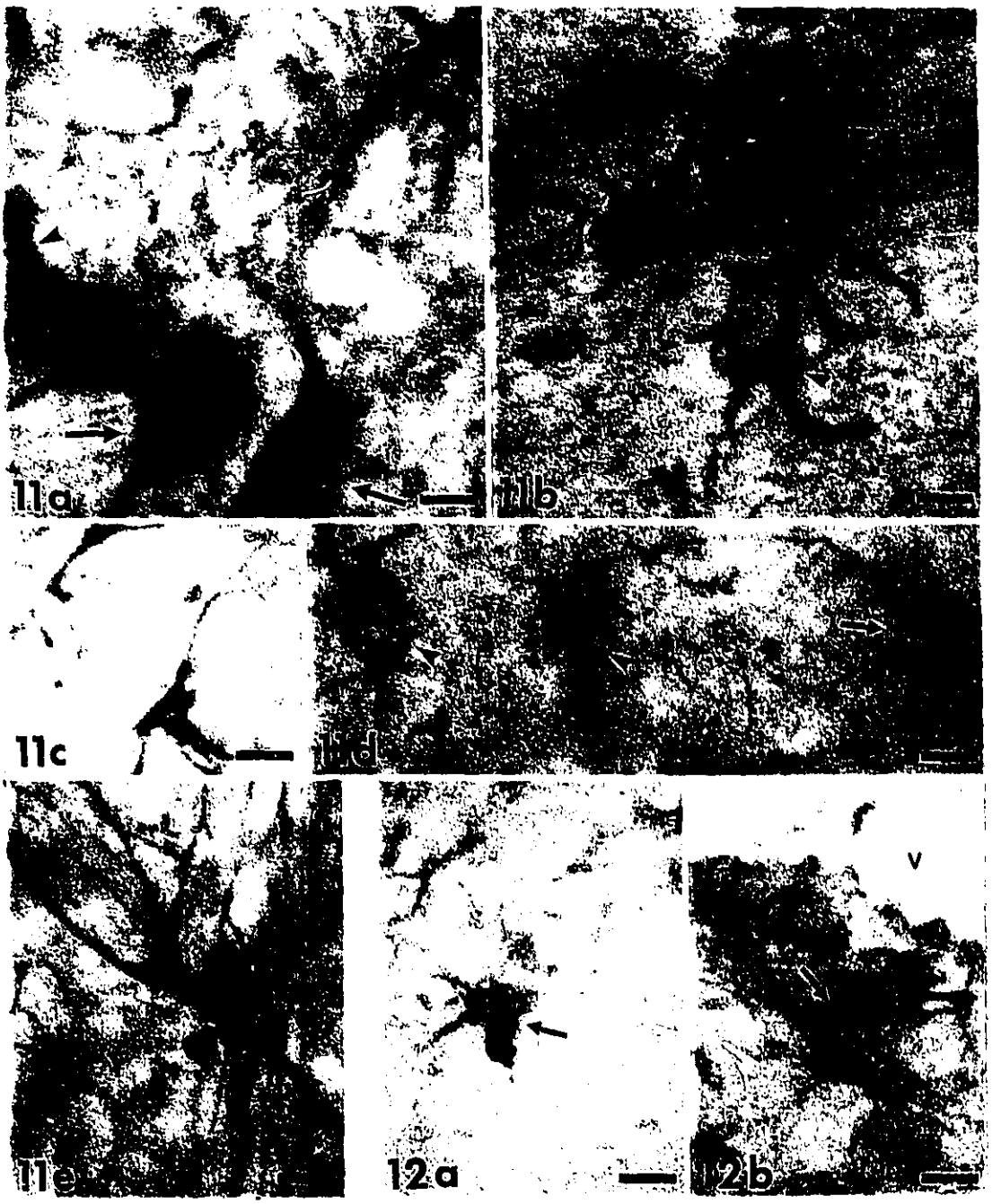

11a
11b
11c
11d
11e
12a
12b
V

TABLE I
Stress Protein Expression in Rat Brain Following Chronic Cysteamine Exposure

| | Astrocytes | | | | | | | | | | | | Ependyma* | | Neurons | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ARC | | 3VS | | HIP | | STR | | CC | | CX | | | | | |
| | C | CSH | C | CSH | C | CSH | C | CSH | C | CSH | C | CSH | C | CSH | C | CSH |
| HSP 27 | +† | ++ | + | ++ | + | ++ | + | ++ | + | ++ | 0 | + | +† | ++ | 0 | 0 |
| HSP 72 | +‡ | ++ | 0 | + | + | ++ | 0 | ++ | 0 | ++ | 0 | + | 0 | 0 | 0 | 0 |
| HSP 90 | 0 | +‡ | 0 | + | 0 | + | 0 | + | 0 | ++ | 0 | 0 | +‡ | ++ | ++ | ++ |
| GRP 94 | 0 | ++† | + | ++ | 0 | + | 0 | + | 0 | ++ | 0 | 0 | +† | ++ | 0 | 0 |

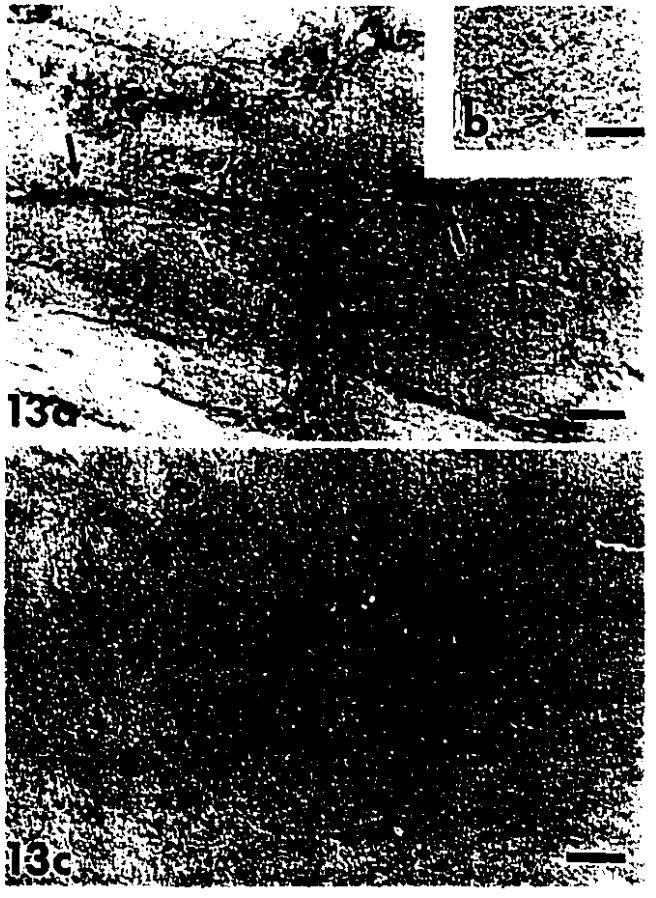
ARC = arcuate nucleus; C = control animals; CC = corpus callosum; CSH = cysteamine-treated animals; CX = cerebral cortex; GRP = glucose regulated protein; HIP = hippocampus; HSP = heat shock protein; STR = striatum; 3VS = third ventricular subependymal zone; 0 = no or very rare staining; + = few positive cells consistently present (<3 cells/400 × field); ++ = numerous positive cells present (≥3 cells/400 × field).

* Third ventricle.

† Diffuse and punctate cytoplasmic staining.

‡ Diffuse cytoplasmic staining.

Fig. 13. Effects of long-term CSH treatment (150 mg/kg) on somatostatin immunoreactivity in the ventral striatum. In untreated animals (a), fiber tracts exhibit abundant somatostatin immunoreactivity (arrows). In contrast, there is significant depletion of somatostatin staining in this region following CSH exposure (c). In the absence of primary antiserum, no somatostatin staining is seen in the striatum of untreated animals (b). Bars = 50 $\mu$m.


b
13a
13c

## DISCUSSION

In rodents, discrete cytopathological changes are observed in sub-populations of periventricular astrocytes in the course of normal aging (6) and in response to X-irradiation (7) and chronic estrogenization (8,9,24). These cells appear to respond to aging and injury by accumulating cytoplasmic inclusions that are histochemically distinct from lipofuscin and exhibit intense orange-red autofluorescence and non-enzymatic peroxidase activity consistent with the presence of porphyrin and heme, respectively (7,9,12). At present, the precise chemical composition of these inclusions, the mechanisms responsible for their biogenesis, and their roles in CNS senescence and disease remain incompletely understood. In immature rat astroglial cultures, CSH and its disulfide form, cystamine, stimulate the accumulation of osmiophilic, membrane-bound inclusions with identical autofluorescent and histochemical profiles suggesting that CSH accelerates the appearance of an "aging" phenotype in these cells (15,17,19). This conclusion is supported by the results of the present study which demonstrate that systemic administration of CSH induces the accumulation of these astrocytic granules in the intact rat brain. In this study, subcutaneous administration of CSH over a 3 week period elicited significant increases in number of DAB-positive astrocyte granules in the dorsal hippocampus, striatum, cerebral cortex, corpus callosum and third ventricular wall. In these regions, the overall astrocytic response to CSH was relatively uniform (with an approximate doubling of granules) regardless of whether the individual areas surveyed were normally densely-populated with DAB-positive astrocytes (e.g. periventricular hypothalamus) or relatively deficient in these cells (e.g. the cerebral cortex). Other regions typically devoid of peroxidase-positive glia, such as the cerebellar cortex, exhibited no granulation in response to CSH, and in many responding regions astrocytes replete with DAB-positive inclusions appeared randomly intermixed with much larger pools of GFAP-positive/DAB-negative glia. Thus, as previously noted in dissociated astroglial cultures (15), there appears to be remarkable heterogeneity with regard to the capacity of astrocytes to granulate in response to CSH. Although there is a propensity for peroxidase-positive astrocytes to reside in circumventricular organs and other blood-brain

barrier-deficient regions (7,22,25), the astrocytic response to CSH bore no overt relationship to the topography of the blood-brain barrier or proximity to cerebral capillaries. These observations are consistent with the fact that CSH freely crosses lipid membranes (26) and systemically-administered CSH induces depletion of neuronal somatostatin immunoreactivity (23, 27, present study) and inhibition of dopamine-ß-hydroxylase (28) in striatum and other regions with intact blood-brain barriers.

The pathologic effects of CSH appeared largely restricted to the induction of DAB-positive inclusions in astrocytes and in ependymal cells of the ventral third ventricular wall. DAB-positive ependymal inclusions have previously been described (29) and predominance of the present CSH effect in *ventral* cells may reflect the distinct ontogeny of the largely tanycytic ependyma that comprises the ventral third ventricular wall (30). Robust hypertrophy of GFAP-positive astrocytes (gliosis) was apparent in the corpus callosum, ventral hippocampal commissure and striatum, but not elsewhere in the chronically CSH-treated animals and not at all in animals exposed to CSH for 24 hours. Moreover, chronic CSH exposure produced no obvious neuronal damage or demyelination in silver-, myelin- and Nissl-stained preparations. Similarly, direct CSH microinjection into rat striatum results in local gliosis and depletion of immunoreactive somatostatin but no overt neuronal cytotoxicity (27). Patients chronically receiving CSH for the treatment of nephropathic cystinosis have been noted to develop neurological morbidity and exhibit basal ganglia necrosis, cortical spongiform changes and patchy demyelination (31). However, the latter are almost certainly due to the disease process itself rather than CSH toxicity because a) pathognomonic cystine crystals and extremely elevated cystine concentrations are present in the affected brain regions (31), and b) some individuals with nephropathic cystinosis who have survived into the second or third decades without CSH treatment have exhibited similar neuropathologic profiles (32,33). The present findings, in conjunction with previous observations that CSH induces granulation of astrocytes but not neurons or oligodendroglia in primary culture (15), strongly suggest that this aminothiol compound produces a unique "gliopathy" characterized by the accumulation of metalloporphyrin-rich cytoplasmic inclusions in sub-populations of astrocytes and

ependymal cells.

The results of the present and earlier *in vitro* studies (15, 18, Mydlarski, Liang & Schipper, submitted for publication) argue that derangements in porphyrin-heme metabolism in the context of a generalized cellular stress response play a fundamental role in the accumulation of peroxidase-positive astrocytic granules during normal aging and in response to CSH exposure. In neonatal astroglial cultures, treatment with CSH for 6 hours induces the expression of HSPs 27, 72, and 90, and the enzyme, HO-1 (18, Mydlarski, Liang and Schipper, submitted for publication). The HO-1 gene has a heat shock element in its promoter region (34) and is induced by CSH along with the other HSPs in a concerted cellular stress response (35-37). Furthermore, CSH-pretreated cells exhibited increased resistance to $H_2O_2$ cytotoxicity in comparison with non-pretreated controls (Mydlarski, Liang and Schipper, submitted for publication) providing physiologic evidence for an antecedent stress response in the CSH-pretreated cultures (38-40). Heme regulates its own formation by feedback repression of Δ-aminolevulinic acid synthase (ALA-S), the rate-limiting enzyme in porphyrin-heme biosynthesis. Thus, whereas acute induction of HO-1 may rapidly degrade heme to biliverdin (41,42), prolonged induction of HO-1 due to chronic CSH treatment may cause sustained reductions in the regulatory heme pool with a resultant de-repression of ALA-S. Rebound induction of the latter, in turn, could conceivably promote metalloporphyrin biosynthesis and sequestration within discrete astrocytic inclusions (15). In the present study, systemic administration of CSH induced HSPs 27, 72, 90, and GRP94 in GFAP-positive astrocytes of the hypothalamic arcuate nucleus, periventricular area, hippocampus, striatum, corpus callosum and, to a much lesser extent, cerebral and cerebellar cortices. Thus, the distribution of the various glial stress proteins stimulated by CSH correlates closely with the topography of the CSH-induced astrocytic granules. Except for HSP90 which exhibited low-level neuronal expression in both CSH-treated and control brains, induction of the other stress proteins resulting from CSH exposure appeared restricted to astrocytes and ependymal cells. Restriction of the cellular stress response to the latter further underscores the notion that CSH induces a primary gliopathy in these animals. Furthermore, CSH elicited similar

patterns of HSP expression as early as 24 hours following the initiation of treatment indicating that cellular stress may be a very proximal event in the biogenesis of the astrocytic inclusions. Finally, whereas HSP72 and 90 exhibited diffuse cytoplasmic staining, staining patterns for HSP27 and GRP94 were both diffuse and punctate. Punctate cytoplasmic staining raises the possibility that the latter stress proteins are constituents of the astrocytic inclusions. Using laser scanning confocal microscopy, we recently confirmed the co-localization of HSP27 and GRP94 (but not HSP72 and 90) to red autofluorescent (peroxidase-positive) granules in the third ventricular wall (Mydlarski and Schipper, in preparation).

A considerable body of circumstantial evidence suggests that intracellular oxidative stress may be the "final common pathway" responsible for the biogenesis of the metalloporphyrin-rich astrocytic inclusions: 1) With the possible exception of GRP94 which responds largely to glucose deprivation and calcium ionophores (43), expression of HO-1 and HSPs 27,72 and 90 are typically induced by heat shock and a variety of oxidative stressors (36). (In contrast to the results of present *in situ* study, we observed considerable constitutive expression of GRP94 in untreated astroglial cultures and the level of expression was not enhanced by CSH exposure (18, Mydlarski, Liang and Schipper, submitted for publication). Perhaps in intact animals CSH indirectly perturbs glial glucose or calcium homeostasis and thereby elicits a GRP94 response.) 2) In the presence of transition metals, CSH undergoes redox cycling with the generation of pro-oxidant species including thiyl radicals, superoxide, $H_2O_2$ and the hydroxyl radical. 3) $H_2O_2$, universal inducer of heat shock proteins and HO-1 in rat astrocytes (37), stimulates the accumulation of peroxidase-positive astrocyte granules in primary culture following prolonged treatment analogous to the effects observed with CSH (18, Mydlarski, Liang and Schipper, submitted for publication). 4) Ionizing radiation, a known generator of intracellular pro-oxidant intermediates, increases numbers of peroxidase-positive glial granules in the rat hypothalamus in a dose-dependent manner (7). If a causal relationship to intracellular oxidative stress is confirmed, determination of the topography and intensity of endogenous glial peroxidase activity may permit accurate "mapping" of CNS regions

particularly prone to chronic oxidative stress during normal aging and under pathologic conditions. For example, reports of HSP72 overexpression (44) and increased lipid peroxidation (45) in Alzheimer cerebral cortex and colocalization of HSPs to senile plaques and neurofibrillary tangles (44) argue that the affected areas may be experiencing chronic oxidative stress. Insofar as the latter is correct, our hypothesis would predict the presence of excessive astrocyte peroxidase activity (metalloporphyrins) in close proximity to foci of Alzheimer-type histopathology. Finally, glial metalloporphyrins may play an active role in the pathogenesis of various neurodegenerative disorders. In the presence of $H_2O_2$, peroxidase-positive astrocytes in primary culture promote the robust oxidation of dopamine and catecholestrogens to neurotoxic semiquinone radicals (16). Thus, the progressive accumulation of these cells in rodent and human striatum could explain, at least in part, the enhanced vulnerability of the senescent nervous system to parkinsonism and other free radical-related neurodegenerative conditions (16,46).

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## Connecting Text #2

In various cell types, repeated exposure to hyperthermic stress induces the formation of perinuclear cytoplasmic aggregates referred to as "heat shock granules" which contain polymerized small HSPs and HSP-protein complexes (Collier et al., 1988; Nover et al., 1989). Based on the finding that CSH treatment, both *in vitro* (Chapter 2) and *in situ* (Chapter 3), elicits astrocyte granulation in the context of a cellular stress response, we set out to determine whether (i) Ub, a small stress protein implicated in the proteolysis of damaged polypeptides, is also activated in astroglial cultures following CSH exposure, (ii) specific stress proteins are actual constituents of the astroglial inclusions, and (iii) patterns of stress protein colocalization to granules in periventricular astrocytes of the normal adult rat brain are recapitulated in CSH-treated glial cultures.

