MECHANISMS OF IRON UPTAKE BY CULTURED BRAIN ASTROCYTES IN RATS

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By

To Yu

SUPERVISOR

Dr. Qian Zhong Ming
Prof. Tang Pak Lai

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Department of Applied Biology and Chemical Technology
The Hong Kong Polytechnic University

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DECLARATION

The work submitted in this thesis is the result of investigations carried out by the author. The material in this thesis has not been accepted in any substance for any degree, and is being concurrently submitted in candidature for any other degree.

Signed _____

To Yu

(Candidate)

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CONTENTS

ABSTRACT

The experiments described in this thesis were performed to investigate the effect of iron on the development of rat brain astrocytes in vitro and the mechanisms of transferrin-bound and transferrin-free iron uptake by cells. This thesis consists of 7 chapters, starting with a general introduction, followed by general methods, then 4 chapters (Chapter 3-6) describing the experimental works involved, and finally concluded with a general discussion.

Chapter 1

Chapter 1 presents a review of the literature. The recent development and current knowledge in some related aspects of brain iron metabolism to this thesis are briefly described. These aspects include: (1) The history of iron in the brain; (2) Brain iron distribution and function; (3) Iron mobilization and storage in the brain; (4) Abnormal brain iron metabolism and central nervous system diseases; and (5) Astrocytes and brain iron metabolism.

Chapter 2

Chapter 2 describes the materials and methods used in this study. Other methods used specially for certain experiments are described within the methods section of the relevant chapter. Apparatuses used in the investigation are described in this chapter as well.

Chapter 3

Chapter 3 describes experiments for examining the effects of ferric nitrilotriacetate (Fe-NTA) at vary concentrations on brain astrocytes in culture. One of the aims of these experiments is to check whether the experimental system to be used for studying membrane transport of iron in astrocytes could induce free radical production and lipid peroxidation. In addition to morphologic studies, 3-(4,5-dimehtylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the cell number. The result demonstrated that high concentration of chelated ferric iron had an adverse effect on astrocytes. Significant reduction of astrocyte population was found at concentrations of 20μM to 5000μM, p values being smaller than 0.05, 0.01, or 0.001 respectively. The effects of Fe-NTA were both concentration and duration dependent. However, no significant difference of morphology was found between 5μM and 10μM groups and the controls. In contrast, when a low iron concentration (5μM) was added to dishes, the cultures could be kept in normal condition for three weeks. Results also showed that the cultured astrocytes were more resistant to high concentration of chelated iron exposure than the cultured cerebellar granule cells. These results demonstrated that the cultured brain astrocytes could be utilized as an experimental model for investigating the mechanisms of iron transport across the membrane of brain glial cells when the concentration used and incubation time are less than 10μM and 3 weeks respectively.

Chapter 4

In this chapter, the binding of iron saturated ^{125}I-transferrin to the cultured rat astrocytes was investigated at pH 7.4 and 4°C. The results demonstrated that cortical astrocytes in suspension bound ^{125}I-transferrin by a saturable and specific manner, indicating the presence of a receptor for transferrin on the membrane of the cells. Scatchard and Hill plot analysis showed that the dissociation constant (Kd) of the binding was about 3.45 x 10^{-8} M and the number of receptor was about 7.09 x 10^4/cell. The receptor was specific for rat and human transferrin. The binding of ^{125}I-rat transferrin could be competitively and specifically inhibited by unlabeled iron saturated rat and human transferrin and no difference was found between the interaction of rat and human transferrin with this receptor. Whereas the interaction of duck or camel transferrin with this receptor was very weak. The Hill coefficient was 0.9877, almost no different from 1, indicating the absence of cooperativity. It suggested that ^{125}I-transferrin bound to a single class site on the membrane of astrocytes.

Chapter 5

The aim of the experiments described in this chapter was to investigate some aspects of mechanisms of transferrin-bound iron uptake by the cultured brain astrocytes in rats. After 15 days of culture, the astrocytes were exposed to 1μM of double-labeled transferrin at 37°C or 4°C for varying time. The cellular transferrin-bound iron and transferrin uptake was analyzed by measuring the intracellular radioactivity with γ-counter. The results showed that transferrin iron uptake kept increasing in a linear manner up to at least 30 min. In contrast to transferrin iron uptake, the internalization

of transferrin into the cells was most rapid only during the first 10 min and then slowed to a plateau level, indicating that transferrin internalization into the cells is a saturable process. Both transferrin and transferrin iron uptake were temperature-dependent. The uptake at 37°C was significantly higher than that at 4°C. The addition of either methylamine or ammonium chloride, blockers of transferrin iron uptake via inhibiting iron release from transferrin within endosomes, significantly decreased the cellular transferrin-bound iron uptake but had no significant effect on transferrin internalization. The pretreatment of cells with trypsin significantly inhibited the cellular transferrin-bound iron and transferrin uptake. These results suggested that transferrin-bound iron transport across the membrane of astrocytes was a transferrin receptor-mediated process and the pattern generally paralleled characteristics of transferrin-bound iron uptake in other mammalian cells outside of the brain.

Chapter 6

In this chapter, the mechanism of transferrin-free iron uptake by the cultured brain astrocytes was investigated. Effects of incubation time, iron concentration, pH, temperature and some other divalent metals on the cellular transferrin-free uptake were determined. After 15 days of plating, the cells were incubated with transferrin-free iron in isotonic sucrose solution at different temperatures for certain time. The cellular transferrin-free iron uptake was analysed by measuring the cellular radioactivity with γ-counter. The result showed that the cultured astrocytes had the capacity to acquire transferrin-free iron. The iron uptake by cells increased with incubation time in a linear manner at a rate 5.776 fmol/10^3 cell per min within the 20

min of incubation period. The uptake was time and temperature dependent, iron concentration saturable, and inhibited by several divalent metal ions, including Co^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+} and Ca^{2+}. The uptake was also pH sensitive, the pH optimum being 6.5. These characteristics of transferrin-free iron uptake by the cultured astrocytes, similar to those obtained from cells outside of the brain and the cultured cerebellar granule cells, implied that a carrier-mediated iron transport system might be present on the membrane of this type of brain glial cells.

Chapter 7

This chapter presents a general discussion of the methods and results of the experiments described in this thesis. Some relevant aspects for future research are suggested.

LIST OF PUBLICATIONS

The following publications have resulted from literature survey and experimental research described in this thesis:

1 Qian Z M, **To Y** and Tang P L

Transferrin receptor on the membrane of the cultured astrocytes in rats

Neuroscience Letter (Submitted)

2 **To Y** and Qian Z M

Iron, transferrin and transferrin receptor in the brain

National Journal of Chinese Medicine (Submitted)

3 Qian Z M, Pu Y M, Wang Q, Xiao D S, **To Y**, Chen W F, and Tang P L

Cerebellar granule cells acquire transferrin-free iron by a carrier-mediated process

Neurosciences (Accepted)

4 **To Y** and Qian Z M

Effect of iron on the development of rat brain astrocytes in culture

(In Preparation)

5 Qian Z M, **To Y** and Tang P L

Transferrin-bound and transferrin-free iron uptake by the cultured brain astrocytes in rats

(In Preparation)

6 **To Y**, Tang P L and Qian Z M (1998)

Expression of transferrin receptor on the cultured astrocyte membrane in rats

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(Proceedings of the IX Biennial Meeting of International Society of Free Radical Research, Sao Paulo, Brazil)

7 Qian Z M, Wang Q and **To Y** (1997)

Why brain iron increases abnormally in neurodegenerative diseases

CPS News Comm. 16: s28-31

8 Xu M F, Tang P L, **To Y** and Qian Z M

Iron absorption (Chapter 3), In Iron Metabolism: Fundamental and Clinical Aspects, ed. Qian ZM, Sciences Press (Accepted)

9 Qian Z M, Pu Y M, Wang Q, **To Y** and Tsoi Y K

Brain iron metabolism and CNS diseases (Chapter 17), In Iron Metabolism: Fundamental and Clinical Aspects, ed. Qian Z M, Science Press (Accepted)

LIST OF FIGURES AND TABLES

ABBREVIATIONS

AD:	Alzheimer's disease
ALAS:	aminolevulinate synthetase
BBB:	blood brain barrier
BSA:	bovine serum albumin
CHO:	Chinese hamster ovary
CNS:	central nervous system
CP:	ceruloplasmin
CSF:	cerebrospinal fluid
cTf:	camel transferrin
DA:	dopamine
DCT1:	divalent cation transporter
DIV:	days in vitro
DMEM:	Dulbecco's modified Eagles medium
dTf:	duck transferrin
EBS:	Earle's balance salt solution
EDTA:	ethylenediaminotetracetic acid
FCS:	heat inactivated fetal calf serum
G-5:	supplemented G-5
GABA:	γ-aminobutyric acid
GFAP:	anti-glial fibrillary acidic protein
GPI:	glycosylphosphatidylinositol
h:	hour
HBSS:	Hanks' balanced salt solution
5-HIAA:	5-hydroxyindoleacetic acid
HSS:	Hallervorden-Spatz syndrome
5-HT:	5-hydroxytryptamine
hTf:	human transferrin