## CHAPTER 4

**Stress protein co-localization to autofluorescent astrocytic inclusions in situ and in cysteamine-treated glial cultures.**

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Key words: Aging; Astrocyte; αB-crystallin; Cysteamine; Glucose regulated protein; Heat shock protein; Ubiquitin

## SUMMARY

In the aging brain, a unique sub-population of limbic and periventricular astrocytes accumulates red autofluorescent, peroxidase-positive cytoplasmic inclusions distinct from lipofuscin. Cysteamine (CSH) exposure rapidly induces identical inclusions in cultured, immature astroglia. CSH induces a cellular stress response prior to astrocyte granulation. To determine whether stress proteins are actual constituents of the autofluorescent granules, 12-week-old rat brain sections and CSH-treated astroglial cultures were immunostained with various anti-stress protein antibodies and evaluated by laser scanning confocal microscopy. We observed intense co-localization of heat shock protein (HSP) 27 and ubiquitin (Ub) to the autofluorescent astrocyte granules *in situ* and in CSH-treated glial cultures. In both preparations, glucose regulated protein (GRP) 94 consistently exhibited partial co-localization to the granule periphery and adjacent cytoplasm. In contrast, HSP72 co-localization to these inclusions was only occasionally seen and the granules appeared entirely devoid of HSP90 and αB-crystallin. Acute exposure of cultured astroglia to CSH induced intense cytoplasmic Ub staining, suggesting that activation of the Ub pathway may be an early event in the biogenesis of these astrocytic granules. Taken together, our results support the notion that the autofluorescent astrocyte inclusions are stress or heat shock granules which progressively accumulate in the aging periventricular brain. Moreover, CSH greatly accelerates the appearance of this senescent astrocyte phenotype in primary culture.

## INTRODUCTION

In all vertebrates examined to date, including humans, a subpopulation of Gomori-positive astrocytes in adult limbic and periventicular brain regions progressively accumulates unique cytoplasmic inclusions that are histochemically distinct from the aging pigment, lipofuscin (reviewed in ref. 27). These glial inclusions exhibit orange-red autofluorescence and non-enzymatic peroxidase activity consistent with the presence of porphyrin and heme, respectively[12,31]. In dissociated fetal and neonatal rat brain cultures, the sulfhydryl compound, cysteamine (CSH), induces the accumulation of histochemically and structurally identical astrocytic organelles[21,34]. We recently observed that short-term administration of CSH to astroglial cultures results in the induction of various stress proteins, including heme oxygenase-1 (HO-1) and heat shock proteins (HSPs) 27, 72 and 90, long before astrocytic granules are visualized by light microscopy. Our observations suggested that sustained induction of the cellular stress response results in derangements of porphyrin-heme metabolism and promotes the sequestration of metalloporphyrins within the nascent astrocytic inclusions in CSH-treated cultures and in the aging periventricular brain[23]. In the present study, immunofluorescent labeling of various stress proteins and laser scanning confocal microscopy were used to determine a) whether ubiquitin (Ub), a polypeptide implicated in the cellular stress response, is similarly over-expressed following CSH treatment, b) if specific stress proteins are actual constituents of the astrocytic inclusions and c) the degree to which patterns of stress protein expression in aging periventricular astrocytes *in situ* are recapitulated in immature cultured astroglia following CSH exposure.

## MATERIALS AND METHODS

Eight-week-old male and one-day-old neonate Sprague Dawley rats were obtained from Charles River breeding farms. Ham's F12, high glucose Dulbecco's modified Eagle medium (DMEM), heat-inactivated horse serum and fetal calf serum were purchased from Gibco, Grand Island, N.Y. Poly-D-lysine, penicillin-streptomycin, cysteamine (2-mercaptoethylamine) and 3,3'-diaminobenzidene (DAB) were purchased from Sigma Chemical Co., St. Louis, MO. Monoclonal antibodies for HSPs 27, 72, 90 and glucose regulated protein (GRP) 94 were obtained from StressGen Biotechnologies Co., Victoria, B.C. (Canada). Polyclonal antisera directed against αB-crystallin were kindly supplied by Dr. J.E. Goldman of Columbia University, New York and Dr. J. Horwitz of UCLA, Los Angeles. Polyclonal anti-Ubiquitin (Ub) antibody which recognizes both free and conjugated $Ub^2$ was kindly provided by Dr. Z. Ali-Kahn of McGill University, Montreal, Quebec (Canada). Monoclonal anti-glial fibrillary acidic protein (GFAP) antibody was kindly supplied by Dr. E. Wang of our institution. Fluorescein isothiocyanate (FITC)-conjugated, goat-derived monoclonal and polyclonal IgG antibodies were purchased from Jackson Immuno Research Laboratories, West Grove, PA. Rhodamine-conjugated, goat-derived monoclonal IgG antibody was purchased from Organon Teknika Cappel, West Chester, PA.

### *Rat brain sections*

Eight-week-old rats were maintained under a 12 hr light: 12 hr dark cycle and allowed free access to standard rat chow and water. At 12 weeks of age, the rats were deeply anaesthetized with sodium pentobarbital and perfused transcardially with 250-300 mL of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS pH 7.4). The brains were immediately removed and immersed in cold fixative for 6 hr. Forty-micron-thick coronal sections were cut on a vibratome (Lancer series 1000) in preparation for immunolabeling.

*Brain cell cultures*

Primary neuroglial cultures were prepared by mechanoenzymatic dissociation of cerebral tissue derived from 2 day old neonate rats as previously described[32]. Cells were grown in Ham's F12 and DMEM (50:50 v/v) supplemented with 10 mM HEPES, 5% heat-inactivated horse serum, 5% fetal calf serum, and penicillin-streptomycin (50 U/mL and 50 µg/mL, respectively). 3.5 x $10^5$ cells/mL were plated on eight-chamber (Labtek) culture slides pre-coated with 0.01% poly-D-lysine. The cultures were incubated at 37°C in humidified 95% air-5% $CO_2$ and culture medium was changed twice weekly. Cultures received 880 µM CSH with each change of culture medium between days 6 and 18 in vitro (DIV). On DIV 6 and DIV 18, cell monolayers were washed in 0.1 M PBS (pH 7.2) prior to fixation in either methanol at -20°C for 5 min, or methanol-acetone (50:50 v/v) at -20°C for 10 min.

*Immunofluorescent labeling*

Free-floating rat brain sections and DIV 18 astrocyte cultures were washed in Tris-buffered saline (TBS; pH 7.6) and 0.1 M PBS (pH 7.2), respectively, and subsequently incubated for 90 min at room temperature with anti-HSPs 27, 72 (inducible), 90, or anti-GRP94 monoclonal antibodies diluted 1:200 in 0.15% bovine serum albumin-PBS (pH 7.2). The secondary antibody consisted of FITC-conjugated goat anti-mouse IgG (1:100 dilution) applied for 30 min at room temperature. For immunostaining with the αB-crystallin and Ub polyclonal antibodies, specimens were first permeabilized with 2% Triton-X in PBS for 5 min. αB-crystallin is a stress protein which shares structural homology with members of the small HSP family and is expressed in reactive astrocytes[17] and in inclusions associated with certain neurodegenerative disorders[13]. The rabbit-derived antisera were applied overnight (Ub, 1:100 dilution; αB-crystallin, 1:50 dilution) in a humidified chamber at 4°C. The secondary antibody consisted of FITC-conjugated goat anti-rabbit IgG (1:200 dilution) applied for 30 min at room temperature. Non-specific mouse ascites fluid and normal rabbit sera served as controls for monoclonal and

polyclonal antibodies, respectively. The brain sections and culture monolayers were rinsed in PBS, mounted in PBS:glycerol (50:50, v/v), and examined and photographed by confocal microscopy.

Short-term exposure of cultured astrocytes to CSH induces a stress response with consequent increases in the expression of HO-1 and HSPs 27, 72 and 90[23]. To determine whether cellular Ub levels are similarly affected, indirect immunofluorescent staining for Ub was performed as described above on DIV 6 following 6 h CSH treatment. Astroglia were identified by subsequent immunolabeling with mouse-derived anti-GFAP monoclonal antibody (1:1000 dilution) for 2 h at 37°C followed by rhodamine-conjugated goat anti-mouse IgG (1:200 dilution) for 30 min at room temperature. Monolayers were rinsed, mounted and examined and photographed under epi-fluorescence using a Leitz Diaplan microscope equipped with appropriate filters.

*Confocal co-localization study*

A BioRad MRC-600 Laser Scanning Confocal Imaging System was used to determine co-localization of FITC (green)-labeled stress proteins to the endogenous, red autofluorescent astrocyte inclusions. The system is equipped with a 15 mW Krypton/Argon laser which can excite the sample with lines at 488, 568 and 647 nm. The excitation filter wheel was used in the dual excitator position which allows only the 488 and 568 nm lines of the laser to reach the sample. The emission wavelengths were detected from the samples using the K1 and K2 filter block set. The latter allows the detection of wavelengths between 522 and 554 nm on the green channel and the detection of wavelengths of 585 or greater on the red channel. Both channels were 70% open. Images scanned on the two channels (red and green) were merged to produce a single profile. In this mode, all regions exhibiting co-localization emit yellow fluorescence. Optical dissection of the stored images and reconstruction of Z-axis (perpendicular) orientations were performed. The Z-axis reconstruction permits differentiation between true co-localization and false co-localization resulting from spurious superimposition of red and green emitters. In the

former, yellow fluorescence persists in the Z-axis orientation whereas it resolves to red and green in the latter. After scanning, the images were collected using COMOS software and stored on optical disks.

## RESULTS

In the intact rat brain, red autofluorescent granules were noted in periventricular astrocytes and, to a lesser extent, in third ventricular ependymal cells as previously described[12]. Treatment of primary astrocyte cultures *in vitro* with CSH induced a massive accumulation of similar red autofluorescent cytoplasmic granules. The inclusions varied extensively in size and appeared to be distributed randomly in the astrocyte perikarya and processes. Smaller inclusions tended to exhibit rounded morphology whereas larger granules were either round or pleomorphic in appearance. Prolonged or intense laser irradiation resulted in pronounced bleaching of the autofluorescence. In the tissue sections and *in vitro* preparations, no yellow or green autofluorescence characteristic of lipofuscin was observed.

### *Stress Protein Immunofluorescence and Co-localization to Autofluorescent Astrocyte Granules*

HSP27: In the rat brain sections, HSP27 exhibited extensive co-localization to the larger autofluorescent astrocyte granules (appearing as bright yellow fluorescence in XY- and Z-axis field orientations; Fig. 1A). Areas of intense co-localization were often surrounded by thin rims of HSP27-positive cytoplasm. As in the brain sections, HSP27 exhibited intense co-localization to large CSH-induced autofluorescent inclusions in the cultured astroglia (Fig. 1B). Some of the smaller autofluorescent inclusions were often noted to be HSP27-negative.

HSP72: In periventricular brain regions, HSP72 immunostaining exhibited occasional co-localization to the autofluorescent astrocyte granules and adjacent cytoplasm (Fig. 2A). In subependymal regions of the third ventricle some unidentified perikarya and processes entirely devoid of autofluorescent granules also exhibited HSP72 immunostaining. In the CSH-treated astroglial cultures, HSP72 staining exhibited infrequent co-localization to the red astrocyte inclusions. When present, staining was usually restricted to the cores of the

inclusions (Fig. 2B). As *in situ*, HSP72 staining of cytoplasm adjacent to the autofluorescent material was occasionally observed (Fig. 2C).

HSP90: The autofluorescent astrocyte inclusions *in situ* were consistently devoid of HSP90 staining. In contrast, immunoreactive HSP90 was often seen in thin tanycyte processes extending from the third ventricular wall to the neuropil of the hypothalamic arcuate nucleus (Fig. 3A). As in the brain sections, there was no co-localization of HSP90 to the autofluorescent inclusions in cultured astroglia. However, diffuse HSP90 immunostaining was commonly observed in nuclei of astrocytes laden with autofluorescent cytoplasmic granules (Fig. 3B). HSP90 immunolabeling of nuclei in CSH-treated astrocytes is consistent with reports of HSP90 translocation from cytoplasm to nucleus in human fibroblasts following heat shock[1].

GRP94: In both the tissue sections and CSH-treated glial monolayers, GRP94 expression consistently exhibited a unique relationship to the autofluorescent astrocyte granules not observed with any of the other stress proteins surveyed (Fig. 4A and B). GRP94 tended to exhibit partial co-localization (yellow) to the periphery or one pole of the (red) autofluorescent inclusions and contiguous granule-free cytoplasm (green). This "tricolour" immunofluorescent pattern was characteristic of the larger autofluorescent granules whereas smaller, perinuclear inclusions were often entirely devoid of GRP94 staining.

Ubiquitin: In coronal sections through the diencephalon, Ub immunolabeling appeared concentrated within the third ventricular subependymal zone and exhibited extensive co-localization to the autofluorescent glial inclusions. Regions of red autofluorescence not associated with at least some Ub-immunoreactivity were uncommon (Fig. 5A). In the cultured material, Ub exhibited consistent co-localization (yellow fluorescence in both the XY- and Z-orientations; Fig. 5D and E) to many of the larger autofluorescent astrocyte granules. In general, ubiquitination was most intense in the central areas of the autofluorescent inclusions. As in the case of HSP27 and GRP94, Ub did not co-localize to many of the smaller autofluorescent granules. Distinct Ub staining of granule-free

cytoplasm was occasionally observed adjacent to pockets of ubiquitinated, autofluorescent inclusions.

αB-crystallin: In the tissue sections, αB-crystallin exhibited a strong band of immunoreactivity in the zona interna of the median eminence and moderate staining of some third ventricular ependymal cells and periventricular astrocytes. However, in the latter, there was no overt co-localization of αB-crystallin to the autofluorescent inclusions (Fig. 6A). In the CSH-treated glial cultures, αB-crystallin often exhibited strong staining of astrocytic perikarya and processes. As in situ, there was no apparent co-localization of this stress protein to the autofluorescent inclusions (Fig. 6B).

Stress protein expression patterns, histochemistry and morphology of Gomori-positive astrocyte granules are summarized in Table I.

*Ub Immunofluorescence Following Short-term CSH Treatment*

Indirect immunofluorescence for Ub and GFAP was performed on DIV 6 on untreated (control) cultures and following 6 h exposure to CSH. A faint, dust-like pattern of Ub immunostaining was observed in the cytoplasm of untreated, GFAP-positive astroglia (Fig. 7A and B). In contrast, following CSH exposure, numerous GFAP-positive astroglia exhibited intense clusters of cytoplasmic Ub immunoreactivity (Fig. 7C and D).

**Figs. 1-6. Immunolocalization of stress proteins to autofluorescent astrocyte granules in rat brain sections and CSH-treated glial cultures**

Fig. 1 HSP27 shows intense co-localization (yellow fluorescence) to astrocyte granules *in situ* and in culture (open arrows; A and B, respectively). Solid arrows indicate smaller granules devoid of HSP27-immunoreactivity [bars = 100 μm for A; 10 μm for B]. C: same field as in B prior to merging of green and red channel images. Image in D is a Z-axis reorientation of the field depicted in B. Yellow fluorescence is preserved indicating true co-localization of HSP27 to the astrocyte granules.

Fig. 2 In the brain sections, HSP72 exhibits occasional immunolocalization to astrocyte granules and adjacent cytoplasm (A, arrow) [bar = 250 μm]. Infrequent co-localization of HSP72 to the autofluorescent material in culture is shown in B (arrow) and immunostaining of cytoplasm adjacent to the autofluorescent inclusions in C [bars = 10 μm for B and C].

Fig. 3 In situ, tanycyte processes (A; arrows) but not autofluorescent granules are immunoreactive for HSP90 [bar = 25 μm]. In the CSH-treated glial cultures (B) HSP90 localizes to the cell nucleus (arrowhead) but not to autofluorescent cytoplasmic inclusions [bar = 10 μm]. When non-specific mouse ascites fluid is substituted for stress protein monoclonal antibodies, the red autofluorescent granules remain visible but no green or yellow fluorescence is observed (B, insert).

Fig. 4 Both *in situ* (A) and *in vitro* (B) GRP94 staining often produced a "tricolour" fluorescent pattern resulting from partial stress protein co-localization to the granule periphery (red-yellow) and prominent staining of adjacent granule-free cytoplasm (green). In these preparations, some of the smaller autofluorescent granules and those proximal to the nucleus exhibit no GRP94 immunoreactivity (arrows, A and B) [bars = 25 μm for A; 10 μm for B]. B (insert): field represented in B prior to merging of green and red channel images.

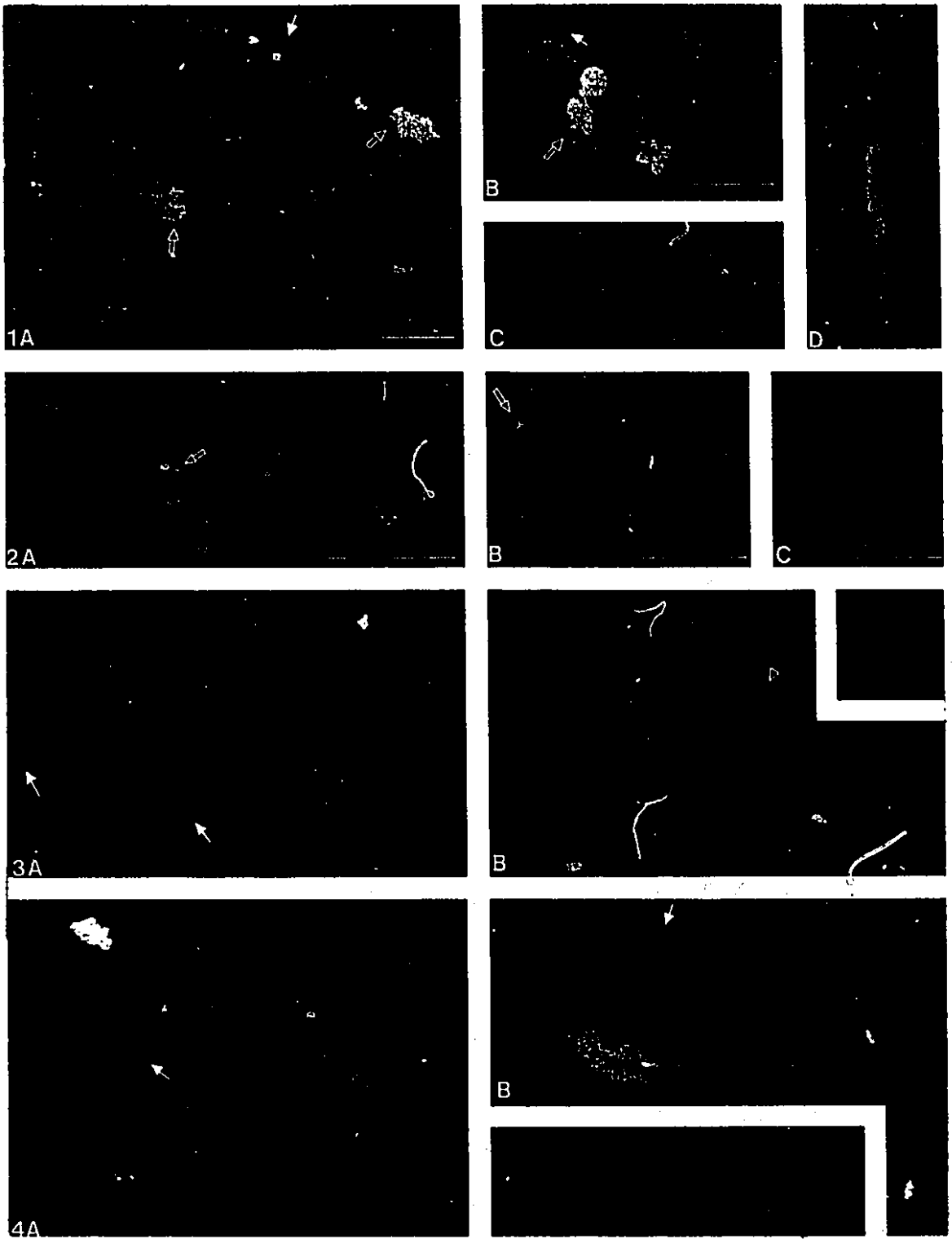
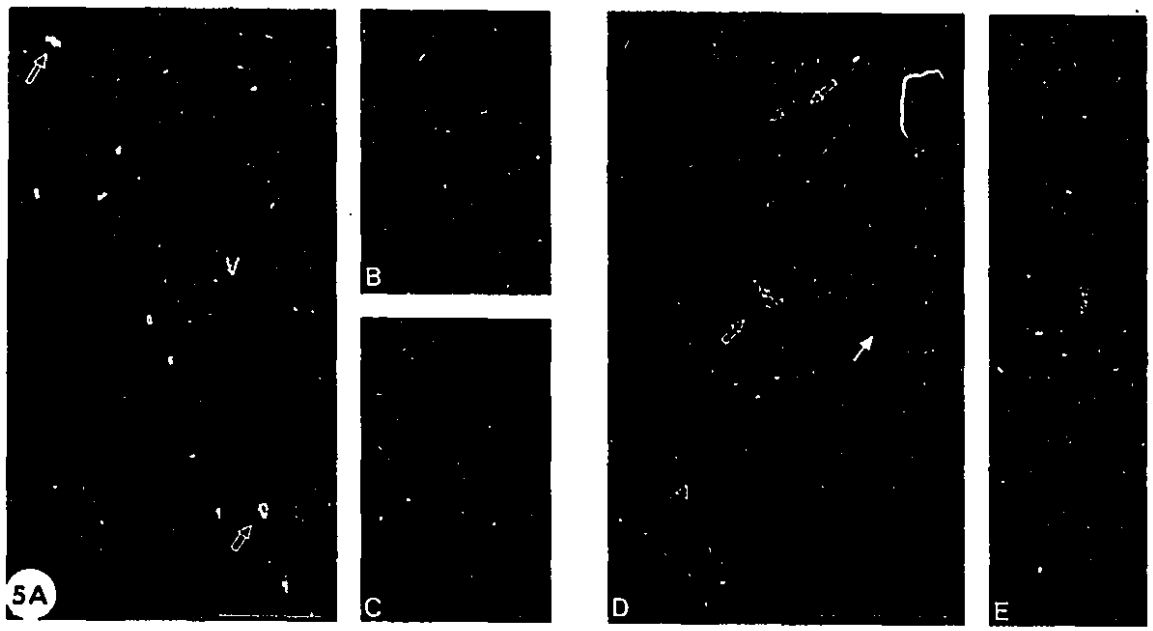

1A
B
C
D
2A
B
C
3A
B
4A
B

Fig. 5 In rat brain, Ub exhibits strong co-localization to the autofluorescent granules situated within the third ventricular subependymal zone (A, arrows) [bar = 25 μm]. B and C: fields shown in A, prior to merging of green and red channel images. V = third ventricle. *In vitro*, the larger autofluorescent granules are ubiquitinated (D, open arrows) whereas smaller granules are not Ub-immunoreactive (D, solid arrow). Occasional Ub-staining of granule-free cytoplasm is shown in D (arrowhead) [bar = 10 μm]. Image in E is a Z-axis reorientation of the field depicted in D. Yellow fluorescence is preserved indicating true co-localization of Ub to the astrocyte granules.

Fig. 6 In the brain sections, strong αB-crystallin staining can be seen in cells along the ventricular wall (A). Both *in situ* and *in vitro*, αB-crystallin manifests strong immunolabeling of astroglia without evident co-localization to the autofluorescent inclusions (A and B, arrows) [bars = 25 μm for A and B]. V = third ventricle. When normal rabbit serum is substituted for stress protein polyclonal antibodies, the red autofluorescent granules remain visible but no green or yellow fluorescence is observed (B, insert).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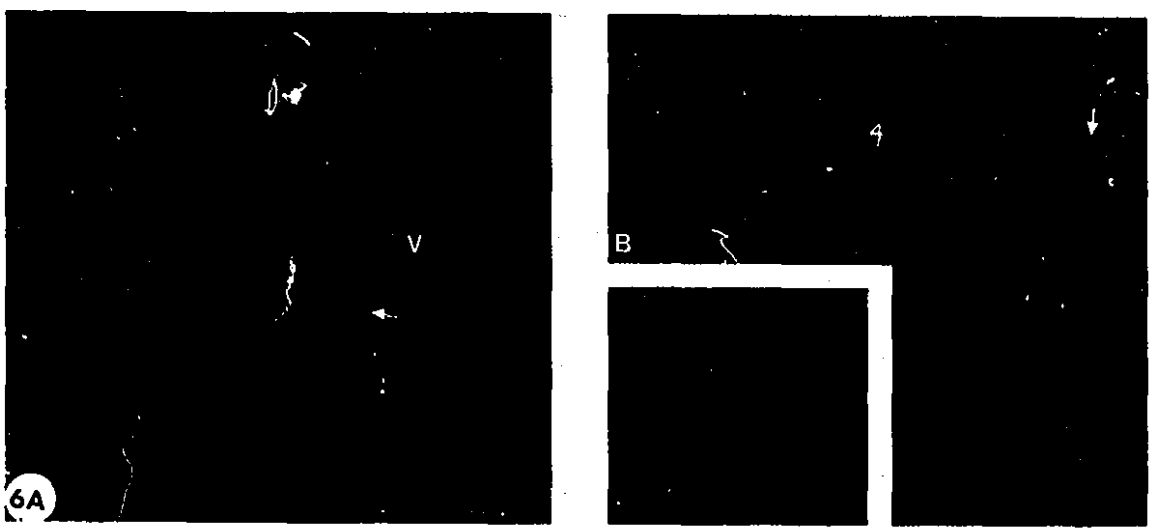
V
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TABLE I

*Gomori-positive astrocyte granules: histochemistry, morphology and stress protein expression patterns*

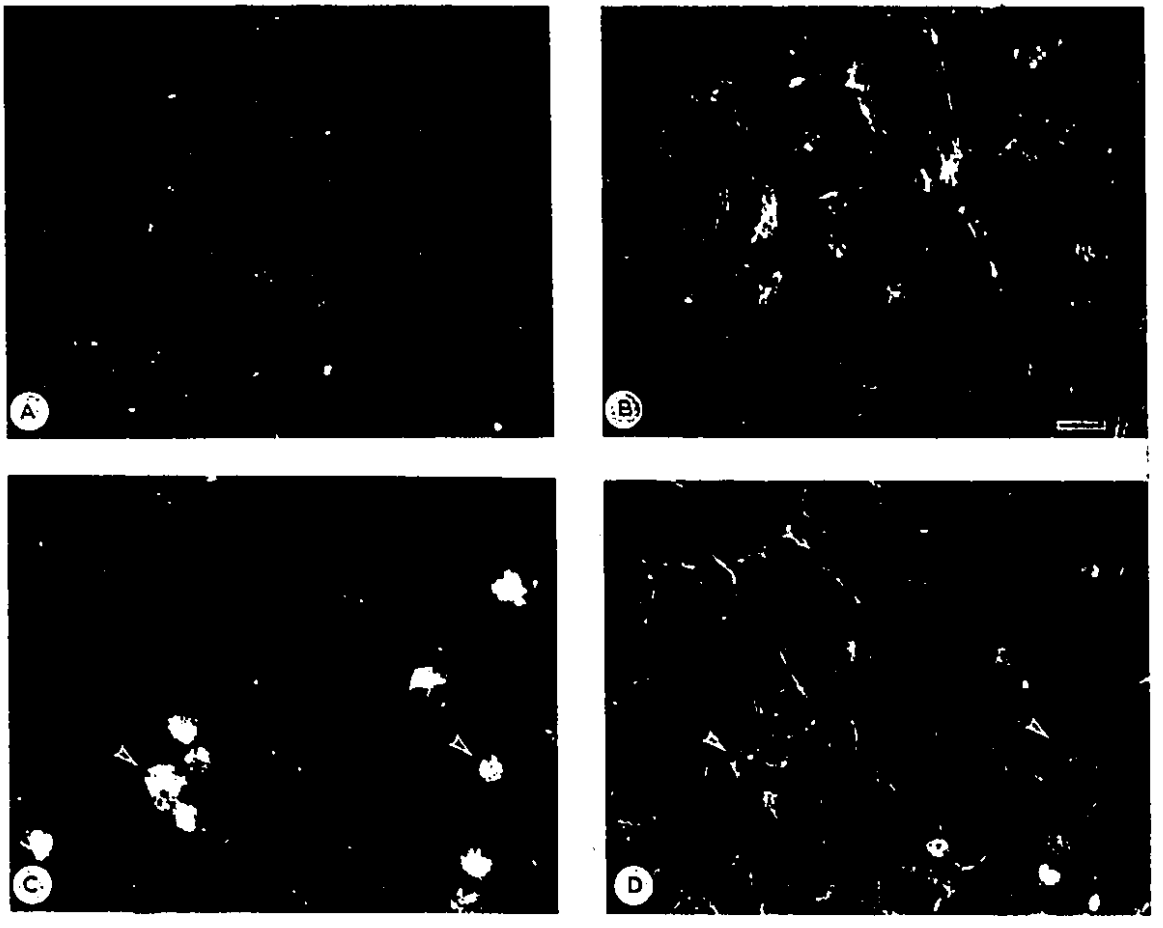
CAH = chrom alum hematoxylin; GRP = glucose regulated protein; HSP = heat shock protein.

| | *Astrocyte granules* | |
|---|---|---|
| | *Rat brain sections* | *Dissociated brain cell cultures* |
| Gomori stains (CAH) | yes[37] | yes[34] |
| Peroxidase activity (non-enzymatic) | yes[31] | yes[34] |
| Lipid | no[31] | no[34] |
| Autofluorescence | yes (red)[12] | yes (red)[34] |
| Ultrastructure and microprobe analysis | osmiophilic; angular/spherical shape; membrane-bound; iron-rich[41,a] | osmiophilic; spherical shape; membrane-bound; iron-rich[21] |
| Increase with aging or time in vitro | yes[28] | yes[34] |
| Cysteamine-induced | yes[33] | yes[34] |
| Estrogen-induced | yes[5,31,b] | ? |
| *Co-localization* | *12-week rat brain* | *12-day cysteamine exposure* |
| HSP27 | intense, larger granules | intense, larger granules |
| HSP72 | occasional | infrequent |
| HSP90 | no | no; nuclear translocation |
| GRP94 | intense, granule periphery | intense, granule periphery |
| Ubiquitin | intense, larger granules | intense, larger granules |
| αB-crystallin | no | no |

[a] Brawer, J.R. and Schipper, H.M. *in preparation*.

[b] accumulation limited to hypothalamic arcuate nucleus.

Fig. 7 Dual immunofluorescent labeling of Ub and GFAP in short-term, CSH-treated (880 μM x 6 h) and control glial cultures. Fields illustrated in A and B and those in C and D are identical. Ub exhibits faint, dust-like cytoplasmic staining (A) in untreated GFAP-positive astrocytes shown in B. After CSH treatment, intense clusters of Ub immunoreactivity (C, arrowheads) are observed in some GFAP-positive astroglia (D, arrowheads). All photographs were taken at the same magnification. [bar in B = 25 μm for A, B, C and D]


A
B
C
D

## DISCUSSION

As previously described[34], CSH treatment of immature astroglia induces the accumulation of unique cytoplasmic inclusions akin to those observed in limbic and periventricular astrocytes of the aging brain. Both *in vitro* and *in situ*, the inclusions exhibit non-enzymatic peroxidase activity and an affinity for the Gomori stains, chrome alum hematoxylin and aldehyde fuchsin[8,31,34,37]. The granules are rich in iron[21,38] and emit an orange-red autofluorescence characteristic of porphyrins[12,34] suggesting that a significant portion of the intracellular iron is sequestered as heme or some other metalloporphyrin. In the present study, laser scanning confocal microscopy revealed virtually identical patterns of stress protein immunolocalization to red autofluorescent astrocytic inclusions in CSH-treated glial cultures and in the subependymal third ventricular region of the adult rat brain. Both *in situ* and in the CSH-treated cultures, a) HSP27 and Ub consistently exhibited robust co-localization to the autofluorescent astrocytic inclusions, b) HSP72 immunostaining was variable with occasional co-localization to red autofluorescence, c) GRP94 immunostaining often appeared restricted to peripheral regions of the autofluorescent granules and adjacent cytoplasm, and d) HSP90 and αB-crystallin did not appear to immunolocalize to the glial inclusions. These observations greatly extend our previous histochemical studies underscoring the identical origin of the CSH-induced astroglial inclusions and those which spontaneously accumulate in the aging periventricular brain. Moreover, the results of the present study, in conjunction with the fact that systemic CSH administration increases numbers of peroxidase-positive astroglial inclusions in various brain regions[33], indicate that this sulfhydryl agent greatly accelerates a normal, aging-related process in subpopulations of astroglia.

We recently hypothesized that sustained intracellular oxidative stress may be a "final common mechanism" responsible for the accumulation of autofluorescent astrocyte granules in CSH-treated cultures and in the aging periventricular brain[23,33]. Evidence supporting this hypothesis is derived from the following observations: 1) CSH, in the presence of transition metals, generates a variety of free radical species including thiyl and

hydroxyl radicals, superoxide anion and $H_2O_2$[22]. 2) Exposure of astroglial cultures to CSH for 6 hours induces HO-1 and HSPs 27, 72 and 90[23], stress proteins typically up-regulated by heat shock and oxidative stress[3,10,11,35]. 3) Co-localization of HSP27, but not αB-crystallin, to the glial inclusions is consistent with a recent report that HSP27-αB-crystallin complexes dissociate in response to cellular stress[43]. 4) $H_2O_2$, a known oxidative stressor, stimulates the accumulation of peroxidase-positive astrocyte granules analogous to the effects of CSH[23]. 5) In our astroglial cultures, CSH rapidly induces a shift from diffuse, low-level Ub-immunostaining to intense, granular Ub deposits (present study). This finding is consistent with the formation of high molecular weight Ub-conjugates observed in a variety of other tissues following heat shock or oxidative stress[6,15]. Moreover, our results indicate that activation of the Ub pathway may be an early event in the biogenesis of peroxidase-positive astrocyte granules analogous to its putative role in the formation of lysosomal, autophagic vacuoles[14]. Thus, the metalloporphyrin-rich astrocytic inclusions appear to represent "heat shock granules" akin to those observed in other cells following hyperthermic or oxidative stress. Heat shock granules containing polymerized HSPs and HSP-protein conjugates may serve to protect various mRNAs and vital proteins from stress-related damage[24,25].

Confocal and electron microscopy have proven useful in delineating the cellular origin of the autofluorescent astrocytic inclusions. Using a panel of FITC-labeled antibodies directed against organelle-specific proteins, we observed partial co-localization of lysosomes, and to a lesser extent early endosomes and the rough endoplasmic reticulum (ER), to red autofluorescent granules induced in cultured astroglia by CSH exposure[29]. We also determined that within 24-72 hours of CSH exposure, many astroglial mitochondria exhibit progressive swelling, rearrangement or dissolution of cristae and sub-compartmental sequestration of redox-active iron (Brawer and Schipper, in preparation). These observations strongly suggest that a macroautophagic process involving abnormal mitochondria, lysosomes and possibly ER cisternae participates in the biogenesis of peroxidase-positive astrocyte granules in CSH-treated cultures and in the aging periventricular brain. Since GRP94 is synthesized in the ER, GRP94 immunostaining of

the granule periphery and adjacent cytoplasm, a pattern observed both *in vitro* and *in situ*, provides further evidence that cisternae of the ER may become incorporated into nascent astrocyte heat shock granules during aging and CSH exposure.