ID:	iron deficent
IRE:	iron-responsive element
IRE-BP:	iron responsice element-binding protein
Lf:	lactoferrin
LfR:	lactoferrin receptor
MAO:	monoamine oxidase
min:	minute
MS:	multiple sclerosis
MTT:	3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl tetrazolium bromide
NE:	norepinephrine
NFT:	neurofibrillary tangles
NTA:	nitrilotriacetic acid
OPD:	*O*-Phenylenediamine dihydrochloride
P97 or MTf:	melanotransferrin
PBS:	phosphate buffered saline
PD:	Parkinson's disease
Pipes:	piperazine-N'-N'-bis (2-ethanesulfonic acid)
PND:	postnatal day
RT:	room temperature
rTf:	rat transferrin
SD:	sprague dawley
SDS-PAGE:	sodioum dodecyl sulfate-polyacrylamide gel electrophoresie
sec:	second
SN:	substantia nigra
SNc:	substantia nigra pars compacta
TCA:	trichloroacetic acid
Tf:	transferrin
TfR:	transferrin receptor
TRIS:	hydrocymethyl-aminoethane

Chapter 1

INTRODUCTION

1.1 INTRODUCTORY STATEMENT

The aim of this chapter is to provide a general introduction to this thesis. The recent development and current knowledge in some related aspects of brain iron metabolism to this thesis are briefly described. These aspects include: (1) The history of iron in the brain; (2) Brain iron distribution and function; (3) Iron mobilization and storage in the brain; (4) Abnormal brain iron metabolism and central nervous system (CNS) diseases; and (5) Astrocytes and brain iron metabolism.

1.2 THE HISTORY OF IRON IN THE BRAIN

In the late nineteenth century, Zaleski began to study the brain iron (1886). He made a quantitative analysis of one human brain and correlated iron levels with observations on stained slices and some microscopic sections. In this systematic analysis of iron in the brain from one patient with fatal 'morbus werlhofii' (idiopathic thrombocytopenic purpura), Zaleski observed that brain contains iron which can be recalculated as 6.5mg/100g wet weight or 36.7 mg/100 mg dry weight. He first reported that the immersion of tissue pieces into solutions of ammonium sulfide or

potassium ferrocyanide gave a much stronger reactions in gray than in white matter. He also noticed that the chemicals he used did not react with iron in hemoglobin and that the reaction differed from those with inorganic iron salts. He hence concluded that tissue iron existed in linkage to organic substances, and proposed in the state of knowledge during the late 19th century that iron was present as 'iron oxide-albuminate'. This little-cited publication in Archaic German clearly anticipated what required many decades of iron research for the final confirmation: iron in tissue, including the brain, is unrelated to hemoglobin ('non-haemin or haematin iron' - Tingey 1938, Hallgren and Sourander 1958); exists in loose linkage to protein (ferritin - Diezel 1955, transferrin - Bloch et al. 1985); is present largely in ferric rather than ferrous form and in higher concentration in gray than in white matter (Tingey 1937, Cumings 1948). Zaleski (1986) also observed that ferrocyanide staining of this section did not reveal many cellular details. This lack of staining is now known to be caused by the extraction of soluble iron-carrying proteins from sections and insufficient sensitivity of the histochemical stains.

Later in 1915, Guizzetti found that the immersion of slices of adult brain in a 2% solution of potassium ferrocyanide, followed by 1% hydrochloric acid promptly causes blue staining of the globus pallidus, substantia nigra, dentate nucleus, red nucleus, and subthalamic nucleus. By using this method, he examined the brains of rabbits, cats, dogs, pigs, sheep, goats, donkeys, horses, cows, guinea pigs, and rhesus monkeys of various ages, and of humans from the neonatal period to the age of 35 years. He first recognized a characteristic pattern of iron distribution in the brains of

various mammals and humans. In 1922, Spatz published a 128-pages important paper on brain iron. In this landmark paper, he discussed the methods of iron histochemistry on gross and microscopic preparations of brain, the development of iron reactivity with growth in animals and humans, and of iron in pathological conditions such as pernicious anemia and general paresis of the insane. He suggested that pathological storage of iron in globus pallidus and substantia nigra had caused a rigid akinetic syndrome with contractures in a 44 years old man. In 1923, Wuth made the first quantitative determinations of iron in selected regions of human brain and found that there was the highest iron concentration was found in globus pallidus, substantia nigra, and red nucleus in the brain.

These early researchers in the area of brain iron metabolism worked with very limited laboratory techniques but reasoned skillfully. They established a solid base on which we can build and made many important findings. However, their important findings received little attention at that time. For several decades, the study on brain iron metabolism remained relatively dormant. In recent two decades, however, there has gradually been an acceleration of investigation in this area. Today, the behavior of iron in the brain has been a hot research topic. This is largely due to a major finding in brains of patients with Parkinson's disease, namely, an increase in the iron content of substantia nigra, and to a related theory concerning the role of iron in the generation of free radicals and hence cell damage. A number of relevant studies on brain iron have been conducted during the past twenty years by

using newly developed methods of histochemistry, biochemistry, immunocytochemistry and molecular biology. The findings obtained from these studies have greatly improved our knowledge on brain iron metabolism.

1.3 BRAIN IRON DISTRIBUTION AND FUNCTION

1.3.1 DISTRIBUTION OF BRAIN IRON

1.3.1.1 The Regional Distribution of Iron

Iron is relatively abundant in brain tissue. Chemical and histochemical analysis demonstrates that the basal ganglia are iron enriched, and have the iron concentration equivalent to that of the liver. (Connor and Benkovic, 1992a; Dwork et al. 1988, Morris et al. 1992). Spatz systematically studied the cerebral iron distribution in human brain by histochemical analysis. He found that the immersion of thick macroscopic sections of human brain tissue into potassium ferricyanide (Turnbull blue) revealed a characteristic pattern of iron distribution. He therefore subdivided CNS into four groups according to the amount of iron present. Group one, with the most intense iron reaction, included the globus pallidus and substantia nigra. The red nucleus, caudate, putamen, subthalamus, and dentate nucleus formed the second group. The third group included the cerebral cortex, anterior thalamus, mammillary

body, midbrain tectum, cerebellar cortex, and central gray matter of the third ventricle and gave a weak iron reaction. The fourth group, in which no staining was observed, was comprised of the medulla oblongata, gray matter of the spinal cord, spinal and sympathetic ganglia and the white matter of the CNS.

Iron distribution changes in brain during development. Hallgren and Sourander (1958) measured the iron in 16 different brain regions and their changes with age. They found that in healthy adult brains, the total iron concentrations in globus pallidus, substantia nigra, and red nucleus are higher than in normal human liver (about 20mg iron / 100g fresh weight for these gray matter regions vs. 13mg iron / 100 mg fresh weight for liver). If total iron concentration of the brain is considered, it is the lowest at birth and increases throughout life. Roskams (1995) analyzed iron in the brain of the same animals throughout the development. In his study the brain was dissected into cerebral cortex, cerebellum-pons, and midbrain. Iron levels increase in each brain region into adulthood, but are lower in the aged group compared to the adult group. In the cerebral cortex, the levels of iron are three times higher at postnatal 2 day than at any other time point. In the adult group, the highest levels of iron are in the cerebellum-pons, whereas during the development, concentrations in the cortex and midbrain are higher than in the cerebellum. The overlapping distribution pattern of iron and γ-aminobutyric acid, enkephalin, and luteinizing hormone - releasing hormone suggest that the distribution of iron is related to its association with the metabolism of one or more neurotransmitters or neuroactive compounds

1.3.1.2 Normal Cellular Distribution of Iron

At the cellular level, iron was found in many cell types of the CNS, including neurons, oligodendroglia, microglia and astrocytes. The majority of the cells which contain iron are oligodendrocytes (Francors et al. 1981, Hill and Switzer 1984, Dwork et al. 1988, Gerber and Connor 1989). Histochemical analysis of the cellular distribution of iron shows the similar results on human, monkey, rat and mice brain tissues. (Dwork et al. 1988, Connor et al. 1990, Levine and Macklin 1990, Morris et al. 1992). Even in iron rich areas, such as the caudate-putamen, substania and deep cerebellar nuclei it is the oligodendrocytes that stain robustly for iron (Benkovic and Connor 1993). The report that 80% of the iron in the brain is found in myelin fractions has lead to the proposal that iron may play an important role in myelination and/or oligodendrocytes are involved in iron regulation in the brain (Michael et al. 1972). Neuronal iron staining is characteristically different from that seen in oligodendrocytes. Neurons, particularly pyramidal neurons in the cerebral cortex and hippocampus have small punctatum of iron reaction product in their somata which increases in density in rats with age (Benkovic and Connor 1992).