Ub-protein conjugates have been described in a host of neuronal and glial inclusions in the normal, senescent brain and in various neurodegenerative conditions. For example, Ub-immunoreactivity is present in corpora amylacea of the aging human brain[7], in neuronal Lewy bodies in parkinsonian subjects[16,20], in Pick bodies[16,19], in paired helical filaments and neurofibrillary tangles associated with Alzheimer's disease[4,16,42] and in astrocytic Rosenthal fibers in Alexander's disease[16,18]. The results of the present study indicate that Gomori-positive astrocyte granules, like corpora amylacea, are ubiquitinated inclusions that progressively accumulate in the normal, aging mammalian nervous system. The Gomori-positive granules are often surrounded by delimiting membranes[21] and exhibit partial staining with lysosomal markers[29]. Ubiquitination of these organelles is therefore consistent with the previous demonstration of free Ub and Ub-protein conjugates within lysosomes[9,36] and further contests the view that Ub-immunoreactivity in neuronal and other tissues is restricted to non-membrane-bound, non-lysosomal inclusions[18].

Chronic or repeated induction of various heat shock genes in response to sustained intracellular oxidative stress may be an important mechanism leading not only to the biogenesis of Gomori-positive astrocyte granules but to other aging- and disease-related CNS inclusions as well. For example, reports of HSP72 overexpression[26] and increased lipid peroxidation[39] in Alzheimer cerebral cortex and co-localization of HSPs to neurofibrillary tangles and senile plaques[26] support the notion that the diseased tissues are experiencing chronic oxidative stress. Finally, in addition to serving as markers of CNS senescence and oxidative stress, metalloporphyrin-rich astrocytic inclusions may actively contribute to the pathogenesis of various neurodegenerative disorders. Heme, a major constituent of the astrocytic inclusions, has been shown to block the energy-dependent degradation of Ub-protein conjugates and may impair the ability of Ub to tag cellular substrates for proteolysis[40]. Sequestration of heme or other metalloporphyrins within these

astrocytic inclusions may therefore interfere with Ub-dependent degradation of damaged, and potentially deleterious, cellular proteins. Furthermore, in the presence of $H_2O_2$, peroxidase-positive astrocytes promote the robust oxidation of dopamine and catecholestrogens to highly reactive semiquinone radicals[30]. Thus, the progressive accumulation of these redox-active glial inclusions in rodent and human striatum may render the senescent nervous system particularly vulnerable to parkinsonism and other free radical-related neurodegenerative conditions[27].

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## Connecting Text #3

We demonstrated that CSH-induced astrocyte granulation in primary glial cultures and in the intact rat brain occurs in the context of a robust cellular stress response (Chapters 2 and 3). In Chapter 4, we showed that patterns of stress protein immunolocalization to cytoplasmic inclusions in CSH-treated glial cultures are homologous to those observed in astrocytes of the aging periventricular brain, further supporting our contention that CSH recapitulates senescence-related changes in these cells. The age-dependent accumulation of peroxidase-positive astrocytic inclusions in the hypothalamic arcuate nucleus of female rodents has previously been shown to result from chronic exposure to circulating ovarian estrogen (Brawer et al., 1978, 1980; Schipper et al., 1981). Furthermore, this estrogen-related effect is readily reproduced in male rats receiving multiple intramuscular injections of estradiol valerate (Brawer et al., 1980). The study described in Chapter 5 was performed to determine whether estrogen-mediated acceleration of astrocyte granulogenesis in the adult rat brain is also dependent upon antecedent induction of a cellular stress response.

# CHAPTER 5

**Estrogen induction of glial heat shock proteins: implications for hypothalamic aging.**

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Key words: Aging, Astrocyte, Estrogen, Heat shock protein, Inclusion

## ABSTRACT

In the aging mammalian hypothalamus, a unique subpopulation of glial cells accumulates peroxidase-positive cytoplasmic inclusions distinct from lipofuscin. In adult rodents, this senescence-dependent glial granulation is accelerated by administration of estradiol valerate. In the present study, brain sections derived from male rats given 3 monthly intramuscular injections of estradiol valerate (0.2mg or 2.0mg) were immunostained for heat shock proteins and glial fibrillary acidic protein to determine whether a glial stress response is implicated in estrogen-induced granulation. Our findings indicate that estrogen elicits a heat shock response and subsequent granulation in astrocytes residing in estradiol receptor-rich brain regions including the arcuate nucleus and the wall surrounding the third ventricle, but not in estradiol receptor-deficient regions such as the striatum and corpus callosum. The heat shock proteins induced by estrogen, namely the 27, 72 and 90 kDa stress proteins, are upregulated in astrocytes in response to oxidative challenge supporting our hypothesis that estrogen mediates senescent changes in the rodent hypothalamus through oxidative mechanisms.

## INTRODUCTION

In the course of normal aging, chronic exposure to ovarian estrogen promotes the development of pathological changes in the hypothalamic arcuate nucleus (ARC), a neuroendocrine locus rich in estradiol receptors and implicated in the regulation of gonadotropin secretion (1,3,23). The appearance of pathological changes in the ARC can be markedly accelerated in male rats following multiple intramuscular injections of estradiol valerate (EV), and in female rats, by EV injection or continuous exposure to high-physiologic levels of estradiol resulting from continuous illumination or steroid-releasing Silastic implants (3,4,18). The estrogen-related ARC lesion is characterized by the accumulation of peroxidase (Gomori-)-positive astrocyte granules that are histochemically and morphologically distinct from the aging pigment, lipofuscin (22,23,26). Loss of ß-endorphin (8), dendritic damage, synaptic remodeling and microgliosis have also been reported in the ARC of EV-treated rats (1,3,5).

The mechanism by which estrogen promotes the accumulation of Gomori-positive astroglial inclusions in the hypothalamus remains unclear. In primary astroglial cultures and in rodent astrocytes *in situ* (28,29), the aminothiol compound, cysteamine (CSH), greatly accelerates the time-dependent accumulation of identical peroxidase-positive astrocytic inclusions. We recently demonstrated that damaged, iron-laden mitochondria are the subcellular precursors of mature, Gomori-positive astrocyte granules both in CSH-treated glial cultures (2) and in the aging periventricular brain (6). Moreover, CSH-induced astrocyte granulation appears to be dependent upon the antecedent induction of a robust cellular stress (heat shock) response characterized by the up-regulation of heat shock proteins (HSP) 27, 72 and 90 in these cells (15,16,28). Studies with CSH, $H_2O_2$ and ionizing radiation, all of which elicit astrocyte granulation (15,28,29,31), strongly suggest that intracellular oxidative stress may be a "final common pathway" promoting both a heat shock response and the biogenesis of cytoplasmic inclusions in these cells. In the present study, we set out to determine whether activation of a cellular heat shock response similarly plays an incipient role in the biogenesis of redox-active astrocyte

granules following chronic estrogenization.

## MATERIALS AND METHODS

Four-week-old male Sprague Dawley rats were obtained from the Canadian Breeding Farms, maintained under a 12 h light: 12 h dark cycle and allowed free access to standard rat chow and water.

Long-term EV study

Beginning at seven weeks of age, the animals received three consecutive monthly intramuscular injections of a) 2.0 mg EV (Delestrogen, Squibb Canada Inc.), b) 0.2 mg EV, or c) sesame oil vehicle (control). Each treatment group contained 4 animals. At 17½ weeks of age (two weeks following the last injection) the rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 100 mL saline followed by 250 mL of 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were immediately removed and immersed in cold fixative for 24 h. Forty-micron-thick coronal sections were cut on a vibratome (Lancer series 1000) in preparation for immunohistochemistry.

48-hour (short-term) EV study

Additional groups of 7-week-old animals were perfused 48 h following a single injection of 0.2 mg EV or sesame oil vehicle.

Peroxidase histochemistry-GFAP immunostaining

Free-floating sections were washed in Tris-buffered saline (TBS; pH 7.6, 4°C) and incubated in modified Karnovsky medium for the demonstration of (brown) endogenous peroxidase activity (10) followed by overnight incubation at 4°C with a rabbit-derived polyclonal antibody directed against glial fibrillary acidic protein (GFAP; 1:40 dilution; kindly provided by Dr. E. Wang of our institution) exactly as previously described (27,28). The tissue was subsequently processed by the Vectastain avidin-biotin-peroxidase

complex (ABC; Vector, Burlingame, CA) technique using goat anti-rabbit IgG (11). GFAP was visualized as a pink precipitate with α-naphthol as chromogen and pyronin B intensification as previously described (27,28). Peroxidase-positive astrocytes in these brain regions were examined and photographed using a Leitz Diaplan photomicroscope. Control sections were processed as above with omission of either a) DAB (with pre-incubation in 3% $H_2O_2$ in TBS for 30 min to eliminate endogenous peroxidase activity), b) the anti-GFAP antibody, or c) $H_2O_2$ in the α-naphthol reaction.

*Quantitation of DAB-positive astrocyte granules*

In the long-term study, numbers of peroxidase-positive astrocyte granules were quantitated in 1000x fields of corpus callosum (CC) and caudate-putamen (CP) with the aid of an ocular grid by a single investigator unaware of the tissue source. Similar counts were performed in the ARC in the short-term study.

Stress protein-GFAP double labeling

Free-floating rat brain sections were pre-incubated in 3% $H_2O_2$ for 30 min to eliminate endogenous peroxidase activity and washed in PBS. Sections derived from the long-term study were subsequently incubated with mouse monoclonal antibodies directed against HSP27, HSP72 (inducible), HSP90, or GRP 94 (StressGen, Victoria, B.C.) as described in full detail elsewhere (28). Antibody dilutions were 1:200 for anti-HSP27 and 1:100 for the other stress proteins. Immunohistochemistry was performed using peroxidase-conjugated goat anti-mouse IgG (secondary antibody) and the ABC kit. Following incubation in DAB-$H_2O_2$, the stress proteins were visualized as brown precipitates. GFAP immunolabeling using α-naphthol-pyronin was subsequently performed on these sections as described above. To control for possible background HSP staining, some sections were processed with non-specific ascites fluid (1:100-1:200 dilutions) in place of the stress protein monoclonal antibody. Rat brain sections from the short-term study were processed for HSP90-GFAP double labeling. Stained sections were examined and photographed

using a Leitz-Diaplan photomicroscope. Analysis was restricted to the ARC, the area surrounding the third ventricle (peri-III), the CC and the CP. For each animal, a minimum area of 0.14 $mm^2$ per region was evaluated and the total number of GFAP-positive astrocytes as well as the percentage of GFAP-positive astrocytes expressing HSP were determined. Differences between groups were compared using Student's two-tailed t-test with $p<0.05$ indicating statistical significance.

## RESULTS

Long-term EV Study

*Peroxidase-positive astrocyte granules*

Peroxidase-positive cytoplasmic granules were visualized as brown (oxidized DAB) deposits surrounded by pink (oxidized α-naphthol-pyronin) GFAP-positive cytoplasm (see reference 27,28 for colour illustrations depicting peroxidase-positive astrocytes). EV treatment has previously been shown to increase numbers of peroxidase-positive astrocyte granules in the ARC and third periventricular region of adult male and female rats (1,3,4,26). In the present study, chronic estrogenization did not increase numbers of peroxidase-positive glial inclusions in the CC or CP, or total numbers of GFAP-positive astrocytes in any of the brain regions surveyed, relative to controls (data not shown). In the absence of anti-GFAP antiserum or $H_2O_2$ in the α-naphthol medium, brown DAB-positive granules were readily visualized but astrocytes remained unlabeled. With the elimination of DAB and following pre-incubation in $H_2O_2$, all GFAP-positive astrocytes appeared devoid of endogenous peroxidase activity.

*Stress protein immunohistochemistry*

Colour illustrations depicting astroglia immunoreactive for GFAP and various HSPs are provided in reference 28 (Chapter 3). The percentages of GFAP-positive astrocytes exhibiting immunoreactivity for each stress protein surveyed following long-term estrogen treatment are shown in Table 1.

HSP27: In animals receiving multiple injections of 0.2 mg EV, there were significant increases in the percentages of astrocytes expressing HSP27 in the ARC and peri-III ($p<0.05$ relative to controls), but not in the other regions examined ($p>0.05$). Increases in the proportion of astrocytes expressing HSP 27 in ARC and peri-III following 2.0 mg EV treatment did not achieve statistical significance ($p > 0.05$ for each comparison relative to controls) due to large standard deviations.

HSP72: Chronic treatment with 2.0 mg EV resulted in a significant increase in the percentage of astrocytes expressing HSP72 in the peri-III region ($p<0.05$) but not in the other brain areas surveyed ($p > 0.05$). A trend towards increased HSP72 expression in the ARC failed to reach statistical significance ($p>0.05$).

HSP90: In the ARC and peri-III regions, low- and high-dose EV treatment induced a robust increase in glial HSP90 staining ($p<0.05$ relative to controls). High-dose EV treatment induced a smaller, but statistically significant ($p<0.05$), increase in glial HSP90 expression the CP, whereas no effect ($p>0.05$) was observed in the CC.

GRP94: A trend towards increased GRP94 immunoreactivity in astrocytes surrounding the third ventricle in EV-treated rats relative to controls was not statistically significant ($p>0.05$). Similarly, administration of EV had no appreciable effects on glial GRP94 expression in any of the other areas examined ($p>0.05$ for each comparison).

48-hour EV Study

As in the case of long-term EV treatment, GFAP expression was unaffected by acute administration of 0.2 mg EV (data not shown). Short-term EV treatment did not significantly alter numbers of DAB-positive astrocyte granules in the ARC (Fig. 1), a locus exhibiting intense granulation after long-term exposure to estrogen (3-5). Additional rat brain sections were processed for HSP90-GFAP double immunolabeling to determine whether an astrocytic stress response precedes EV-induced granulation. In the ARC, peri-III region and CC, there were significant increases in the percent of glia expressing HSP90 after short-term EV treatment (Fig. 1). In the CP, an increase in the proportion of astrocytes expressing HSP90 after short-term estrogen treatment failed to achieve statistical significance ($p<0.05$).

Table 1. Percent of GFAP-positive astrocytes (mean ± standard deviation) per region expressing HSP 27,72 or 90 or GRP 94 after long term EV treatment.

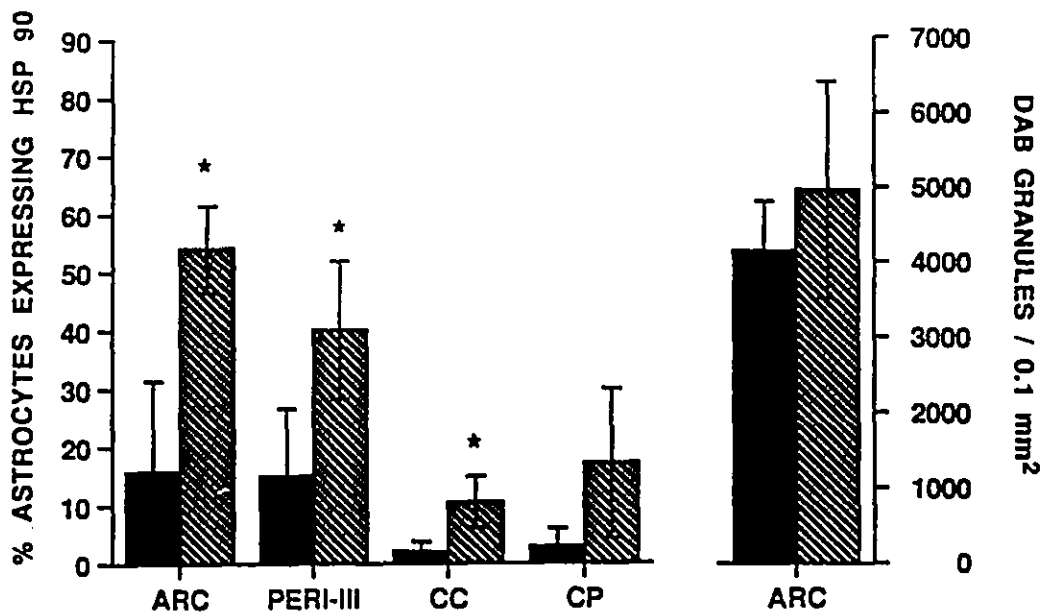
| | ARC | | | Peri-III | | |
|---|---|---|---|---|---|---|
| | CONTROL | EV 0.2 mg | EV 2.0 mg | CONTROL | EV 0.2 mg | EV 2.0 mg |
| HSP 27 | 16.7±6.9 | 68.2±7.1* | 56.2±27.2 | 28.5±4.4 | 58.6±14.2* | 55.9±29.3 |
| HSP 72 | 7.6±7.8 | 17.2±18.1 | 23.8±11.7 | 4.4±3.4 | 16.8±10.9 | 30.6±5.9* |
| HSP 90 | 2.7±1.9 | 37.8±15.5* | 37.1±6.4* | 6.1±7.9 | 35.5±9.0* | 32.8±6.0* |
| GRP 94 | 16.0±14.4 | 23.5±24.4 | 15.6±18.6 | 12.0±13.9 | 24.5±17.0 | 25.7±25.2 |

| | CC | | | CP | | |
|---|---|---|---|---|---|---|
| | CONTROL | EV 0.2 mg | EV 2.0 mg | CONTROL | EV 0.2 mg | EV 2.0 mg |
| HSP 27 | 26.4±26.0 | 38.2±24.9 | 46.8±22.3 | 33.0±4.5 | 36.6±12.7 | 43.3±8.4 |
| HSP 72 | 13.2±16.6 | 9.3±9.4 | 15.2±4.8 | 7.4±10.2 | 4.6±4.8 | 2.7±4.0 |
| HSP 90 | 19.5±15.4 | 12.2±8.1 | 14.9±9.1 | 3.7±3.1 | 4.8±6.0 | 12.9±5.3* |
| GRP 94 | 16.1±22.4 | 13.1±11.0 | 5.8±2.9 | 8.3±4.1 | 9.8±5.6 | 7.6±4.0 |

Abbreviations: ARC = arcuate nucleus; EV = estradiol valerate;
CC = corpus callosum; CP = caudate-putamen;
HSP = heat shock protein;
Peri-III = third periventricular region

* = significantly increased from control values ($p < 0.05$).