One additional cell type staining prominently for iron is tanycyte that lines the third ventricle. The epithelial cells of the choroid plexus within the ventricles also stain intensely for iron. These cells may be involved in iron transport between the brain and the cerebrospinal fluid. The possibility of iron transport into or out of the brain via tanycytes and chorois plexus needs to be investigated because the knowledge of this aspect is severely lacking.

1.3.1.3 Altered Cellular Distribution of Iron

Iron is found throughout the brain but the iron uptake into the brain is maximal during rapid brain growth. The iron transport protein, Transferrin (Tf), and the iron storage protein, ferritin have been examined immunohistochemically along with iron in a number of brain regions from normal and aged humans. There is an age-related alteration in cell labeling: astrocytes in both gray and white matter contain Tf in the oldest age group, whereas in the younger group the subcortical Tf immunoreactivety is confined mostly to oligodendrocytes (Connor et al. 1990).

Ferritin in subcortical brain regions is also present in astrocytes but is primarily confined to those in the gray matter, even in the oldest age group (Connor et al. 1990). Under normal conditions, as mentioned earlier iron is found predominantly in oligodendrocytes. In myelin deficient rat, iron staining is confined to astrocytes and microglia (Connor and Menzies 1990). Disruptions in the histological distribution of iron have been reported in some disease states. In Alzheimer Disease (AD) and multiple sclerosis iron staining is associated with plaques (Connor et al. 1992b, Craelius 1982). Neuromelanin-containing neurons of the substantia nigra accumulate iron in Parkinson Disease (PD) (Good et al. 1992).

Altered cellular distribution of iron, Tf and ferritin has also been reported. Iron and ferritin immunoreacitivity has been observed in cells, which are associated with neuritic plaques in AD (Connor 1992c). Astrocytes containing Tf have been found

in AD, multiple sclerosis and central pontine myelinolysis (Esiri et al. 1976, Gocht and Lohler 1990).

1.3.1.4 Comparison of Iron and Transferrin Distribution in the Brain

The distribution of iron and Tf has some similarities and differences. Using the avidin–biotin immunoperoxidase technique and a diaminobenzidin intensification of the Prussian Blue method, basal ganglia, substantia nigra, and dentate nucleus stain relatively heavily for both, pallidum contains more of both than does putamen, and cerebral white matter contains more of both than does cortex. However, in basal ganglia, the radially oriented fiber tracts contain considerably more iron and less transferrin than does the neuropil. Similarly, in the substantia nigra, the pars reticulata, which contains the striatonigral terminals, is stained more heavily for iron and less heavily for Tf than the remainder of the neuropil (Hill 1985). The red nucleus stained more heavily for iron than the surrounding structures for iron, but less heavily for Tf. Other discrepancies included Tf immunoreactivity but little or no iron staining in cranial nerve nuclei, inferior olivary nuclei, choroid plexus, leptomeringes, ependyma, peripheral nerve, and numerous identifiable neurons and astrocytes. On the other hand, the iron-positive cells may be mostly involved in iron regulation whereas the Tf -positive cells may be myelinating oligodendrocytes. It is possible that iron-positive cells secrete Tf at a high level so that intracellular amount at any given time is below the limits of detectability immunohistochemically (Espinosa de los Monteros 1990).

The distribution of Tf positive oligodendrocytes in gray matter is similar to the iron containing cells, these cells are in perineuronal and perivascular positions (Connor and Fine 1986, Connor et al. 1990, Connor and Benkovic 1992a). In the white matter, the Tf positive and iron containing cells do not share a similar distribution but do share a common morphology. The iron containing cells occur in patches of cells while the Tf positive cells are more homogeneously distributed throughout white matter. Both Tf and iron containing cells are aligned in rows as classically described interfascicular oligodendrocytes.

1.3.2 FUNCTIONS OF IRON IN THE BRAIN

A number of physiological functions, some of which are listed in Table 1, have been attributed to brain iron. Much of this information is derived from animal studies, particularly in rats made iron deficient nutritionally. Because iron is a component of many enzymes, its role in oxidative and amino acid metabolism has been extensively investigated. Iron deficiency (ID) leads to reduced activity of succinate dehydrogenase and aconitase (Pollitt and Leibel 1976, Dallman et al. 1978), two important enzymes of the tricarboxylic acid cycle. Iron is associated with some enzymes involved in amine neurotransmitter metabolism. Monoamine oxidase (MAO) was formerly thought to be iron dependent (Youdim and Sourkes 1966), but recent work do not support this finding (Mackler and Finch 1982), and the association of iron with MAO remains unclear (Oreland et al. 1971). Non-heme iron is catalytically necessary for the function of aldehydeoxidase (Bray 1975), an

enzyme necessary for the oxidation of 5-hydroxyindoleacetaldehyde to 5-hydroxyindoleacetic acid (5-HIAA). ID, therefore, leads to an increase in the concentration of 5-hydroxytryptamine (5-HT) and other 5-hydroxindole compounds in animal study (Mackle and Finch 1982). The turnover of 5-HT in the rat brain is, however, unaffected by ID (Youdim et al. 1989). The concentrations of some neurotransmitters such as dopamine (DA) and norepinephrine (NE), and the precursor tryptophan and tyrosin, are not affected by ID (Youdim et al. 1982). The inhibitor, neurotransmitter GABA and the excitatory, glutamate are both decreased in ID rats, and there is a reduction of two GABA shunt enzymes and NAD^+-linked isocitrate dihydrogenase (NAD^+ = oxidized nicothinamide adenine dinucleotide) (Taneja et al. 1986). These effects of ID on the neurotransmitter lead to some behavioral and neuroendocrine consequences, as discussed below.

In addition to its role in oxidative and neurotransmitte metabolism, iron is important for dopamine D_2 receptor function. Iron deprivation in rats leads to reduction in the Bmax (maximum number of binding sites) for D_2 receptors measured in homogenates of caudate nucleus membrane (Ashkenazi et al. 1982, Youdim et al. 1983). There is concomitant reduction in the behavioral response to apomorphine (Ashkenazi et al. 1982). ID also reduces the hypothermic action of amphetamine, an effect similar to that of haloperidol and pimozide (Youdim et al. 1982). ID, therefore, leads to the subsensitivity of the D_2 receptor, which is reversible with the restoration of brain iron, except perhaps in the young (immature) brain. The exact mechanism of this interaction is not known. Iron could be part of the receptor site, for it is well recognized that DA agonists (apo-morphine) and

antagonists (phenothiazines) are iron chelators, and iron chelators (desferrioxamine, o-phenanthroline, and 2',2' dipyridyl) inhibit the binding of DA antagonist ^{3}H-spiperone to the D_2 receptor (Ben-Shachar et al. 1985). Alternatively, iron may be important for the structure of the membrane and therefore the conformation of the membrane-bound DA receptor (Youdim et al.1989). It is also possible that iron is necessary for the formation of the D_2 receptor protein (Youdim et al. 1983). Injection of $FeCl_3$ directly into the brain (Czernansky et al. 1983) or the ventricles (Ben-Shachar and Youdim 1987) leads to the development of D_2 receptor supersensitivity. The effect of ID is reportedly specific to the D_2 receptor, and other neurotransmitter sites such as the alpha- and beta-adrenergic, muscarinic (Youdim et al. 1983), serotonergic, benzodiazepine (Ben-Shachar et al. 1985), and dopamine D_1 receptors (Youdim and Gree 1977) are not affected.

The catalytic role of iron and ascorbic acid in oxidation, hydroxylation, and peroxidation reactions are well established (Gutteridge et al. 1979). When Fe^{2+} ions are added to liposomes or rat liver microsomes, lipid peroxidation begins after a short lag time (Aruomae et al.1989). Iron-facilitated lipid peroxidation leads to the formation of cytotoxic aldehydes, hydrocarbon gases, and other products. Lipid peroxidation is a major mechanism of cell injury in organisms subjected to oxidative stress (Zaleska and Floyd 1986, Evans et al.1991). The injection of Fe^{2+} or Fe^{3+} salts into the amygdala-hippocampus complex leads to the induction of epileptic foci, probably through lipid peroxidation (Czernansky et al. 1983). The basal ganglia and the hippocampus are particularly susceptible to iron-mediated lipid peroxidation

(Lozoff et al. 1982, Spatz 1922). Iron also promotes the generation of hydroxyl radicals form hydrogen peroxide via Fenton the reaction:

$$H_2O_2 + Fe^{2+} \leftrightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

The implications of this reaction for the role of free iron in the pathogensis of some neuropsychiatric disorders will be discussed later.

TABLE 1. Biological functions of brain iron

1. Catalytic role in enzymatic processes

A. Tricaboxylic cycle enzymes

Succinate dehydrogenase [a]

Aconitase [a]

B. Oxiative phosphorylation enzymes, e.g., cytochrome oxidase C [a]

C. Amino acid and neurotransmitter metabolism enzymes

Phenylalanine hydroxylase [a]

Monoamine oxidase (MAO) [a]

Aldehyde oxidase

Aminobutyric acid transaminase

Glutamate dehydrogenase

2. Effect on D2 receptor function

3. Effect of other neurotransmitters

γ-Aminobuturic acid

Serotonin

Opiate-peptides

4. Role in peroxidation, oxidation, and hydroxylation reactions

5. Other possible functions (not established)

	Role in protein synthesis
	Role in maintenance of blood-brain barrier

[a] Brian enzyme levels remain unchanged in iron deficiency.

1.4 IRON MOBILIZATION AND STORAGE IN THE BRAIN

1.4.1 PROTEINS INVOLVED IN BRAIN IRON METABOLISM

1.4.1.1 Ferritin

Ferritin is generally considered as an intacellular iron storage protein. At least one-third of iron in the brain is stored as ferritin (Hallgren and Sourander 1958, Octave et al. 1983, Connor et al. 1992d). Connor et al. (1992) have recently determined that ferritin is ten times more abundant than Tf in the human brain, although in the rat brain ferritin levels are only one-quarter of the Tf levels. Ferritin is a 450 kDa protein with an ability to sequester up to 4,500 atoms of iron. Ferritin is formed by the assembly of 21 subunits of two isoforms; L chain (19kDa) and H chain (21kDa). These isoforms are functionally distinct and exist in different ratios resulting in isoferritins that are H or L chain rich. In adult monkey brains, L-chain-rich ferritin is predominantly present in microglia, while the mixture of the isoferritins is present in oligodendrocytes (Kalaria et al. 1992).

Immunohistochemical studies differentiating the isoferritins have not been performed in rodents, but ferritin has been demonstrated in microglia and oligodendrocytes in rat and humans. Whereas in mice the astrocytes are the predominant ferritin-positive cells with little found in the other neuroglia cell types (Connor et al 1990, Benkovic and Connor 1993). Ferritin can be conceptualized to have two roles: (1) it can function in the sequestration of excess iron, lead to excess iron loss as the function in the regulation of iron kinetics, and (2) it can function in the storage of intracellular iron (Last and Salleem 1995).

1.4.1.2 Transferrin and Transferrin Receptor

Tf is an 80 kD glycoprotein that is capable of binding 2 iron atoms. This protein functions primarily as an iron transporter, but is capable of transporting other metals such as manganese and aluminum (Aschner 1990, Roskams and Connor 1990). Tf is relatively abundant in the cerebrospinal fluid (CSF). It is made in the brain by the choroid plexus and oligodendrocytes, which is supported by evdience that both have the ability to secrete Tf (Kluge et al. 1986, Walsh et al. 1984, Bloch et al. 1985, Aldred et al. 1987, Dwork et al. 1990).

The brain obtains iron via a Tf - TfR interaction at the level of the blood-brain barrier, and both neurons and glia acquire iron via Tf (Swainman and Machen 1984,1985, Pardridge et al. 1987, Fishman et al. 1987, Crowe and Morgan 1994).

TfR is a key component of the iron regulatory system. It has received relatively little attention in the adult nervous system and even less information exists on the expression and distribution of the TfR during development. The density of TfRs on the brain microvasculature is 6-10 fold higher in adult human brains as compared to the brain parenchyma. Autoradiographic studies reveal a heterogeneous distribution of TfRs in the adult brain; areas associated with motor function express a relatively higher density than non-motor areas (Hill et al. 1985, Mash et al. 1990, Kalaria et al. 1992).

In a preliminary analysis of TfR density during development, TfR density was low at birth (15.2 fmol/mg protein) and increased 4 fold (67 fmol/mg protein) by postnatal day (PND) 18 where the level of receptor stabilized into adulthood (3 months of age). The low level of TfRs at birth in the presence of high concentration of Tf and iron suggests a negative feedback relationship. The cellular distribution of the TfR and factors which regulate TfR expression require considerable additional investigation because of changes which occur with age and following injury and the possibility that TfR density may be an indicator of neuronl respiratory activity (Mash et al. 1990).

1.4.1.3 Control of Expression of Ferritin and Transferrin Receptor

Expression of ferritin and transferrin receptor is controlled in many cells by the intracellular concentration of free ionic iron. Very tight control is achieved by

modulating the rate that specific mRNA is translated or degraded, allowing for rapid adjustments to changing intercellular iron concentration. In this way, low iron concentration triggers a decreased translation of iron storage protein, ferritin and of the key regulatory enzyme in heme synthesis, aminolevulinate synthetase (ALAS). On the other hand, iron depletion triggers an increased translation of TfR, making extracellular iron more available to the cell. But the importance of transitional control of iron in each type of brain cell has not been elucidated.

1.4.1.4 Other Iron Transport Proteins

Recently, at least three four proteins (melanotransferrin [P97 or MTf], lactoferrin [Lf]/lactrferrin receptor [LfR], ceruloplasmin [CP] and divalent cation transporter [DCT1]) have been reported to have an association with the binding and transportation of iron in the brain, but their exact natures are unknown. Their possible roles in brain iron transport are described in 1.4.2. Because the brain resides behind a barrier and has a heterogeneous population of cells, its iron management may be unique. Moreover, the timely delivery of appropriate amounts of iron is critical to normal brain development and function. Mismanagement of cellular iron can result in decreased metabolic activity and increased vulnerability to oxidative damage. Therefore, in addition to Tf/TfR and ferritin, the physiological importance of these proteins in brain iron homeostasis need further study.

1.4.2 MECHANISMS OF IRON UPTAKE BY BRAIN CELLS

1.4.2.1 Transferrin - Bound Iron Uptake

The predominant iron uptake mechanism involves the acquisition of Tf-bound iron in mammalian cells. In 1963, Jandl and Katz first reported the role of the membrane TfR in the uptake of Tf-bound iron by reticulocytes (Dautry-Varsat 1986, Qian and Tang 1995). Today, the membrane TfR-mediated endocytosis has been considered to be the main route for iron transport from plasma Tf into the cells under normal physiological conditions. In general, the Tf-dependent iron transport process can be divided into seven main steps:

1) Binding: Fe-Tf binds to the extracellular portion of TfR on the cellular membrane. It is a simple physiological process independent of cellular metabolism;
2) Internalization or endocytosis: Fe-Tf-TfR complexes are clustered together and localized in clathrin-coated pits, which eventually bud off to form coated vesicles, called endosomes or receptorsomes;
3) Acidification: The intravesicular pH of the endosomes is lowered to about 5-6 by the activity of H^+-ATPase on the membrane of the endosomes;
4) Dissociation and reduction: The intravesicular acidification induces the releasing of iron (Fe^{3+}) from Tf and reduction of $Fe^{3+}$to Fe^{2+} with the endosomes;

5) Translaction: After releasing from Tf, iron (Fe^{2+}) is transported through the membrane of the endosomes to the cytoplasm. The mechanism of this process remains unclear, probably an iron carrier-mediated process;
6) Cytosolic transfer of iron into intracellular compounds: After translocation from endosome to cytoplasm, iron is shuttled by ATP or AMP, which transfers iron to the sites that need iron for physiological activity, e.g. haem synthesis, electron transport in mitochondria etc., or to ferritin for storage;
7) Return of Tf-TfR to the plasma membrane: After the releasing of iron, Tf-TfR containing endosomes return to the plasma membrane. The apotransferrin is replaced by new Fe-Tf molecules from extracellular fluid and the uptake process is repeated. Apotransferrin released form the receptors returns to the plasma or surrounding solution (Qian and Tang 1995).

The feature of this system is that iron can regulate uptake through the iron-responsive element-binding protein (IRE-BP) to the TfR mRNA (Kuhn and Hentze 1992). This translative regulationcan is controlled by modulating the rate that specific mRNA is translated or degraded, allowing for rapid adjustments to changing intracellular iron concentrations. The mRNA form, at least three iron-related proteins (ferritin, TfR, and delta aminolevulinate synthetase (ALAS)), contain a short untranslated nucleotide sequence known as the iron responsive element (IRE). The IRE forms a stable stem-loop binding site for an iron-sensing protein named the IRE-BP. When cellular iron storages are critically depleted, the IRE-BP binds with high affinity to the IRE on mRNA and represses translation in the 5' upstream position. Using this mechanism, iron depletion triggers the increased translation of TfR,

making extracellular iron more available to the cell. In this elegant homeostatic arrangement, cellular iron concentration controls the synthesis of key iron-regulated proteins with great speed. It is known that the IRE-BP is synthesized in brain tissue (Patino and Walden 1992, Gelman 1995).