FIG. 1. The graph on the left shows the percent of GFAP-positive astrocytes (mean ± S.D.) expressing HSP90 per unit area 48 h following a single injection of 0.2 mg EV. The graph on the right indicates number of DAB-positive granules (mean ± S.D.) per unit area of ARC after identical, short-term EV exposure. Controls = solid bars. EV-treated = hatched bars.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## DISCUSSION

We previously demonstrated that estrogen administration or withdrawal accelerates or attenuates, respectively, the aging-related accumulation of peroxidase-positive astrocyte granules in the rodent periventricular brain (3,4,22,23). The pharmacological doses of EV utilized in this study induce many pathological changes in the ARC of male rats akin to those which arise spontaneously in senescent female rats and in castrated younger females exposed to high-physiological concentrations of exogenous estradiol (1,3,4,23). In the present study, we observed that estrogen-mediated granulation is restricted to regions rich in estrogen-receptors such as the ARC and the third ventricular subependymal zone, and does not occur in estrogen-receptor-deficient areas such as the CP and CC. We recently reported that in primary glial cultures and in the intact periventricular brain, the sulfhydryl agent, CSH, induces the appearance of identical glial inclusions in the context of an antecedent cellular stress response (15,16,28). Moreover, certain stress proteins up-regulated by CSH, such as HSP27 and ubiquitin, become incorporated within the nascent astrocytic inclusions (17).

The results of the current study further support our contention that induction of a cellular stress response is a pre-requisite for astrocytic granulation in the aging rat brain. Using immunocytochemical double labeling, we observed marked enhancement of HSP 27 and 90 staining in GFAP-positive astrocytes of the ARC and peri-third ventricular regions of long-term EV-treated rats relative to controls. We also observed augmented expression of HSP 72 which was limited to the peri-third ventricular region following prolonged EV treatment. Olazábal and co-workers have reported the induction of neuronal, but not glial, HSP90 and HSP70 in rat hypothalamus following estrogen treatment (12,19,20). However, they did not specifically assess HSP expression in GFAP-positive astrocytes by dual label immunohistochemistry. To our knowledge, the present study is the first to demonstrate estrogen-related upregulation of HSPs in astrocytes. This estrogenic effect on HSP expression (and subsequent granulation) may be direct given the high concentration of estrogen-receptor-positive astrocytes residing in the hypothalamic-median

eminence region of rodents (14).

Unlike HSP27, HSP90 expression following long-term EV treatment was also increased in the striatum, a region refractory to EV-induced granulation. The paucity of estrogen-receptors in the striatum (13,30,32) suggests that induction of glial HSP90 in this region may not represent a direct genomic effect of estrogen in this cell population. Estrogen induces HSP90 in various neuronal populations (12,19) where it may serve as a steroid receptor chaperone (21). Conceivably, estrophilic neurons residing outside the caudate-putamen may, upon innervating the latter, evoke a striatal glial HSP90 response. Similarly, estrophilic neurons projecting through the CC may mediate transient upregulation of glial HSP90 in this region after short-term EV exposure. Enhanced HSP90 expression in striatal (and callosal) astrocytes may, on the other hand, represent a direct, non-estrogen-receptor-mediated action of the steroid on these cells.

In all regions surveyed, GFAP expression appeared unchanged by chronic EV exposure. In contrast, long-term CSH administration induces a strong heat shock response and widespread granulation in astrocytes along with *enhanced* GFAP staining (28). Taken together, the results of the CSH and estrogen experiments indicate that the accumulation of Gomori-positive astrocyte granules may occur within the context of, or entirely independent of, classical astrocyte hypertrophy (gliosis).

Astrocytes exposed to EV (present study) or CSH (15) synthesize HSPs 27, 72 and 90, stress proteins which are highly responsive to oxidative challenge. In contrast, astrocytic expression of GRP94, a stress protein responsive to glucose deprivation and calcium ionophores but not to oxidative stress (9,33), remained unchanged following CSH or EV treatment (15; present study). These observations support the notion that intracellular oxidative stress is the mechanism responsible for estrogen-related HSP expression and granulation in aging periventricular astrocytes. The fact that heat shock protein expression rapidly occurs in astrocytes without evidence of concomitant granulation in the 48-hour EV experiment (present study) indicates that stress protein expression is not a consequence

of the accumulation of the iron-rich (redox-active) cytoplasmic inclusions. Nonetheless, the latter may oxidize catecholestrogens and catecholamines to free radical intermediates (25) and thereby promote further oxidative injury within the aging hypothalamus.

We recently observed that the administration of potent antioxidants such as vitamin E (7) or 21-aminosteroids (24) prevents estrogen-induced depletion of hypothalamic beta-endorphin, anovulatory sterility, and the development of polycystic ovaries in adult, female rats. These observations further support our hypothesis that estrogen-related degeneration within the aging rodent hypothalamus-pituitary-ovarian axis is mediated, at least in part, by oxidative stress.

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## Connecting Text #4

At the time the study described in Chapter 4 was nearing completion, Brawer and Schipper determined that abnormal mitochondria are the subcellular precursors of Gomori-positive astroglial inclusions *in situ* and in CSH-exposed glial cultures (Brawer et al., 1994a,b). Ultrastructural and X-ray microprobe analyses have revealed that shortly after exposure to CSH, astroglial mitochondria exhibit swelling, distortion of cristae, and accumulation of chromium (Brawer et al., 1994a). With additional exposure time, mitochondrial profiles accumulate redox-active iron and exhibit non-enzymatic peroxidase activity. Finally, the abnormal, iron-laden mitochondria fuse with lysosomal elements in a complex autophagic process to form mature Gomori granules (*ibid*).

Both CSH- and EV-dependent increases in astrocyte granulation occur in the context of a generalized cellular stress response (Chapters 2 - 5). The stress proteins induced by these treatments are commonly upregulated in response to hyperthermic challenge as well as oxidative stress. In the presence of transition metals, CSH is known to generate several prooxidant species including $H_2O_2$, and the superoxide, hydroxyl, and thiyl free radicals (Munday, 1989). In the mammalian hypothalamus, estradiol can be converted to catecholestrogen which, in turn, autoxidizes to generate $H_2O_2$ and superoxide (Kalyanaraman et al., 1985). Based on these and the aforementioned observations, we hypothesized that sustained intracellular oxidative stress is the "final common pathway" responsible for the transformation of normal glial mitochondria to mature Gomori-positive inclusions. In support of this, direct exposure of astroglial cultures to the oxidant, $H_2O_2$, results in a robust accretion of peroxidase-positive cytoplasmic inclusions akin to the effects of CSH. The study described in the Appendix was performed to determine whether CSH is directly responsible for mediating oxidative injury to isolated astroglial mitochondria (Gomori granule precursors).

## APPENDIX

The study presented here constitutes one component of a larger collaborative manuscript entitled:

**Redox perturbations in cysteamine-stressed astroglia: implications for inclusion formation and gliosis in the aging brain.**

Authors: Manganaro F, Chopra VS, Mydlarski MB, Bernatchez G, Schipper HM

Free Rad Biol Med (1995) in press.

# APPENDIX

## Introduction

Both *in situ* and in CSH-treated astrocyte cultures, peroxidase-positive glial inclusions are derived from swollen, acristic mitochondria that participate in a complex macroautophagic process (Brawer et al., 1994a,b; Schipper et al., 1993). We have hypothesized that intracellular oxidative stress may be the final common pathway leading to astrocyte granulogenesis. This study was performed to determine whether oxidative mechanisms play a role in the selective toxicity of CSH to the mitochondrial compartment.

## Methods

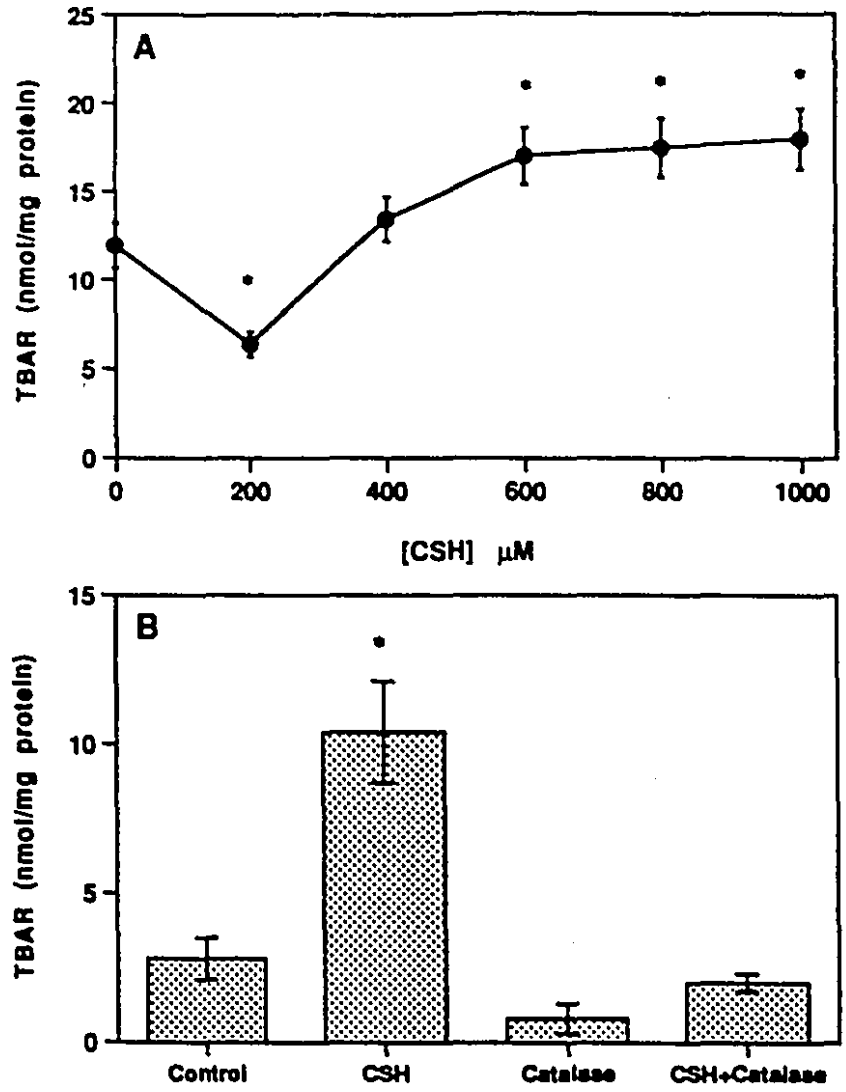
Primary astrocyte cultures were prepared as previously described (Chapter 2). 6-day-old untreated astrocyte monolayers were washed twice with cold phosphate buffered saline, scraped, centrifuged, and resuspended in 10 volumes of lysis buffer containing 2 mM $MgCl_2$. 2 mM Tris-HCl pH 7.4, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma Chemical Co., St. Louis, Mo). The cells were sonicated (Sonics & Materials, Danbury, CT) at a power level of 10 for 5 min in a cooled water bath. The cell sonicates were centrifuged at 1250 g for 20 min, and the mitochondria-rich supernatants were collected. The mitochondrial fraction was pelleted by centrifugation at 25,000 g for 25 min (Mickelson et al., 1980). Mitochondrial purity was increased 65-fold relative to the whole cell preparations as determined by cytochrome C oxidase activity (Madden and Storrie, 1978). The mitochondrial fractions were collected and subjected to varying concentrations of CSH in the presence (Minotti and Aust, 1987) and absence of 100 μM $FeCl_3$ (Sigma) in 150 mM NaCl (pH 7.0). Mitochondrial lipid peroxidation was assessed in the presence of exogenous iron to compensate for loss of this transition metal during the isolation procedure (Schipper, unpublished results). The effects of CSH (880 μM) on mitochondrial lipid peroxidation were also assessed in the presence of bovine liver catalase (0.02%; Sigma) to determine the role of CSH-derived $H_2O_2$ in our system. The mitochondria were incubated with the above reagents for 1 h at 37°C

followed by heating to 96°C for 1h in the presence of 12 volumes of thiobarbituric acid (TBA) solution (0.4% SDS, 8% acetic acid, 0.3% TBA). After cooling, lipid peroxidation in the mitochondrial incubates was estimated spectrophotometrically by measuring the formation of thiobarbituric acid-reactive products (TBAR) at 532 nm (Ohkawa et al., 1978) relative to the hydrolysis of 1,1,3,3,-tetramethoxypropane (Sigma) in standard preparations.

**Results and Discussion**

CSH in the range of 600-1000 μM significantly stimulated TBAR formation in isolated astroglial mitochondria in the presence (Fig. 1A) and absence (Fig. 1B) of $FeCl_3$. In the absence of exogenous iron, abundant mitochondrial heme ferrous iron may sustain the autoxidation of CSH with concurrent generation of oxyradicals (Munday, 1989) thereby promoting the peroxidation of mitochondrial lipids. In support of this, earlier studies have demonstrated that CSH-induced lipid peroxidation of isolated rat liver mitochondria is prevented when CSH is maintained in reduced form (Skrede and Christophersen, 1966). In our study, TBAR augmentation by CSH (at 880 μM) was entirely abolished by coincubation with catalase (Fig. 1B) indicating that CSH-derived $H_2O_2$ may be largely responsible for mitochondrial injury in this system. These results suggest that antioxidant therapy may prove useful in attenuating the accumulation of redox-active astroglial inclusions in the aging nervous system.

Fig. 1. Effects of CSH on TBAR formation in suspensions of astrocyte mitochondria. (A) Mitochondrial suspensions in the presence of 100 μM $FeCl_3$. Vertical bars represent the standard deviation of three to six observations. (B) Mitochondrial suspensions in the presence of CSH (880 μM) with and without exogenous catalase (0.02%). Asterisks denote statistically significant differences relative to untreated controls ($p < 0.05$).


A
TBAR (nmol/mg protein)
25
20
15
10
5
0
0
200
400
600
800
1000
[CSH] µM
B
TBAR (nmol/mg protein)
15
10
5
0
Control
CSH
Catalase
CSH+Catalase

# CHAPTER 6

# CONCLUSION

## 1. CELLULAR STRESS AND THE BIOGENESIS OF ASTROCYTIC INCLUSIONS

A distinct subpopulation of neuroglia in the hippocampus, striatum and periventricular brain regions of aging vertebrates accumulates cytoplasmic granules which are morphologically and histochemically distinct from lipofuscin (Schipper, 1991). The cytoplasmic granules are Gomoriphilic, exhibiting an affinity for chrome alum hematoxylin and aldehyde fuchsin. The granules emit an orange-red autofluorescence characteristic of porphyrins or oxidized flavoproteins and contain a non-enzymatic peroxidase activity thought to be largely mediated by ferrous iron (Brawer et al., 1994a; Schipper et al., 1991).

In the rodent brain, experimental induction of Gomori-positive astrocytes occurs in periventricular brain regions in response to X-irradiation (Srebro, 1971) and chronic estrogenization (Brawer et al., 1980, 1983; Schipper et al., 1990a; Schipper, 1993). In astrocyte cultures derived from dissociated fetal and neonatal rat brain, identical Gomori- and peroxidase-positive granules accumulate with time *in vitro* (Schipper et al., 1990c). Moreover, addition of CSH to the culture medium massively accelerates the accretion of these glial inclusions and provides an excellent model for investigating the mechanisms responsible for their biogenesis (Schipper et al., 1990c).

Peroxidase-positive astroglia in culture have been shown to oxidize neutral catechols, generating semiquinone radicals (Schipper et al., 1991) with proven cytotoxic activity (Horning et al., 1978; Kalyanaraman et al., 1985). In view of this latter property and the fact that peroxidase-positive astrocyte granules progressively accumulate in the aging brain, Schipper postulated that these cells may contribute to the pathogenesis of parkinsonism and other age-associated, free radical-related neurodegenerations (Schipper, 1991; Schipper et al., 1991). The studies described in this thesis were performed in an attempt to elucidate the mechanisms responsible for the accumulation of peroxidase-positive astrocytic inclusions in primary culture and in the aging periventricular brain.

## 1.1 Induction of the Cellular Stress Response Precedes Astrocyte Granulation *In Vitro*

In the first study (Chapter 2), the CSH model for accelerated astrocyte granulation *in vitro* was employed to determine whether CSH induces stress protein expression in astroglia as had been previously shown in rat liver (Peterson et al., 1989). We demonstrated that exposure of primary astrocyte cultures to CSH provokes a robust cellular stress response, as characterized by the enhanced expression of HO-1 and HSPs 27, 72 and 90, before increases in numbers of DAB-positive cytoplasmic granules become apparent at the LM level. The immunofluorescent staining patterns exhibited by the HSPs in CSH-pretreated cells were characteristic of those displayed in other cell types undergoing heat shock. Furthermore, in comparison to control cultures, CSH-pretreated cells exhibited enhanced resistance to both mechanoenzymatic trauma and $H_2O_2$, providing physiologic evidence that CSH induces a cellular stress response in cultured astroglia. Taken together, these findings suggest that activation of a cellular stress response may be a functional prerequisite to the formation of Gomori-positive astrocyte granules in CSH-treated glial cultures. In this respect, Gomori-positive astrocytic inclusions may represent a type of "stress granule" akin to "heat shock granules" which have been shown to arise in other cell types following sustained stress (Collier et al., 1988; Nover et al., 1989).