Few findings are reported about the Tf-iron uptake by brain cell and the effect of various known blocker on brain cells iron transfer. Swaiman and Machen (1985) demonstrated that iron uptake exists in neuronal and glial cell cultures, as well as iron uptake can be inhibited by the lysosomotropic agents ammonium chloride and methylamine. The data from their experiments demonstrates the possibility that iron transport to brain cells is Tf-mediated just like in other tissues. But according to previous description, different glial cell types in the brain have different roles in iron metabolism (Beard et al. 1993). In addition, the cortical glial cultures have many cell types which maybe show the difference in iron uptake. Descamps et al (1996) and Fishman's (1987) in vivo and in vitro studies suggest that the TfR-mediated iron uptake across the BBB. However, the difference between the transport rates of iron and Tf suggests that at least some iron transport across the BBB is independent of Tf and its receptor. Thus, Tf-dependent iron uptake is not the only route in brain cell. Recently, Qian et al. (1998) investigated some aspects of Tf-bound iron uptake by the cultured cerebellar granule cells. Their results showed that (1) Tf uptake by the cells increased rapidly at the first 5 min, reaching its maximum after about 20 min of incubation; (2) Tf-Fe uptake kept increasing in a linear manner during the whole period of incubation; (3) the addition of either NH_4Cl or CH_3NH_2, the blockers of Tf-Fe uptake via inhibiting iron release from Tf within endosomes, decreased the

cellular Tf-Fe uptake but had no significant effect on Tf uptake; (4) trypsin and unlabelled Tf-Fe inhibited the uptake rate of Tf-Fe as well as Tf. The results suggested that Tf-Fe transport across the membrane of this type of neuron, much like other mammalian cells, be mediated by Tf-TfR endocytosis.

1.4.2.2 Transferrin-Independent Iron Uptake

Cells may acquire iron from other routes. This possibility is supported by the following findings: (1) Photomicrographs of brain sections stained for iron show that there is little overlap in the distribution of TfR and iron. Except for the interpeduncular nucleus, the areas with dense TfR have little or no stainable iron (Hill 1988); (2) Cerebrospinal fluid (CSF) iron concentration exceeds the iron-binding capacity of Tf present in CSF, thus, other proteins may bind and transport iron in the brain (Ueda et al. 1993); (3) Hypotransferrinemic mice have been shown to have higher than normal iron uptake into the brain and a significant amount of iron is transported into brain by a route independent of Tf/TfR (Takeda et al. 1998); (4) Nontransferrin-bound iron readily cross the BBB at low Tf level (Beard et al. 1993, Gelman 1995). Therefore, it is reasonable to believe that there are other pathways of iron uptake by brain cells in addition to Tf/TfR mediated endocytosis (Qian and Wang 1997).

In a recent study (Qian et al. In press), we investigated the mechanism of Fe^{2+} (Tf-free iron) uptake by brain neuronal cells using the cultured cerebellar granule cells. Effects of incubation time, iron concentration, temperature and other divalent metals

on the cellular uptake were determined. After 5 days of plating, the cells were incubated with different concentration of Fe^{2+} in isotonic sucrose solution at different temperatures for certain time. The cellular Fe^{2+} uptake was analysed by measuring the cellular radioactivity with γ-counter. Our result showed that the cultured cerebellar granule cells had the capacity to acquire Fe^{2+} at pH 6.5, at which it was demonstrated that Tf binds iron very poorly and only very little Tf can be internalized by reticulocytes (Morgan 1988) and Hela cells (Sturrock et al. 1990). The iron uptake by cells increased with incubation time in a linear manner at a rate of 0.1.76 pmol/μg protein per min within the first 10 min. The uptake was time and temperature dependent, iron concentration saturable, and inhibited by several divalent metal ions, such as Co^{2+}, Zn^{2+}, Mn^{2+} and Ni^{2+}. These characteristics of Fe^{2+} uptake by the cultured cerebellar granule cells observed in this study, similar to those obtained from cells outside of the brain, implied that a carrier-mediated iron transport system might be present on the membrane of this type of brain neuronal cells. It is the first report on Tf-free iron uptake by brain cells. At present, it is completely unknown what are the physiological functions of this iron transport system. Another important question needs to be answered is what is the nature of the suggested iron carrier on the membrane of cerebellar granule cells. Some possible candidates for the membrane iron carrier in somatic cells have been reported or suggested, including p97 (melanotransferrin) (Kennard et al. 1995, 1996, Rothenberger et al. 1996), K^+-ATPase (Watkins et al. 1991, Li et al. 1994, 1995), integrin-mobilferrin pathway (Conrad and Umbreit 1993, Conrad et al. 1992, 1994), TfR (Qian and Morgan 1992, Qian et al. 1997) and some other molecules (Qian et al.

1997). Besides, the possible roles of p97 (Rothenberger 1996, Jefferies et al. 1996b) and LfR (Faucheux et al. 1995, Bonn 1996) as well as divalent cation transporter (DCT1) (Gunshin et al. 1997) in brain iron transport were recently reported (Qian and Wang 1998). Further studies on the relationship between these molecules and the suggested carrier are needed. In addition, more studies should also be conducted to found out whether there is a so-called "iron carrier" on the membrane of other types of brain cells.

1.4.2.3 Other Iron Transport Proteins in Cellular Iron Uptake in the Brain

1.4.2.3.1 Lactotransferrin Receptor/Lactotransferrin

Lactotransferrin receptor, also called lactoferrin receptor (LfR) is a monomeric glycoprotein of 105 kDa which was previously identified only in monocytes and intestinal cells (Iyer and Lonnerdal 1993), has been demonstrated to express in the brain. The overproduction of this protein has been linked with increased intraneuronal iron levels and degeneration of nigral dopaminergic neurons in PD (Faucheux et al. 1995, Leveugle et al. 1996, Bonn 1996).

The well-established process of iron in mammalian cells is mainly mediated by the Tf/TfR pathway (Qian and Tang 1995, Qian et al. 1997). Hence, Faucheux et al. (1995) investigated the changes in this pathway in PD, using binding techniques and autoradiography. They found that the density of Tf binding sits (TfR) in

mesencephalon was not higher in patients with PD than in controls. This finding was consistent with that obtained from the investigation of the distribution and density of TfR in normal and PD midbrain by Morris et al. (1994). These results suggested that the excessive accumulation of iron found in PD does not result form the increased expression of TfR. Therefore, Faucheux et al. turned their attention to another iron-binding protein, LfR, to look for an additional route for iron to gain access to neurons. They investigated LfR expression in the mesencephalon to determine whether neuronal death in PD was associated with changes in the amount of LfR on cellular membrane (Faucheux et al. 1995b). Immunohistochemical staining of postmortem human brain tissue showed that LfR was localized on neurons (perikarya, dendrites, axons), cerebral microvasculature, and, in some cases, glial cells. In the brain from Parkinsonian patients, LfR immunoreactivity on neurons and microvessels was increased and more pronounced in those regions of the mesencephalon where the loss of dopaminergic neurons was severe. Moreover, in the substantia nigra (SN), the intensity of immunoreactivity on neurons and microvessels was higher for patients with higher nigral dopamineric loss. Subsequently, they used quantitative immunohisochemical methods (Leveugle et al. 1996) to analyze the distribution of lactotransferrin (or lactoferrin, Lf), a 80 kDa iron binding glycoprotein composed of 703 amino acid residues, in the mesencephalon of neurologically normal individuals and patients affected with PD. Lf was observed in a large population of neurons in the substantia nigra of control cases. Lf-positive neurons were severely affected by the neurodegenerative process that occurred in PD, as indicated by a significant decrease in the number of immunolabeled neurons in all of these cases. Quantitative analysis also demonstrated higher immunolabeling

levels of Lf in the surviving neurons in the substantia nigra and ventral tegmental area of PD cases compared to control cases. The increased Lf expression found in PD brain has also been demonstrated to occur in the brain of AD and other neurodegenerative disorders (Osmand and Switzer 1991, Leveugle et al. 1994, Rebeck et al. 1995). However, the evidence on whether there is an overexpression of LfR in these disorders is not available.

These results suggest that the LfR may have a role similar to that of TfR on the surface of brain capillary endothelial cells and neurons. Furthermore, Lf may have a role similar to that Tf in iron transport in the brain, ensuring that an adequate amount of iron crosses the BBB as well as the membrane of brain cells under normal circumstances. A study of LfR expression in leukaemia cell lines shows that there is a significant difference between the control mechanisms of TfR and LfR expression. TfR expression is regulated by intracellular iron concentration as mentioned before, but LfR expression appears not to be controlled in this way (Bonn 1996). Therefore, it is very important to find out how the expression of LfR is controlled and how Lf-bound iron crosses the cellular membrane by a LfR-mediated process.

1.4.2.3.2 Melanotransferrin

Melanotransferrin (MTf), an iron binding protein recently identified in the brain tissue, may play a physiological role in iron transport within the human brain. The overexpression of this protein may be involved in excess iron accumulation in the

brain and hence associated with the development of AD (Kennard et al. 1995, 1996, Jefferies et al. 1996a, b, Rothenberger 1996).

The MTf molecule, also called p97 (human melanoma tumor-associated antigen), was first identified on the surface of melanoma cells (Brown et al. 1981). This protein belongs to the important group of iron binding proteins that inclulde serum Tf, Lf and ovotransferrin (Baker et al. 1987). Although MTf has a striking structural similarity to human serum Tf and Lf, its function has not been well determined MTf, like Tf and TfR, is a sialoglycoprotein and is encoded on chromosome 3 in humans (Plowman et al. 1983). In addition to expression on melanoma cells, MTf has been reported to be expressed by a wide range of cultured normal cell types such as liver cells and intestinal cells (Sciot et al. 1989, Alemany et al. 1993). Baker et al. (1992) has shown that MTf has only one iron binding site. It has also been demonstrated that there are two distinct molecular forms of MTf. The first one is a plasma membrane-associated form which contains a glycosylphosphatidylinositol (GPI) anchor, allowing it to be inserted into the cell membrane, while an additional form also exists, which is soluble in the serum or CSF and does not contain a GPI anchor. In all cases where cells have expressed GPI-anchored MTf, they have also expressed a secreted soluble form of MTf (Food et al. 1994). Whether the two forms of the molecule participate in separate functions has not been determined. However, it has been suggested that the GPI-anchored form of MTf could perform the joint function of both Tf and TfR and that the soluble form could also bind iron and then mediate its uptake by cells via a receptor system (Food et al. 1994).

Recently, Jefferies et al. (1984, 1996), Kennard et al (1995, 1996) and Rothenberger et al. (1996) reported some important findings on putative functions of MTf in iron transport and the role of MTf overexpression in the development of AD. These findings showed: (1) MTf is able to bind and internalize iron into cells (Jefferies et al. 1995, 1996). The studies using CHO (Chinese hamster ovary) cell lines demonstrated that GPI-anchored MTf on the membrane provides a novel route for cellular iron uptake that does not depend upon Tf and its receptor. They have provided evidence that MTf has ability to bind and internalize iron into cells from Fe-citrate, but not from Tf-bound iron, at a rate equivalent to iron uptake form Tf/TfR endocytosis. The internalization of iron is temperature sensitive, time dependent and saturated at a media concentration of 2.5μg/ml with a Vmax of 0.1 pmol Fe/10^6 cells /min and a Km of 2.58 μM for MTf. These results suggested that this alternative pathways to the Tf/TfR mediated endocytosis, MTf mediated iron internalization, in mammalian cells is probably an energy-dependent carrier-mediated process rather than occurring by passive diffusion or simple fluid phase pinocytosis (Kennard et al. 1995). (2) MTf is present in normal brain capillary endothelium (Rothenberger et al. 1996). In an immunohistochemical study, they showed that MTf is highly localized in capillary endothelium in all brain tissues examined. The distributions of MTf and TfR are remarkable similar, but quite different form that of Tf that is mainly localized in glial cells. This data suggested that MTf may play a role in iron transport within the human brain.(3) MTf is selectively expressed on reactive microglial cell in AD patients (Jerreries 1996). This expression was associated with amyloid plaques in postmortem brain tissue. All other microglia not associated with the senile AD plaques and those found in brain

tissue form patients with other neuropathologies, including PD and amyotrophic lateral sclerosis (ALS), did not express detectable levels of MTf, indicating that the distribution of MTf is unique and that MTf is a marker of AD. The reactive microglia in senile plaques express high levels of ferritin. The MTf iron delivery pathway is probably the provider of the iron present in the ferritin. (4) MTf concentration was found to be significantly elevated in CSF of AD patients compared with the CSF of subjects suffering from other neuropathologies (Kennard et al. 1996). Furthermore, when the data for the AD patients were plotted against the time since the patient was first observed with symptoms of AD, linear regression analysis showed that there was a significant correlation between the increase in serum concentration of MTf and the progression of the disease.

These findings suggest that GPI-anchored MTf may deliver iron across the BBB by a mechanism analogous to that mediated by the TfR. Dysregulation of MTf may be one of the causes of excess iron deposition in AD brain tissue. The likely importance of this molecule in normal physiological process has been proposed by Jefferies et al. (1996) based on their results. However, information on the distribution and functional characterization of this protein in the brain is very limited. It remains to be determined whether different types of brain cells have the ability to express MTf, and whether and how this protein is involved in iron uptake by brain cells such as neurons and glia under physiological conditions. Further studies on the distribution of MTf in differint types of brain cells in different regions, the role of this protein in iron uptake by different types of brain cells, and the mechanism of homeostatic control of MTf expression in the brain will be valuable in understanding the causes

of MTf overproduction, its relationship with or its possible role in the development of abnormally high levels of tissue iron in the brain and pathogenesis of AD. With regard to the functions of two forms of MTf, another possibility worth of consideration is that soluble MTf functions as an iron transporter in CSF or brain extracellular fluid delivering iron to GPI-anchored MTf that acts as a membrane receptor and mediates iron internalization in brain cells. Although the high affinity of MTf for the iron makes this seem unlikely, substances secreted by brain cells, such as citrate, may mediate iron exchange between MTf moleculs.

1.4.2.3.3 Divalent Cation Transporter

Divalent cation transporter (DCT1) is a new mammalian proton-coupled metal-ion transport protein. Its presence in the brain and other tissues has recently been reported by Gunshin et al. (1997). The possible role of DCT1 in physiological iron transport in the brain has been suggested and the defects in this membrane protein has been associated with brain iron imbalance and neuronal death in neurodegenerative diseases (Gunshin et al. 1997).

This integral membrane protein consists of 561 amino acids with 12 putative membrane-spanning domains. It is the most recently identified member of the Nramp (natural-resistance-associate macrophage protein) family (Gruenheid et al. 1993, Cellier et al. 1995, Vidal et al. 1995a, b). Other members of this family are Nramp 1 and Nramp 2 and their role in divalent cation transport have recently been identified as well. The study conducted by Bunshin et al. (1997) shows that diet-

induced iron deficiency (ID) may increase DCT1 expression in the brain and other tissues although the enhanced extent is different, suggesting that DCT1 mRNA expression is probably regulated by dietary and/or tissue iron concentration. It has been found that DCT1 cDNA contains in its 3' untranslated region a putative iron-response element which forms a stem-loop containing the consensus sequence found in the 3' and 5' iron-responsive elements of the TfR and the ferritin mRNAs, respectively (Klausner et al. 1983, Casey et al. 1988, Gunshin et al. 1997).

This protein has an unusually broad substrate range including Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+}. The Fe^{2+} transport mediated by DCT1 is active and H^{+}-dependent. The investigation on cellular localization of DCT1 mRNA in different tissues including brain, using non-radioactive in situ hybridization with digoxigenin-labelled cRNA probe, revealed the existence of DCT1 mRNA in neurons and epithelial cells of the choroid plexus but not glial or ependymal cells in each region examined in the brain. A qualitative examination of sagittal sections indicated that most neurons expressed DCT1 mRNA at a low level. More prominent labeling was present in densely packed cell groups, such as the hippocampal pyramidal and granule cells, cerebellar granule cells, the preoptic nucleus and pyramidal cells of the pirifiorm cortex. Moderate amounts of DCT1 mRNA were present in the SN. One cell group, the ventral portion of the anterior olfactory nucleus, contains large amounts of DCT1 mRNA, at least an order of magnitude more than elsewther in the brain (Gunshin et al. 1997). The functional characterization of DCT1 and its cellular localization in brain suggests that DCT1 may play a role in physiological iron

transport in the brain. It has also been proposed that the defects in DCT1 are likely to contribute to the etiology of certain neurodegenerative diseases by promoting the generation of reactive oxygen species by divalent cations (Gundhin et al. 1997). However, at present, the functions and functional mechanisms of DCT1 in central nervous system have not been fully understood. Moreover, no information is available on the possible role of dysfunction of DCT1 in neurodegenerative diseases. Further exploration is needed.

1.4.3 CERULOPLASMIN AND IRON EFFLUX FROM BRAIN CELLS

Ceruloplasmin (CP), a blue copper-containing oxidase that is mainly synthesized in hepatocytes, is also synthesized in the CNS and probably involved in brain iron metabolism. The decreased or absent expression of this protein may induce excessive iron accumulation in brain cells in acerulplasminemia and perhaps in a variety of neurodegenerative diseases where abnormalities in iron metabolism have been demonstrated (Connor et al. 1993, Harris et al. 1995, Kaplan and O' Halloran 1996, Klomp and Gitlin 1996, Klomp et al. 1996).