## 1.2 The Biogenesis of Peroxidase-positive Astrocyte Granules *In Situ*

The second study (Chapter 3) was undertaken to determine whether subcutaneous CSH administration to young adult rats accelerates the aging-related accumulation of peroxidase-positive astrocyte granules *in situ*, and whether this also occurs in the context of a glial cellular stress response. Long-term CSH treatment produced significant increases in DAB-positive granule densities in all regions surveyed except the arcuate nucleus. In the latter, there was a clear trend towards increased granulation following CSH exposure which failed to reach statistical significance. Other regions largely devoid

of peroxidase-positive glia, such as the cerebellum, exhibited no CSH-induced granulation. Thus, systemic CSH administration significantly accelerates the appearance of a normal "aging" phenotype in subpopulations of rat astroglia. In addition, both acute and prolonged exposure to CSH treatment induced HSPs 27, 72 and 90 and GRP 94 in GFAP-positive astrocytes residing in virtually all brain regions susceptible to CSH-induced granulation. The upregulation of GRP94 *in situ* may represent an indirect effect of CSH (perhaps mediated by perturbations in glucose or calcium homeostasis) because this aminothiol does not appreciably alter patterns of GRP94 expression in primary astrocyte cultures (Chapter 2). Thus, as in primary astroglial cultures, upregulation of the cellular stress response is a proximal event in the biogenesis of peroxidase-positive astrocyte granules *in situ*.

1.3 CSH Elicits a Unique Gliopathy

As described in Chapter 3, long-term CSH exposure reduced somatostatin immunoreactivity in fiber tracts of the ventral striatum akin to its effects in short-term studies (Ceccatelli et al., 1987). However, this finding may not represent true depletion of somatostatin levels or loss of somatostatin-containing neurons. Rather, by virtue of disulfide bond interactions, CSH may chemically alter somatostatin molecules rendering them undetectable by conventional immunohistochemistry (Kwok et al., 1992). Moreover, there was no evidence of neuronal damage or demyelination in Nissl-, myelin-, or silver-stained sections derived from rats subjected to long-term CSH exposure. The development of neuropathological features, including basal ganglia necrosis and patchy demyelination, has been documented in patients chronically receiving CSH for the treatment of nephropathic cystinosis (Vogel et al., 1990a). However, these deleterious changes probably derive from the disease process itself and seem unrelated to CSH toxicity because similar neuropathologic profiles are apparent in some individuals with nephropathic cystinosis who have survived into the second or third decades without CSH treatment. Nevertheless, our findings suggest that brain autopsy material derived from subjects who received CSH treatment for nephropathic cystinosis should be examined for

the accumulation of Gomori-positive glial inclusions. In the present study, prolonged exposure to CSH elicited robust astrocytic hypertrophy (gliosis) in the corpus callosum, ventral hippocampal commissure and striatum of rats. These findings, in conjunction with observations that CSH-induced HSP expression and cytoplasmic granulation are restricted to astrocytes (and certain third ventricular ependymal cells) *in situ* and in pure neuroglial cultures (Chapter 2) strongly suggest that this aminothiol compound elicits a primary "gliopathy" independent of any antecedent neuronal injury.

1.4 Gliosis is Not Essential for Astrocyte Granulation

Both CSH exposure (Chapter 3), and EV treatment (Chapter 5) induce overexpression of HSPs and peroxidase-positive granulation within subpopulations of astrocytes in the adult rat brain. In contrast, chronic CSH, but not long-term EV, treatment resulted in enhanced GFAP expression *in situ*. Taken together, these results indicate that the accumulation of Gomori-positive astrocyte inclusions may occur within the context of, or entirely independent of, classical astrocyte hypertrophy (gliosis).

1.5 Gomori Astrocyte Inclusions Contain HSPs

The third study (Chapter 4) was performed to ascertain whether HSPs are actual constituents of the Gomori astrocyte granules in adult rat brain sections and in CSH-treated astrocyte cultures. Using immunofluorescence-conjugated antibodies directed against a host of stress proteins in conjunction with laser scanning confocal microscopy, we determined that HSP27 exhibits intense colocalization to the red autofluorescent astrocyte inclusions both *in situ* and *in vitro*. GRP94 exhibited partial colocalization to peripheral regions of the astrocytic inclusions in both preparations. Colocalization of GRP94, an ER-derived stress protein, suggests that elements of the ER may participate in the biogenesis of peroxidase-positive astrocyte granules. Using a panel of antibodies directed against various cellular organelles, it was recently demonstrated that the ER exhibits occasional colocalization to the astrocyte inclusions in CSH-treated cultures

(Schipper et al., 1993). Furthermore, ultrastructural studies of astrocyte inclusions in periventricular regions of aging and estrogen-treated rats (Brawer and Sonnenschein, 1975; Srebro and Lach, 1987) and in CSH-treated glial cultures (McLaren et al., 1992) revealed that the granules occasionally appear contiguous with electron-dense cisternal elements, possibly of ER origin. Accordingly, these results suggest that HO-1, another ER-derived stress protein, would similarly exhibit partial colocalization to astrocyte granules. Indeed, partial immunolocalization of HO-1 to autofluorescent astrocyte granules in CSH-treated cultures and in the intact rat brain has been observed (Mydlarski and Schipper, unpublished results). In contrast, HSP72 was only infrequently found to be a constituent of astrocytic inclusions in brain and in culture, and the granules appeared consistently devoid of HSP90 and αB-crystallin. Thus, patterns of stress protein immunolocalization to astroglial inclusions in the third periventricular region of the adult rat brain and in CSH-treated glial cultures are virtually indistinguishable. This finding greatly extends previous histochemical and morphological data underscoring the identical origin of the CSH-induced astroglial inclusions and those which spontaneously accumulate in the aging periventricular brain.

1.6 Ubiquitin Activation is an Early Event in Gomori Granulation

In contrast to untreated, control astroglial cultures which exhibited weak, diffuse Ub-immunostaining, short-term (6h) CSH treatment induced a shift to intense, granular deposition of Ub suggestive of the formation of Ub-protein conjugates (Chronopoulos et al., 1992). The induction of Ub and the formation of high molecular weight conjugates is known to occur in a variety of tissues following heat shock or oxidative stress (Carlson et al., 1987; Finley et al., 1987; Haas and Bright, 1985). This indicates that activation of the Ub system in our model may represent an *early* event in the biogenesis of these inclusions. Moreover, these results contest the "common view of ubiquitin being involved in [neuropathological] reactions that are both secondary and late" (Landon et al., 1994). Furthermore, our results demonstrate that the Ub system is activated shortly after CSH exposure (6h), concomitant with the upregulation of HSPs 27, 72, 90 and HO-1 (Chapter

2). This finding argues against the view that activation of the Ub system is contingent upon prior failure of the HSPs in their attempt to renature damaged polypeptides and reconfer protein homeostasis (Wilkinson, 1994).

## 1.7 Gomori Granules are Ubiquitinated Inclusions

Gomori-positive astrocyte granules often appear to be delimited by membranes under transmission EM (McLaren et al., 1992) and the larger inclusions are heavily labeled with lysosome-specific markers as determined by immunofluorescence confocal microscopy (Schipper et al., 1993). Ubiquitination of mature, autofluorescent inclusions (Chapter 4) is therefore consistent with earlier studies demonstrating the accumulation of free Ub and Ub-protein conjugates within lysosomes (Doherty et al., 1989; Laszlo et al., 1990; Schwartz et al., 1988), and further contradicts the view that Ub-immunoreactivity in neural and other tissues is restricted to non-membrane-bound, non-lysosomal inclusions (Manetto et al., 1989). Immunoelectron microscopy may be required to determine whether Ub or other specific HSPs colocalize to aberrant mitochondria prior to autophagy, or if incorporation of the stress proteins into astroglial inclusions occurs during or after lysosomal fusion.

Ub has been associated with a number of senescence- and disease-related inclusions including granulovacuolar bodies, Marinesco bodies, granular degeneration of myelin, dystrophic axons and neurites, and corpora amylacea (reviewed in Dickson and Yen, 1994; Cissé et al., 1993). Of these inclusions, only corpora amylacea have previously been shown to predominate in astrocytes of the normal aging human brain (Anzil et al., 1974; Palmucci et al., 1982; Ramsay, 1965). The results presented herein, and those of Schipper and Cissé (1995) documenting the presence of Gomori granules in aging human neural tissues, clearly establish ubiquitinated, Gomori granules as a second, highly consistent biomarker of astrocyte senescence in the mammalian brain. Moreover, recent work from Schipper's laboratory provides strong evidence implicating Gomori-positive glial

inclusions as structural precursors of corpora amylacea in subpial and periventricular regions of the aging and Alzheimer-diseased human brain (Cissé and Schipper, 1995; Schipper and Cissé, 1995)

## 1.8 Estradiol-related Astrocyte Granulation

Administration of estrogen to adult female rats hastens the development of senile persistent estrus and produces a lesion in the hypothalamic ARC nucleus characterized by dendritic damage, microgliosis, loss of β-endorphin, and an accretion of peroxidase-positive astrocyte granules (Brawer and Sonnenschein, 1975; Brawer et al., 1983; Schipper et al., 1981; Schipper et al., 1990a; reviewed in Brawer et al., 1993 and Schipper, 1993). The study described in Chapter 5 was performed to determine whether estrogen, like CSH, accelerates age-dependent Gomori granulation in the context of a cellular stress response. We determined that three monthly intramuscular injections of estradiol valerate (EV) resulted in the overexpression of HSPs 27, 72, and 90 and augmented cytoplasmic granulation in GFAP-positive astrocytes of the ARC and third ventricular subependymal zone, areas rich in estrogen receptors. In contrast, long-term EV treatment did not induce HSP upregulation coupled with astrocyte granulation in estrogen receptor-deficient brain regions such as the CP and CC. Although Olazábal and coworkers have previously reported the induction of HSPs 70 and 90 in hypothalamic neurons with estrogen treatment (Kleopoulos et al., 1991; Olazábal et al., 1992a,b), this study represents the first to demonstrate estrogen-related upregulation of HSPs in astrocytes. Moreover, short-term administration of EV (48h) induced HSP expression, but failed to cause concomitant astrocyte granulation in the ARC. These findings, in conjunction with results derived from CSH- and $H_2O_2$-treated astrocyte cultures (Chapters 2 and 4), and CSH-exposed rats (Chapter 3), lend further support to the notion that astrocytes must first engage in a cellular stress response *en route* to accumulating peroxidase-positive cytoplasmic inclusions.

## 1.9 Peroxidase-positive Astrocyte Granules Derive From Damaged Mitochondria

Ultrastructural examination of cultured astrocytes following variable lengths of exposure to CSH has recently been performed in an effort to delineate the subcellular precursors of Gomori astrocyte inclusions (Brawer et al., 1994a). Shortly after exposure to CSH, astroglial mitochondria undergo a dramatic sequence of morphological changes leading to Gomori granule development. In untreated, control astroglia, mitochondria exhibiting tightly packed cristae abound. Within 6h of CSH treatment, many (but not all) mitochondria exhibit swelling, distortion of cristae, and accumulation of chromium as detected by electron microprobe analysis, but are devoid of peroxidase activity. After 24h of exposure to CSH, mitochondrial profiles surrounded by concentric sheaths of membrane and altogether devoid of organized cristae become apparent. In contrast to normal mitochondria and smaller nascent glial inclusions, many of the larger, membrane-bound, acristic profiles contain iron in addition to chromium. Furthermore, the larger iron-laden inclusions are DAB-positive, attesting to the role of iron as the source of peroxidase activity in these cells. After 72h of CSH exposure, increased numbers of peroxidase-positive, acristic mitochondria, but very few swollen profiles characteristic of the shorter treatment periods, are observed. Clustering of the aberrant mitochondrial profiles in the vicinity of normal mitochondria becomes more pronounced with CSH exposure time in culture. Interestingly, related changes including swelling and loss or shortening of cristae have been shown to occur in mitochondria as a function of normal aging (Wilson and Franks, 1975), further substantiating the notion that CSH accelerates a senescence-dependent mitochondrial dystrophy in subpopulations of astroglia. Peroxidase-positive inclusions observed at 12 days of CSH treatment occasionally abut cisternal elements reminiscent of rough ER (Brawer et al., 1994a), and exhibit the lysosomal protein, lgp120 (Schipper, et al., 1993), and acid phosphatase activity (Brawer et al., 1994a). These results indicate that CSH treatment provokes a macroautophagic response in astroglia involving the sequestration of damaged, iron-laden mitochondria (and, to a far lesser extent, other subcellular constituents) within electron-dense, membrane-bound cytoplasmic inclusion bodies. Various mitochondria-specific markers consistently colocalize to

peroxidase-positive astrocyte inclusions in the aging rat (Brawer et al., 1994b) and human (Schipper and Cissé, 1995) brain. These findings indicate that a similar autophagic process initiated by mitochondrial degeneration may represent a fundamental, senescence-related change in subpopulations of limbic and periventricular astroglia within the mammalian brain.

## 1.10 Metal Sequestration by Peroxidase-positive Glial Inclusions

Iron influx has been shown to increase in cells undergoing oxidative stress (Halliwell et al., 1992). Recently, Wang et al. (1995) demonstrated that CSH treatment of cultured astrocytes selectively enhances the sequestration of inorganic $^{59}FeCl_3$ by mitochondria but does not alter iron distribution in either the whole-cell or lysosomal compartments. Similarly, CSH-induced alterations of mitochondrial structure coincide with enhanced uptake of radiolabelled $^{51}$chromium by astroglial mitochondria (Brawer et al., 1995). Interestingly, elemental X-ray microprobe analysis of the Gomori inclusions in cultured astrocytes and in the estrogen-treated arcuate nucleus reveals certain differences between the two systems. In culture, CSH-induced Gomori granules contain chromium and iron (Brawer et al., 1994a; McLaren et al., 1992), but copper is not detectable (Brawer and Schipper, unpublished observations). In contrast, astrocyte inclusions in estradiol-treated animals appear to contain abundant copper and small amounts of chromium but fail to exhibit X-ray emission peaks indicative of iron (Brawer et al., 1994b). In the latter, the presence of iron has, however, been demonstrated histochemically using Perl's method with DAB-intensification (Hill and Switzer, 1984). These apparent incongruities may (i) reflect the different availabilities of transition metals in cultured astroglia versus the intact brain, (ii) arise from loss of loosely-bound metals during tissue handling, or (iii) result from variable sensitivities of the methods employed in demonstrating intracellular metals. In any case, both iron and copper are capable of mediating non-enzymatic peroxidase reactions (Goldstein and Czapski, 1986; Munday, 1989; Simpson et al., 1988; Sinha et al., 1990; Stadman, 1990) and could thereby account for the redox-active properties of

Gomori astrocyte inclusions. Furthermore, the finding that porphyrin-heme biosynthesis is suppressed in CSH-treated cells indicates that *nonheme* iron and/or related transition metals, but not metalloporphyrin iron as initially conjectured (Schipper et al., 1990a), are responsible for the peroxidase-activity of these astrocytic inclusions (Wang et al., 1995). Although also capable of participating in redox reactions (Mikalsen et al., 1989; Rossi and Wetterhahn, 1989; Sugiyama 1992), chromium is an unlikely candidate for the peroxidase activity of Gomori granules because in CSH-treated astrocyte cultures, chromium peaks are readily detectable by microprobe well in advance of the appearance of DAB staining (Brawer et al., 1994a). Nonetheless, these results suggest that CSH not only injures mitochondria, but also promotes the sequestration of redox-active transition metals within the mitochondrial compartment. Finally, the orange-red autofluorescence typically emitted by the peroxidase-positive inclusions may represent the presence of oxidized mitochondrial flavoproteins (Duchen and Biscoe, 1992; Kohler and Fromter, 1985), rather than porphyrin accumulation as had previously been proposed (Goldgefter et al., 1980).

## 1.11 Intracellular Oxidative Stress: A "Final Common Pathway" for the Biogenesis of Astrocytic Inclusions

Oxidation of CSH in the presence of transition metals generates several prooxidant species including $H_2O_2$, and the superoxide, hydroxyl, and thiyl free radicals (Munday, 1989). HO-1, Ub, and the various HSPs induced by CSH (Chapters 2 and 4) are commonly upregulated in response to hyperthermic challenge as well as oxidative stress. In contrast, addition of CSH to the cultures did not enhance the expression of GRP94, a stress protein known to respond to glucose deprivation and calcium ionophores, but not to heat shock or oxidative stress (Lee, 1987; 1992). Subcutaneous administration of CSH also resulted in overexpression of these redox-sensitive HSPs in GFAP-positive astroglia (Chapter 3) providing further indirect evidence of a free radical mechanism of CSH action.

The gene coding for manganese superoxide dismutase (MnSOD) is modulated in bacteria, and to a lesser extent in mammalian cells, by oxidative stress (Liochev and Fridovich,

1992; Wong et al., 1992). Conceivably, this mitochondrial enzyme, which catalyzes the dismutation of superoxide anion to $H_2O_2$, protects mitochondria from inordinate or inadvertent superoxide radical generation during the normal electron transport process and following exposure to mitochondrial toxins. MnSOD activity is significantly enhanced in CSH-treated glial cultures and in the intact diencephalon of rats given subcutaneous injections of CSH relative to respective controls (Manganaro et al., 1995). Increased MnSOD activity in liver mitochondria derived from aged humans has been proposed as a mechanism whereby senescent tissues cope with an increased oxidative burden (Yen et al., 1994). Elevated MnSOD levels in the Parkinsonian substantia nigra have been documented (Saggu et al., 1989) and are felt to indicate the occurrence of excessive oxidative stress in this condition (Jenner, 1992). CSH-induced oxidative stress may similarly provoke the observed increases in astroglial MnSOD gene transcription and enzymatic activity in our model (Manganaro et al., 1995).