CP is an abundant serum α_2-glycoprotein and has a molecular mass of approximately 132 kDa. It consists of a single polypeptide chain of 1046 amino acid residues and belongs to a family of multi-nuclear 'blue' copper oxidases which includes ascorbate oxidase and laccase. The X-ray structure of human serum CP has been solved at a

resolution of 3.1Å (Zaitseva et al. 1996). The molecule is comprised of six plastocyanin-type domains arranged in triangular array. There are six copper atoms; three form a trinuclear cluster sited at the interface of domains 1 and 6, and there are three mononuclear sites in domains 2, 4 and 6. Each of the mononuclear copper atoms is coordinated to one cysteine and two histidineresidues, and those in domains 4 and 6 also coordinate to a methionine residue; in domain 2, the methionine is replaced by a leucine residue which may form van der Waals type contacts with the copper. The trinuclear center and the mononuclear copper in domain 6 form a cluster essentially the same as that found in ascorbate oxidase (Zaitseva et al. 1996)

At least four functions have been attributed to CP: copper transport, ferroxidase activity, amine oxidase activity and antioxidant functions in the prevention of the formation of free radicals in serum (Zaitseva et al. 1996). The dominant role of CP may be as a ferrooxidase, facilitating the release of iron from cells prior to binding by apotransferrin. The evidence that CP contributes to mammalian cell iron transport was presented by Osaki et al. (1966) about 40 years ago. Pigs made copper-deficient by dietary restriction had low levels of CP and displayed impaired release of iron from tissue storage sites (Lee et al. 1968). Studies using hypoceruloplasminemic swine by copper deprevation showed that CP is essential to the normal movement of iron from cell to plasma (Roeser et al. 1970). It is now well recognized that CP is critical for iron egress form some cell types (Harris 1995, Kaplan and O'Halloran 1996, Takahashi et al. 1996) although the transport system responsible for iron release form cells has not been determined. Recently, the genetic characterization of aceruloplasminemia, a newly recognized disorder of iron metabolism resulting form

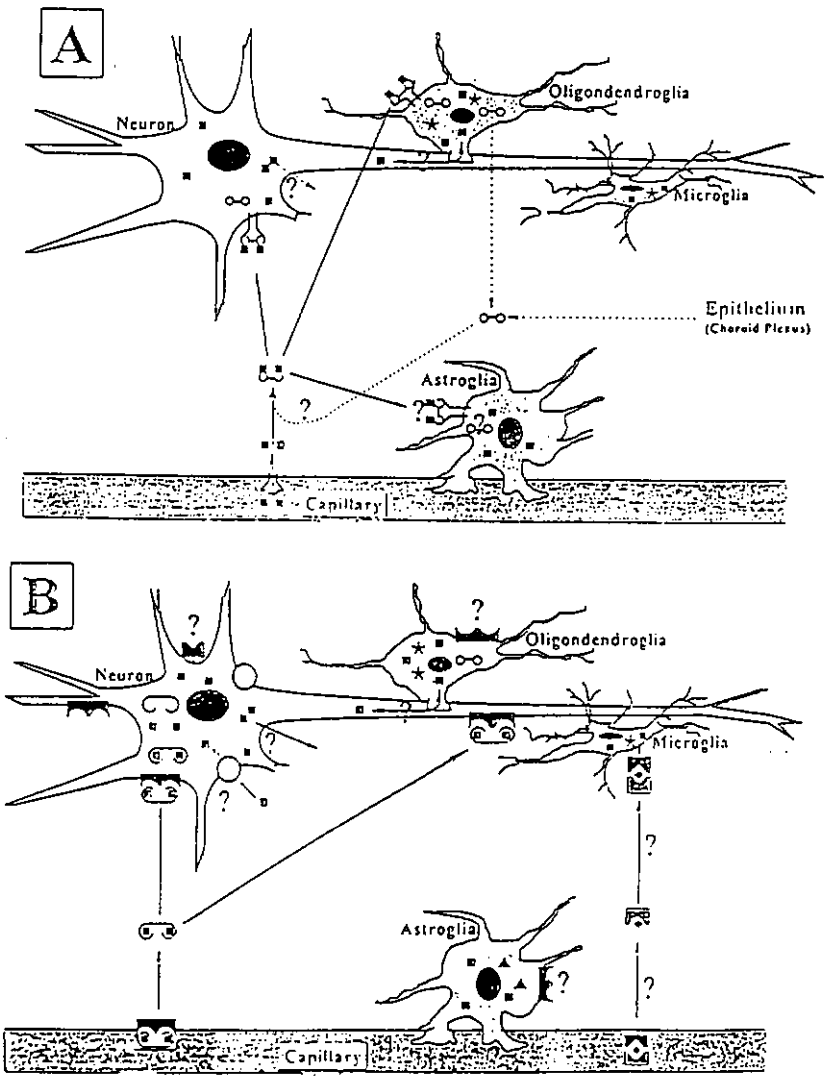
inherited mutations in the CP gene, provided insight into the role of CP in iron metabolism in the brain. The most striking and unique feature of this disorder is the progressive neurodegeneration of the retina and basal ganglia in association with accumulation of iron in these tissues (Yoshida et al. 1995, Harris et al. 1995, 1996, Kawanami et al. 1996, Logan 1996, Takahashi et al. 1996). In order to define the mechanisms of CNS iron accumulation and neuronal loss in aceruolplasminemia, Klomp et al (1996) investigated ceruloplasmin gene expression in the murine CNS. This study is very important in determining whether CP is involved in brain iron metabolism since very little CP synthesized and released by liver can cross the BBB under physiological circumstances. Their results showed that in the CNS abundant CP gene expression could be detected in specific populations of astrocytes within the retina and the brain, as well as in the epithelium of the choroid plexus. Analysis of primary cell cultures confirmed that astrocytes expressed CP mRNA. Biosynthetic studies revealed synthesis and secretion of CP by these cells. The data from pulse-chase studies revealed that murine CP was synthesized as a precursor polypeptide of 130 kDa apparent molecular mass which was then converted to a mature peptide of 132 kDa (Klomp et al.1996). In addition to astrocytes and the epithelium of the choroid plexus, the study on immunocytochemical staining for CP in human brain suggests that CP synthesis may occur in neuron (Loeffer et al. 1996). Klomp and Gitlin (1996) also examined CP gene expression in human brain and retinal tissue. They demonstrated CP-specific transcripts in multiple regions of human brains using RNA bolt analysis and RNAse protection studies, and CP synthesis and secretion in these same regions using biosynthetic methods. Their study also revealed abundant CP gene expression in specific populations of glial cells associated with the brain

microvasculature, surrounding dopaminergic melanized neurons in the SN and within the inner nuclear layer of the retina. These findings provided further support for the view that CP is essential for normal iron metabolism in the CNS. CP may function to promote iron efflux from brain cells via ferroxidase activity. If so, in the absence of CP expression in patients with aceruloplasminemia, iron would be unable to move form brain cells into the brain extracellular fluid and CSF for eventual excretion, resulting in iron accumulation in related brain tissues.

Intracellular iron balance is dependent not only upon the amount of iron taken up and sequestered but also upon the amount of iron released from cells. However, we know very little about the molecular mechanisms by which iron is released from cell. This question has been considered as one of the major unresolved issues in iron metabolism at present (Kaplan 1996). In addition, the molecules involved in regulating the rate of iron efflux from cells under physiological conditions have not been well determined (Logan 1996). During the past years, very little research effort has been devoted to investigating these aspects. The findings presented by Gitlin et al. are the first which suggest a role for CP in iron efflux from brain cells under physiological conditions. Further studies on the mechanism involved in the process of CP-mediated iron release from brain cells and a more detailed analysis of functions of CP in iron transport and of the homeostatic control of CP expression within the CNS are needed. In addition, the possibility that iron exits the brain cells through the same transport system used for iron accumulation (De Silva et al. 1996) is worth investigating. These studies will be of value in understanding normal brain iron metabolism.

1.4.4 HYPOTHETICAL SCHEME FOR BRAIN IRON TRANSPORT

On the basis of these findings reviewed in the preceding chapters, the possible involvement of some proteins in brain iron transport is summarized diagrammatically in Fig. 1. Take as a whole, it seems appropriate to conclude that there may be multiple proteins including Tf/TfR, Lf/LfR, MTf, CP, DCT1 and possibly other iron carriers or transporters on the membrane of different types of brain cells which are responsible for iron metabolism in the brain, although they may vary in physiological importance.