As in the case of CSH, EV-related astrocyte granulation occurs in the context of an antecedent cellular stress response (Chapter 5). Originally, Schipper and colleagues (1990a) hypothesized that estrogen may promote the accumulation of redox-active, metalloporphyrin-rich glial inclusions by stimulating δ-aminolevulinic acid synthase, the rate-limiting enzyme in porphyrin-heme biosynthesis, as is known to occur in other estrogen-target tissues (Ellefson, 1982). However, recent studies in Schipper's laboratory (Wang et al., 1995) contest this view insofar as (i) porphyrin-heme biosynthesis is *suppressed* by CSH treatment, and (ii) CSH stimulates the sequestration of free, *nonheme* iron within nascent Gomori-positive granules. Since CSH-stimulated astrocyte granules are morphologically and tinctorially indistinguishable from those which arise in brains of aging and estrogen-treated animals, it is reasonable to assume that enhanced porphyrin-heme biosynthesis does not account for astrocyte granulation in these cases either. The studies presented in this thesis offer a novel mechanism implicating the cellular heat shock response in the biogenesis of estrogen-induced astrocytic inclusions. HSPs 27, 72 and 90 appear to chaperone steroid receptors, and HSP27 is known to contain a steroid-responsive element in its promoter region (Cohen and Meselson, 1985; Hoffman et al., 1987). In

addition, subpopulations of hypothalamic astroglia have been shown to contain estradiol receptors (Langub and Watson, 1992). Thus, induction of these HSPs in hypothalamic ARC astrocytes may simply represent a steroid-specific action in these cells. However, in many cell types, these HSPs are also commonly upregulated in the face of oxidative stress raising the possibility that estrogen promotes a concerted HSP response in ARC astroglia via the generation of prooxidant intermediates. The mammalian hypothalamus contains the enzyme estrogen 2-hydroxylase which catalyzes the conversion of estradiol to 2-hydroxyestradiol (catecholestrogen; Ball and Knuppen, 1978). Peroxidase-catalyzed reactions transform catecholestrogens to highly reactive semiquinone radicals (Kalyanaraman et al., 1985). Spontaneous autoxidation of catechol groups may, additionally, generate oxyradicals including $H_2O_2$ and superoxide anion (*ibid*). Thus, estradiol-derived free radical species may mediate a cellular stress response and subsequent granulation in a subpopulation of hypothalamic astroglia.

Schipper et al (1990a) hypothesized that the redox-active iron which accumulates in Gomori astrocyte inclusions may oxidize catecholestrogens to prooxidant intermediates and thereby promote oxidative stress to the surrounding neuropil. The fact that stress protein expression rapidly occurs in astrocytes without evidence of enhanced granulation in the 48-hour EV experiment suggests that initial HSP induction is not a consequence of the accumulation of iron-rich (redox-active) glial inclusions. Rather, a vicious cycle may ensue whereby oxidation of catechol moieties within astroglia produces oxyradicals which stimulate HSP overexpression and provoke the accumulation of redox-active cytoplasmic inclusions. Collectively, the findings described in this section provide indirect evidence that prolonged or repeated exposure to oxidative stress may be the "final common pathway" responsible for activation of the cellular stress (heat shock) response and subsequent biogenesis of peroxidase-positive astroglial inclusions *in vitro* and in the intact, aging nervous system. This hypothesis is further supported by Srebro's earlier demonstration that X-irradiation, a known generator of free radicals, increases numbers of Gomori-positive glial granules in the rat hypothalamus in a dose-dependent manner (Srebro, 1971).

### 1.11.1 Prooxidant Effects of CSH on Astroglial Mitochondria

Mitochondrial swelling is considered a marker of oxidative stress (Castilho et al., 1995) and occurs with aging (Wilson and Franks, 1975), following heat shock (Welch and Suhan, 1985), and under conditions of increased osmotic pressure due to compromised membrane integrity (Skrede, 1966). Using transmission electron microscopy, astrocyte mitochondria exhibit swelling and dissolution of cristae shortly after CSH treatment (Brawer et al., 1994a). Increases in prooxidant species affecting cellular and subcellular systems can also be detected by evaluating the accumulation of oxidized proteins and lipids (Gutteridge and Halliwell, 1990). The potential for peroxidation of lipids, or the "peroxidizability index" of the inner mitochondrial membrane has been shown to increase with age (Laganiere and Yu, 1993; Yu et al., 1992). Moreover, significant age-dependent increases in the levels of lipid peroxides have been documented in human liver mitochondria (Yen et al., 1994). Indeed, of all subcellular compartments, mitochondria normally represent the greatest source of intracellular prooxidants (Shigenaga et al., 1994). Hemoproteins, abundant constituents of the mitochondrial electron transport system, sustain thiol oxidation with concomitant generation of cytotoxic prooxidant species (Munday, 1989). The experiments described in the Appendix were performed to directly determine whether oxidative stress is an important mechanism mediating CSH-related injury to isolated astroglial mitochondria (Gomori granule precursors). In the presence of exogenous $FeCl_3$, incubation of purified mitochondrial suspensions derived from cultured rat astroglia with CSH (600-1000 μM), resulted in significant mitochondrial lipid peroxidation relative to non-CSH-treated, control preparations. When $FeCl_3$ was removed from the incubation medium, mitochondrial suspensions exposed to CSH continued to exhibit significantly enhanced levels of lipid peroxidation relative to control suspensions. Conceivably, abundant mitochondrial heme ferrous iron sustains the autoxidation of CSH with concurrent generation of reactive oxygen species (Munday, 1989), thereby promoting oxidative damage to the mitochondrial compartment. These results are consistent with earlier data demonstrating that inhibition of CSH autoxidation completely prevents the CSH-induced lipid peroxidation of rat liver mitochondria (Skrede and Christophersen,

1966). Moreover, addition of catalase to our astroglial mitochondrial incubates completely abrogated CSH-related mitochondrial lipid peroxidation, attesting to the role of CSH-derived $H_2O_2$ in the development of mitochondrial injury and Gomori granulation in our system (Appendix). Finally, as described in Chapter 2, $H_2O_2$ exposure promotes the accumulation of (mitochondria-derived) peroxidase-positive inclusions in cultured rat astroglia.

## 1.12 CSH Paradoxically Confers Cytoprotection to Astroglia Concomitant with Mitochondrial Injury

Although purified astroglial mitochondria exhibited enhanced sensitivity to lipid peroxidation in the presence of CSH (Appendix), whole cell lysates derived from CSH-treated astroglial cultures consistently manifested lower levels of lipid peroxidation relative to untreated controls (Manganaro et al., 1995). This latter observation is consistent with findings that CSH *reduces* lipid peroxidation in rat liver microsomes *in vitro* (Haenen et al., 1989). The apparent discrepancy between a selective prooxidant effect to the mitochondrial compartment and a concomitant antioxidant effect to the whole cell may be accounted for by the fact that aminothiols act either as oxidizing or reducing agents depending on the redox status of their microenvironment (Munday, 1989; Weiss and Kumar, 1994). Redox potentials of different subcellular compartments are known to vary within the same cell (Hwang et al., 1992). In support of this, mitochondrial MnSOD, but not cytosolic CuZnSOD, exhibited increased activity following CSH exposure relative to untreated controls (Manganaro et al., 1995), suggesting that CSH-related generation of superoxide anion in the cytosol may be blunted relative to that which occurs within the mitochondria. Thus, in cellular compartments containing relatively low amounts of redox-active transition metals, the antioxidant properties of CSH may prevail over its oxyradical-

generating capacity. Indeed, CSH has been used for many years in experimental oncology to protect against excessive radiation-induced tissue damage (Bacq, 1955). It is thought that CSH confers protection by scavenging free radicals generated by ionizing radiation (Weiss and Kumar, 1994). Numerous thiol-containing compounds, including CSH, chelate catalytic transition metals (Knoblock and Purdy, 1961) and may thereby attenuate the redox-associated generation of prooxidant species (Skrede and Christophersen, 1966). Thiols also reduce tissue injury resulting from free radical assault by directly quenching the latter via hydrogen atom donation (Halliwell et al., 1992).

Short-term exposure of cultured astroglia to CSH confers enhanced resistance to mechanical stress (trypsinization) relative to untreated controls. Prolonged treatment additionally protects the astrocytes from a subsequent exposure to oxidative stress ($H_2O_2$) in comparison with controls (Chapter 2). Since CSH is no longer detectable by HPLC in CSH-pretreated astroglial cultures following a 24h washout period, these results argue against the possibility that the aminothiol serves as a *direct* protectant in the cytotoxicity assays. Other mechanisms must therefore mediate the cytoprotective effects of CSH in our system.

In rat liver, CSH upregulates the activity of HO-1 (Peterson et al., 1989) which catalyzes the breakdown of redox-active heme to the antioxidant bile pigment, biliverdin (Stocker, 1990). Thus, rapid induction of HO-1 by CSH in primary astrocyte cultures (Chapter 2) may confer some degree of cytoprotection to these cells by augmenting the degradation of prooxidant metalloporphyrins and thereby promoting the restoration of cellular redox homeostasis.

Sulfhydryl reactive agents are potent inducers of the cellular stress response (Morimoto et al., 1990). CSH rapidly induces HSPs 27, 72 and 90, Ub, and HO-1 in astrocytes of dissociated brain cell cultures (Chapter 2). Similarly, in the intact rat brain, CSH upregulates the expression of all HSPs examined in astrocytes but not in neurons (Chapter 3). Thus, CSH appears to mediate cytoprotection by yet another mechanism, namely by

inducing a host of HSPs which may function to prevent deleterious aggregation of unfolded or aberrant proteins and protect lipid membranes and the translational apparatus from stress-induced damage (Ananthan et al., 1986; Burdon et al., 1987; Finley et al., 1984; Liu et al., 1992). Moreover, the CSH-induced accumulation of HSPs may accelerate recovery from stress-induced changes given the putative role of certain HSPs (especially those belonging to the HSP70 family) in protein folding, assembly, aggregate dissolution and nucleolar renaturation. Furthermore, rapid activation of Ub in CSH-exposed astroglia may promote glial recovery and survival by assisting in the degradation of unresolvable denatured protein complexes and the reestablishment of normal protein homeostasis.

## 1.13 Does CSH Invoke a Cytoprotective Stress Response By Altering Native Polypeptides?

Cells exposed to heat shock, oxidative stress, amino acid analogues, or sulfhydryl reactive agents have been shown to undergo a cellular stress response (see Chapter 1, Section 7.2). In spite of their apparent diversity, the ability to alter or damage cellular proteins seems to be a common feature shared by these treatments (Hightower, 1980). Moreover, intracellular injection of aberrant proteins triggers HSP expression (Ananthan et al., 1986). These findings have led to the consensus that the cellular stress response system is ultimately "responsive" to the intracellular accumulation of denatured proteins. CSH, a sulfhydryl compound, may cause alteration of protein conformation by a number of mechanisms. (i) The activity of protein disulfide isomerase, which catalyzes formation or cleavage of protein disulfide bonds, is influenced by local thiol/disulfide redox potentials (Freedman, 1984). Sulfhydryl reducing agents promote the reduction of protein disulfide bonds by this enzyme. Accordingly, the thiol group of CSH may disrupt nascent protein synthesis by interfering with disulfide bond formation. The sulfhydryl moiety of CSH could potentially reduce and cleave disulfide bridges present in mature proteins and

enzymes by a similar mechanism, or by thioltransferase-mediated thiol-disulfide exchange, and result in unfolding of the native polypeptide (Mannervik et al., 1983; Terada, 1994). (ii) As mentioned above (Section 1.12), CSH-mediated free radical-scavenging requires donation of its hydrogen atom. However, this results in the generation of the highly reactive thiyl radical which, if not reduced, can complex with oxygen to produce thiyl peroxyl radicals (Asmus, 1990; Sevilla et al., 1989). Oxysulfur radicals such as these have been shown to inactivate enzymes and promote denaturation of various cellular proteins (Aruoma et al., 1989). (iii) Autoxidation of CSH, which readily occurs in the presence of transition metals at neutral pH (Skrede and Christophersen, 1966), produces many cytotoxic reactive oxygen species capable of initiating peroxidative damage of DNA, enzymes, and lipoproteins (Munday, 1989). As per the prevailing model for induction of the stress response (see Chapter 1, Section 7.7.1.2), damaged proteins which accumulate in the cytosolic compartment (as a result of CSH exposure) compete effectively for binding to constitutive HSP70c, which, under normal circumstances, maintains HSFs in their inactive states. Release of HSFs from HSP70c enables the former to oligomerize and become activated, bind to DNA, and initiate transcription of diverse HSPs.

## 1.14 Sensitivity of the Ub System to Oxidative Stress May Promote the Accumulation of Protein Aggregates Within Astroglia

The accumulation of aberrant proteins and protein aggregates within a variety of cytoplasmic inclusions is characteristic of many senescence- and disease-related neurodegenerative changes (Dickson and Yen, 1994). Shang and Taylor (1995) recently demonstrated that $H_2O_2$-related oxidative stress compromises the Ub conjugation activity of cultured mammalian cells with a resultant reduction in proteolytic activity. Moreover, the results indicated that activation of the Ub system only occurs following removal of the stress and cell recovery. The activities of the Ub- activating and conjugating enzymes, E1 and E2, respectively, are dependent on their constituent free thiol groups (Haas et al., 1982; Hershko et al., 1983). The authors suggested that oxidative modification of these

thiol groups may inactivate Ub-dependent proteolysis and contribute to the intracellular accretion of damaged proteins. In a similar fashion, unremitting oxidative stress in the aging and diseased nervous system, and in primary astrocyte cultures repeatedly exposed to CSH, may prevent Ub-dependent degradation of aberrant proteins and promote their accumulation within astrocytic inclusions. Furthermore, Ub-dependent proteolysis, and the release of renatured target proteins from most HSPs requires ATP hydrolysis (Becker and Craig, 1994; Welch, 1992). Although we have not measured ATP concentrations in CSH-treated cells, others have shown that ATP levels in mammalian cells decline rapidly following heat shock (Findly et al., 1983; Stevenson et al., 1981; Weitzel et al., 1985). Thus, ATP depletion as a direct consequence of cellular stress, or resulting from the progressive aging-related decline in mitochondrial function could contribute to the accrual of HSP-bound proteins and engender a deficit of free HSPs available to bind newly damaged polypeptides. CSH-treated astroglia should provide a useful model to investigate further the role of oxidative stress and the Ub system in the accumulation of aberrant proteins and protein aggregates in the aging and degenerating nervous system.

## 2. A CELLULAR STRESS MODEL FOR THE BIOGENESIS OF REDOX-ACTIVE ASTROCYTE INCLUSIONS

Astrocyte proliferation and hypertrophy (reactive gliosis) in response to CNS injury have been viewed alternately as either beneficial or detrimental to the recovery process (see Chapter 1, Sections 2.6.1 and 2.7.1). Specific enhancement of astroglial cytoprotective mechanisms, stimulated by as yet unknown factors in the aging and diseased brain (and simulated by CSH exposure), may allow astrocytes to survive and participate in reactive gliosis in the face of attendant neuronal degeneration resulting from oxidative stress. Moreover, while conferring a cytoprotective advantage to stressed astroglia, the elaboration of various HSPs and other defense mechanisms may ultimately prove deleterious to certain regions of the nervous system as a whole by promoting the persistence and accumulation of mitochondria-derived glial inclusions replete with redox-active transition metals. The studies described in this thesis, in conjunction with recent

data obtained in Schipper's laboratory, suggest a model whereby repeated or sustained exposure of aging astroglia to oxidative stress initiates the development of peroxidase-positive cytoplasmic inclusions which, in turn, may augment the oxidative burden borne by the aging and degenerating nervous system (Diagram 1):

Stage 1: (A) In the course of normal aging, unidentified factors (simulated by CSH and its free radical derivatives) promote oxidative and/or sulfhydryl stress within limbic and periventricular brain regions. In response, subpopulations of indigent astroglia exhibit mitochondrial swelling, distortion of cristae, and increased mitochondrial permeability to chromium (Brawer et al., 1994a, b, 1995). Intramitochondrial reduction of hexavalent chromium to its pentavalent state generates free radicals which further augment mitochondrial membrane injury (Brawer et al., 1995). The affected mitochondria emit orange-red autofluorescence due to oxidation of abundant flavin-containing proteins (Wang et al., 1995). (B) Concomitant sulfhydryl or oxidative stress within the cytosolic compartment alters native protein conformations resulting in disengagement of HSF from HSP72c, translocation of HSF to the nucleus, and concerted induction of stress protein gene transcription.

Stage 2: (A) With unremitting oxidative injury, swollen mitochondrial profiles become acristic, or the cristae rearrange in concentric stacks around the inclusion perimeters forming multilamellar bodies (Brawer et al., 1994a). (B) Increased expression of HSPs 27, 72, and 90 help stabilize and promote renaturation of vital cellular constituents including structural proteins, enzymes and the translational apparatus, and protect the affected astrocytes from a host of subsequent stressors. In addition, HSP90 translocates to the nuclear compartment where it may mediate generalized suppression of gene transcription and protein synthesis characteristic of the "heat shocked" state (Akner et al., 1992). Overexpression of HO-1, an ER-derived stress protein, further reduces intracellular oxidative injury by degrading redox-active hemoproteins into antioxidant bile pigments (Stocker, 1990). Early activation of the Ub system promotes the proteolysis of irreversibly damaged proteins and removal of denatured protein aggregates.

Stage 3: (A) Redox-active transition metals, such as non-heme ferrous iron and copper, are sequestered within the damaged mitochondria (McLaren et al., 1992; Brawer et al., 1994a, b). As a result, the latter appear increasingly osmiophilic (electron-dense) under transmission EM (Brawer and Sonnenschein, 1975; Brawer et al., 1994a; Srebro and Lach, 1987). Stress-induced inhibition of porphyrin-heme biosynthesis in these cells (Wang et al., 1995) may further facilitate the translocation of non-transferrin-bound, non-heme iron from the cytosolic to the mitochondrial compartment (Adams et al., 1989). (B) HSP27, HSP60 (Schipper and Cissé, 1995), and Ub may recognize and bind to unfolded and hydrophobic polypeptide sequences within the damaged mitochondria. Alternatively, stress proteins may be conveyed to the nascent glial inclusions during lysosomal fusion (Stage 4). MnSOD is induced in an attempt to mitigate superoxide-related mitochondrial injury (Manganaro et al., 1995).

Stage 4: (A) Lysosomes fuse with effete mitochondria and other damaged subcellular constituents in a complex macroautophagic process (Brawer et al., 1994a; Schipper, et al., 1993). Multiubiquitinated mitochondrial proteins may, in part, spur lysosomal fusion (Mayer et al., 1991b). The incorporation of ER cisternae within the autophagic vacuoles "delivers" ER-derived stress proteins, such as HO-1 and GRP94, to the nascent Gomori-positive inclusions. HO-1 may thus facilitate hemoprotein degradation within the mitochondrial subcompartment of the inclusion. (B) In the presence of $H_2O_2$, the transition metals (Fe, Cu) sequestered within mature Gomori inclusions behave as a non-enzymatic peroxidase activity capable of oxidizing catecholamines and catecholestrogens to neurotoxic semiquinone radicals (Schipper et al., 1991). Thus, in the aging and degenerating nervous system, a vicious cycle of oxidative injury to glial mitochondria and the surrounding neuropil may be perpetuated long after dissipation of an initiating neurotoxic insult.

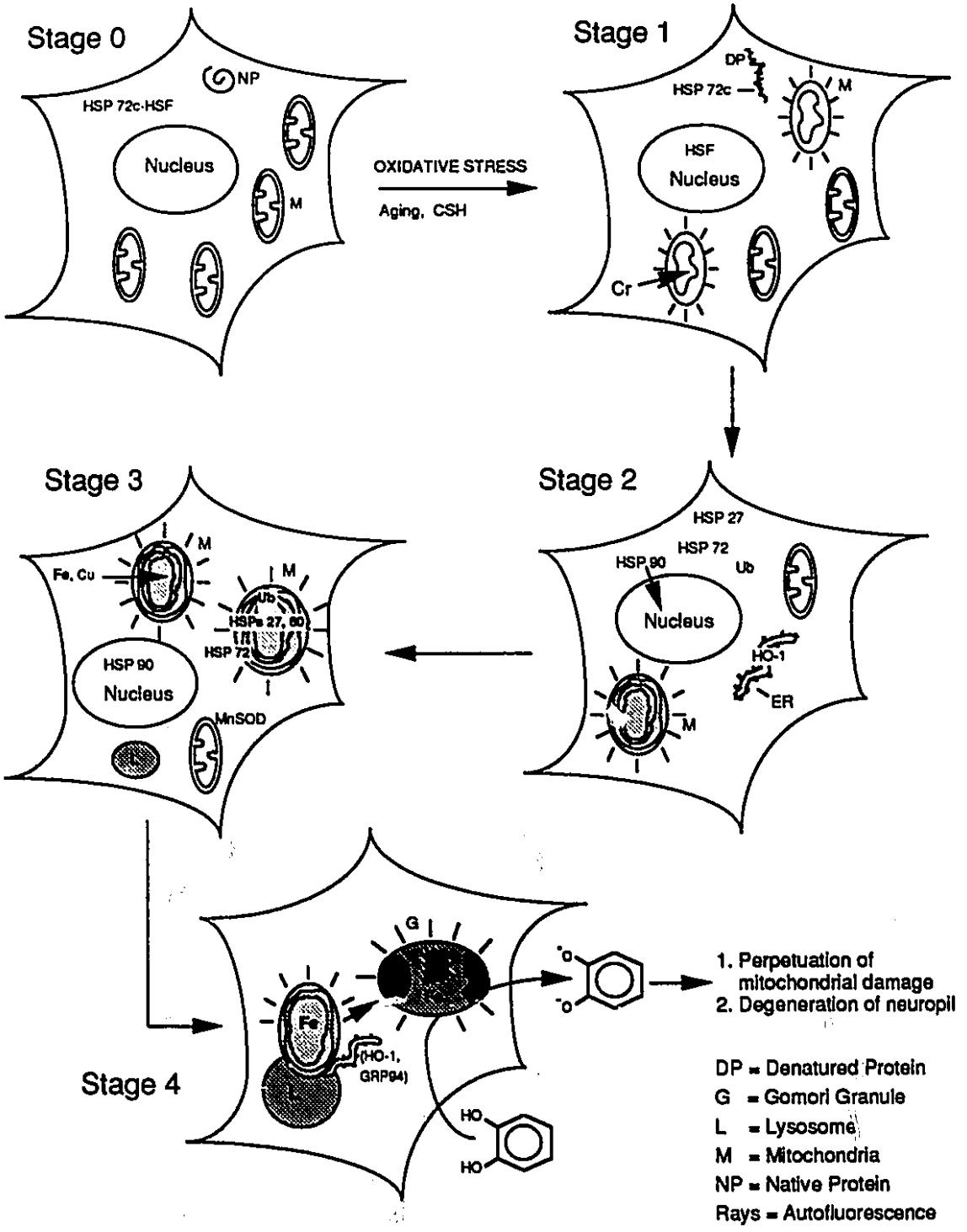



Diagram 1. A model for the biogenesis of Gomori-positive inclusions in senescent astroglia.

## 3. IMPLICATIONS FOR BRAIN AGING

Many of the cytopathological alterations associated with the aging and diseased brain represent end-stage or "graveyard" pathology. In these cases, there is considerable difficulty discerning cause and effect relationships and establishing the temporal sequence of events which lead to the neuropathology. Recently, Dickson and Yen (1994) reflected upon the need to develop animal and tissue culture experimental systems which adequately model the changes progressively occurring in the senescent and diseased nervous system. In an attempt to achieve such a model, we have employed both CSH and estradiol to accelerate the aging-related accumulation of Gomori-positive astrocytic inclusions observed in limbic and periventricular brain regions. Our ability to significantly enrich the population of peroxidase-positive astrocytes in primary brain cell cultures by the addition of CSH has provided an excellent opportunity to determine the subcellular origins of the glial inclusions, the mechanisms governing their formation, and the role(s) these cells may play in the aging and degenerating nervous system.

### 3.1 The Senile Persistent Estrus Rat

As previously described, chronic estrogen administration to young adult rats hastens the development of anovulatory acyclicity. In this model of senile persistent estrus, a lesion develops in the hypothalamic arcuate nucleus of EV-treated rats consisting of the accretion of peroxidase-positive astroglial inclusions, degeneration of neuronal processes, microgliosis, and loss of β-endorphinergic neurons in the context of an antecedent cellular stress response. Conceivably, metal-laden Gomori granules accelerate the production of free radicals (Schipper et al., 1991) which are somehow preferentially neurotoxic to local β-endorphinergic neurons implicated in the regulation of gonadotropin secretion (Desjardins et al., 1993). Disruption of the latter may, in turn, interfere with generation of the pre-ovulatory gonadotropin surge and produce sterility, polycystic ovaries, and other

features of the senile persistent estrus state (Brawer et al., 1993). Evidence supporting a role for free radicals as mediators of estrogen-related neurotoxicity in the aging rodent hypothalamus has recently been obtained: Dietary supplementation with $\alpha$-tocopherol (vitamin E; Desjardins et al., 1992) or the 21-aminosteroid antioxidant, U74389F (Schipper et al., 1994) completely abrogated the estrogen-induced decline of hypothalamic $\beta$-endorphin concentrations in adult female rats. Furthermore, vitamin E prevented the development of multicystic ovaries and anovulatory sterility in EV-treated rats (Desjardins et al., 1992). Work is currently in progress (Brawer and Schipper, unpublished) to determine whether antioxidant treatment also prevents the induction of a cellular stress response and subsequent astrocyte granulation in hyperestrogenized rats.

3.2 The Senescent Human Brain

The progressive accumulation of peroxidase-positive astroglial inclusions represents a highly consistent feature of the aging process in the vertebrate CNS (Schipper, 1991). Since the formation of redox-active astroglial inclusions appears to result from unremitting oxidative stress, it may follow that CNS regions exhibiting an abundance of the inclusions are those at increased risk for chronic oxidative cellular injury during senescence. In addition, during aging, oxidatively-damaged astroglial mitochondria (nascent Gomori inclusions) which sequester redox-active transition metals could potentially propel the development of free radical-related neural damage long after removal of an initial cytotoxic insult. A multitude of aging-related alterations in mitochondrial structure and function have been documented including impairments of oxidative phosphorylation and increases in free radical production and MnSOD activity, decreased fluidity of the inner membrane due to oxidative injury, and enhanced oxidative damage to mitochondrial DNA (reviewed in Shigenaga et al., 1994; Yen et al., 1994). The prevailing Mitochondrial Hypothesis of Aging states that these age-related, free radical-associated changes result in bioenergetic insufficiency of tissues containing affected mitochondria, and a compensatory increased workload for healthy mitochondria, thereby exposing the latter to an elevated oxidative burden. This, in turn, permits the establishment of a vicious

cycle of oxyradical generation, mitochondrial injury and loss of tissue viability (Linnane et al., 1992). Our model for mitochondrial injury and astrocyte senescence appears to conform to many features of this hypothesis. We have demonstrated that MnSOD is increased in CSH-treated astroglial mitochondria, and that the latter undergo swelling and lipid peroxidation in the presence of the aminothiol. In addition, in our model, CSH administration appears to damage only a minority of astroglial mitochondria, while others remain intact. This observation correlates well with the proposal that "tissue bioenergy mosaics" result from heterogeneous damage to mitochondrial subpopulations (Linnane et al., 1992). Thus, sporadic degeneration of mitochondria within aged Gomori astrocytes may similarly account, in part, for the reported mosaicism for selective mitochondrial DNA mutations in the senescent human brain (Soong et al., 1992).

Punctate Ub-immunoreactivity is known to increase in an age-dependent manner in glia of white matter (Dickson et al., 1990) and has also been reported to colocalize to vacuole-containing astrocytes in the normal, senescent, human globus pallidus (Abe et al., 1994). Although the origin of the vacuoles is unknown, those reports may, in the light of the present study, reflect the ubiquitination of Gomori-positive astrocyte granules.

Corpora amylacea constitute a fundamental biomarker of aging in the human brain. Corpora amylacea predominate within periventricular astrocytes, share many tinctorial and histochemical features in common with Gomori-positive astrocyte inclusions, and are immunoreactive for HSPs 27, 72, Ub (Cissé et al., 1993), HO-1 (Schipper et al., 1995) as well as several mitochondrial epitopes (Schipper and Cissé, 1995). Moreover, Cissé and Schipper (1995) demonstrated, for the first time, that corpora amylacea can be induced in cultured rat astroglia by very long-term (90 days) CSH exposure, and that they derive from Gomori astrocyte inclusions (damaged mitochondria) which become progressively glycosylated. On the basis of these findings, Schipper conjectured that excessive oxidative stress in Alzheimer brain (see following section) exacerbates senescence-related injury to astroglial mitochondria which, in turn, give rise to the overabundance of corpora amylacea reported in this condition (Cissé and Schipper, 1995).

## 4. IMPLICATIONS FOR NEURODEGENERATION

### 4.1 Alzheimer's Disease

Over the last decade, evidence from numerous studies has implicated oxidative stress in the pathogenesis of AD, PD and amyotrophic lateral sclerosis. With respect to AD, there are reports of increased levels of nonheme iron in the vicinity of senile plaques and neurofibrillary tangles which are hallmark pathological features of this dementing condition (Perl et al., 1991; Youdim, 1988). AD brain tissue also exhibits greater lipid peroxidation relative to that of age-matched, non-demented controls (Palmer and Burns, 1994; Subbarao et al., 1990). Furthermore, a host of redox-sensitive cellular stress proteins, including HSPs 27, 72, Ub and HO-1 occur in association with neurofibrillary pathology and corpora amylacea in the AD brain (Cissé et al., 1993; Perez et al., 1991; Renkawek et al., 1994a; Schipper et al., 1995; Wang et al., 1991). Finally several laboratories have reported deficiencies in oxidative phosphorylation and other mitochondrial enzyme abnormalities in Alzheimer-affected brain tissues (Kish et al., 1992; Parker et al., 1994; Wallace et al., 1995) which, according to the Mitochondrial Hypothesis of Aging (Ames et al., 1995; Linnane et al., 1992; Miquel and Fleming, 1986; Shigenaga et al., 1994), may be the cause, or the consequence, of excessive oxidative stress. CSH-treated rats and primary astrocyte cultures may, at least partly, recapitulate some of the pathological changes occurring in AD. Thus, in both AD brain and in our CSH models there is (i) robust hippocampal astrogliosis, (ii) the accumulation of iron-rich inclusions (Perl et al., 1991; Youdim, 1988), (iii) oxidative mitochondrial injury, (iv) the upregulation of redox-sensitive stress proteins (HSPs 27, 72, HO-1 and Ub), (v) increased lysosomal activity (Diedrich et al., 1991), (vi) development of corpora amylacea, (vii) somatostatin depletion (Beal and Martin, 1986), and (viii) cognitive deficits (Justino et al., 1995). It remains to be determined whether prolonged CSH exposure will also result in abnormalities of ß-amyloid metabolism, cytoskeletal protein hyperphosphorylation, and neuronal degeneration characteristic of AD.

## 4.2 Parkinson's Disease

In idiopathic PD, selective degeneration of dopaminergic neurons in the pars compacta of the substantia nigra is correlated with an excessively high level of iron deposition in this region. Redox-active ferrous iron may contribute to the pathogenesis in this disorder by reducing $H_2O_2$, derived from MAO-catalyzed oxidative deamination of dopamine, to the potently neurotoxic hydroxyl radical. In addition, ferrous iron may exhibit nonenzymatic peroxidase activity and convert catechol moieties to cytotoxic semiquinone radicals (Goldfischer et al., 1966; Graham, 1978; Schipper et al., 1991). Based on the studies presented in this thesis and recent evidence from Schipper's laboratory (Wang et al., 1995), we have developed a model for iron sequestration within stressed astroglia of the aging and degenerating nervous system. As discussed above (Section 2), stress-induced perturbation of glial porphyrin-heme biosynthesis and direct oxidative damage to mitochondrial membranes promote the incorporation of nonheme iron within glial mitochondria. This mechanism may play an important role in the pathologic accumulation of redox-active iron in affected PD brain regions. In support of this contention, others have reported that the abnormal deposition of iron in the PD brain may be occurring, at least in part, within astroglial mitochondria (Connor et al., 1990; Jellinger et al., 1990; Olanow, 1992). Defective activity of the initial portion of the electron transport chain (Complex I), and a preponderance of mitochondrial DNA deletions in PD substantia nigra are further evidence that dystrophic mitochondria play a key role in the pathogenesis of this disease (Parker et al., 1989; Schapira et al., 1990; Wallace et al., 1995). Delineation of similar impairments in mitochondrial function and genomic damage in CSH-treated astrocytes would further implicate Gomori-positive astrocytes in the pathogenesis of PD.

In MPTP-induced parkinsonism, astroglial MAO-B catalyzes the oxidation of the protoxin, MPTP, to the dopaminergic neurotoxin, MPP+ (Chiba et al., 1984). MAO-B inhibitors (eg. deprenyl) have proven useful in attenuating the *in vitro* conversion of MPTP to MPP+, and reducing the cytotoxicity observed in experimental models *in vivo* (Di Monte

et al., 1991; Langston et al., 1984; Markey et al., 1984). However, inhibition of MAO-B (and MAO-A) does not completely abrogate the generation of MPP+, and increasing the dose of MPTP can overcome the cytoprotection conferred by administration of deprenyl (Fuller and Hemrick-Luecke, 1989). In contrast, addition of iron and copper chelators to astrocyte cultures in conjunction with MAO inhibition further reduced the residual bioactivation of MPTP to MPP+ (Di Monte et al., 1995). Finally, it has been shown that dopaminergic toxicity resulting from a given dose of MPTP is augmented in older mice relative to younger controls (Tsai et al., 1994). Taken together, these diverse observations suggest that Gomori-positive astrocytes, replete with metal-containing cytoplasmic inclusions, may participate in the non-enzymatic bioactivation of MPTP and other xenobiotics to neurotoxic intermediates and thereby render the senescent nervous system prone to PD and other free radical-related neurodegenerations. Interestingly, the age-related sequestration of redox-active transition metals in Gomori astrocytes occurs in concert with an age-dependent increase in brain MAO-B activity (Sparks et al., 1991). Conceivably, this situation promotes oxyradical generation because augmented *astroglial* MAO-B activity (Levitt et al., 1982) oxidizes dopamine, thereby generating $H_2O_2$ which, in turn, may be (i) used as cofactor for the peroxidase-mediated conversion of dopamine to semiquinone radicals, or (ii) reduced by catalytic metals to the cytotoxic hydroxyl radical. If Gomori-positive astrocytes play a significant role in the pathogenesis of PD, antioxidant therapy, in concert with pharmacological inhibition of metal sequestration by "stressed" astroglial mitochondria, may constitute an effective strategy in the management of this, and perhaps other, neurodegenerative afflictions.

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