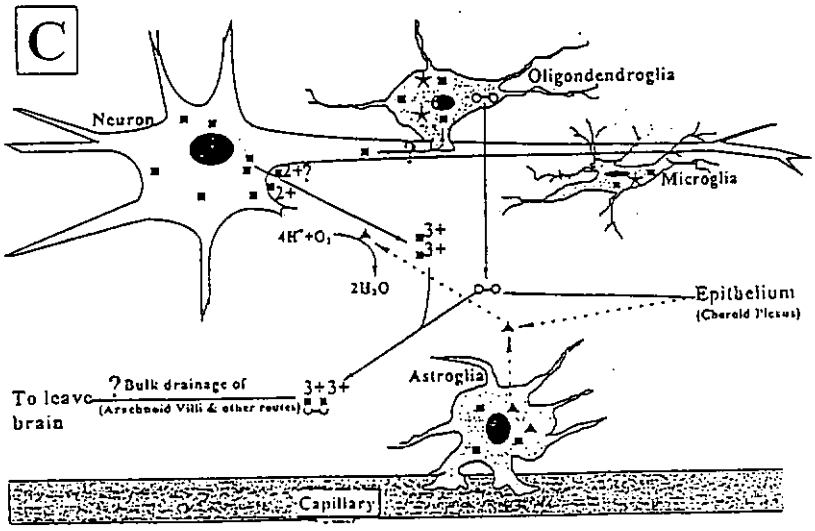


Fig. 1. Hypothetical scheme for the possible role of Tf/TfR, Lf/LfR, MTf, DCT1 and CP in brain iron transport (Qian et al 1998). (A) Hypothetical mechanism of Tf/TfR-mediated brain iron transport. The existence of TfR has been demonstrated in neuronal cells, oligodendrocytes and brain capillary endothelium. As in other tissues outside of the brain, Tf/TfR-mediated endocytosis is probably the main mechanism of iron uptake by most brain cells and Tf-bound iron is the major iron transport form in the brain. It has been suggested (Brown et al. 1981, Taylor and Morgan 1990, Ueda et al. 1993, Connor and Menzies 1996) that Tf-bound iron (O--O) is endocytosed into brain capillary endothelial cells by TfR (⌝ ⌜) mediated process on luminal surface. Iron (■) is separated from Tf (O--O) within the cells, presumable by the general mechanism of acidification within the endosome, and then might be transported alone, perhaps as Fe^{2+}, across the abluminal membrane of the endothelium into brain interstitial fluid. Majority of Tf is recycled back into blood and does not cross into brain. Within brain interstitial fluid iron will bind with any unsaturated Tf synthesized either in choroid plexuses or in oligodendrocytes (Connor

1992c) or transported into the brain CSF system (Bradibury 1997). Tf-bound iron then bind to TfR on neuron or other brain cells and then endocytosed. Many important questions concerning this process have not been answered, especially there is the higher degree of uncertainty regarding the role of endogenous brain Tf in brain extracellular iron transport (Dickinson and Connor 1995) as well as the transport form of iron (Fe^{2+}) across the abluminal membrane of the endothelium. (B) Suggested role of Lf/LfR, MTf and DCT1 in brain iron transport. The presence of Lf/LfR, MTf and DCT1 in the brain has been demonstrated (Faucheux et al. 1995, Gunshin et al. 1997, Jefferies et al. 1996, Leveugle et al. 1996, Rothenberger et al. 1996). The possible role in physiological brain iron transport has been suggested. The mechanism of Lf/LfR, MTf and DCT1 involved in brain iron transport is not completely detailed. However, in the case of Lf/LfR pathway, it is suggested that LfR (▬) might have a role similar to that of TfR on the surface of brain capillary endothelial cells and neurons and that Lf (⊂) might have a function similar to that of Tf in iron transport in brain interstitial fluid (Bonn 1996). Lf-bound iron (⊂●) is probably one of the transport forms of iron in CSF and extracellular fluid. As regards to MTf-mediated iron uptake system, very little is known about its mechanism. Some possibilities have been proposed. One of them is that the GPI-anchored MTf could perform the joint function of both Tf and TfR and that soluble MTf could also bind iron and then mediates its uptake by cells via a receptor system (Food et al. 1994). Another possibility worthy of consideration is that soluble MTf (▬) functions as an iron transporter (Soluble MTf-bound iron:) in brain extracellular fluid, delivering iron to GPI-anchored MTf (▬◆) which acts as a membrane receptor, mediating iron internalization in brain cells. While DCT1 (●) mediated

iron transport is probably an active and H^+-dependent process (C). Proposed role of CP in brain iron metabolism. CP is believed to be critical for iron egress from brain cells to extracellular fluid. It has been hypothesized that iron, probably in the form of Fe^{2+} ($\blacksquare^{2+}$), is presented to the surface of brain cells, and then oxidated to Fe^{3+}($\blacksquare^{3+}$) and combined with Tf in extracellular fluid. CP (▲) is thought to be required for the conversion of Fe^{2+} to Fe^{3+}. It has been demonstrated that CP could be synthesized and secreted by astrocytes and the epithelium of the choroid plexus (Klomp et al. 1996) and probably also by brain neuron (Loeffler et al. 1996). (★: Ferritin; ?: To Be confirmed)

1.5 ABNORMAL BRAIN IRON METABOLISM AND CENTRAL NERVOUS SYSTEM (CNS) DISEASES

1.5.1. EFFECT OF IRON DEFICIENCY IN THE BRAIN

Iron deficiency is common, although the prevalence estimates depend upon the economic state of the population studied and the methods used for evaluation. In developing countries, as many as 20-40% of infants and pregnant women, the groups most at risk, may be affected, whereas studies of these groups in Sweden and the United States put the figures at 5-10% (Hallberg et al. 1979)

What constitutes ID has been extensively debated, and the answer depends greatly on the context in which the concept is being used (Dallman 1982). The main biochemical and hematological tests used to determine ID are presented in Table 2. Investigations of ID have traditionally concentrated on the hematological consequences. The symptoms related to tissue ID has long been considered to be late and unusual complications (Finch and Huebers 1982). It is now recognized that early ID may affect the tissue functions before or in concert with decrease in hemoglobin production (Pollitt and Leibel 1982). ID leads to a 30-40% reduction in brain non-heme iron in the rat (Dallman et al. 1975). However, the turnover of iron is low in the brain compared with the liver, and the brain has a greater tendency to resist iron depletion, and a reduced rate of repletion upon therapy (Pollitt 1982). This changes during ID, so that the BBB is significantly open and the iron stores can be restored, which in turn leads to a closure of the BBB so that iron overload does not occur in the brain (Youdim et al. 1989). The iron stores in the young (immature) brain are particularly vulnerable to ID and are not restored in the case of the rat even after up to 6 months of iron therapy (Dallman et al. 1975).

A number of factors influence the absorption of dietary iron, some of which can have an independent direct action on brain function, thus confounding the role of ID. One factor of note is low ascorbic acid intake (Hallberg 1989). Another possibility to consider is that ID may sometimes be associated with zinc deficiency e.g., in the case of high phytate intake, which inhibits the absorption of both metals. ID also results in the increased absorption not only of iron but also of other trace elements

like lead and cadmium (Watson et al. 1986). Increased lead is known to have adverse effects on CNS function (Hallberg 1989).

TABLE 2.Laboratory tests to assess body iron status

1. Erythrocyte indices
   - Hemoglobin (Hb)
   - Erythrocyte count (RBC)
   - Hematocrit (Hct)
   - Mean corpuscular volume (MCV)
2. Erthrocyte morphology
   - Stained-blood film for microcytosis, poikilocytosis, and anisocystosis
3. Serum ferritin
4. Serum iron (Fe), total iron-binding capacity (TIBC), and transferrin saturation (Fe/TIBC)
5. Bone marrow examination for stainable iron
6. Free erythrocytic protoporphyrin
7. Ferrokinetic studies using ^{59}Fe
8. Iron tolerance tests
9. Urinary ironfollowing administration of iron-chelating agents
10. Needle liver biopsy for stainable iron

ID may adversely affect brain function directly. Children with ID suffer from apathy, short attention span, and inability to concentrate, and usually get low scores on intelligence tests (Walter et al. 1983, Lozoff 1989). Since iron is essential for the biosynthesis and metabolism of several neurotransmitters, emotional changes have

been proposed as co-factors of cognitive dysfunction in ID in early childhood (Shoham et al. 1996).

Several studies on the effects of iron deficiency in rat brain metabolism show that the activities of aldehyde oxidase, a key enzyme in the pathway of serotonin degradation, are significantly reduced. Moreover, the concentrations of serotonin and total 5-hydroxyindole compounds are elevated in brain tissue of iron deficient animals. Perinatal iron deficiency significantly alters myelination of the spinal cord and white matter of cerebellar folds. Dallman (1975) demonstrated in early postnatal life that the iron is also resistant to the restoration of normal brain iron.

1.5.2 IRON OVERLOAD AND NEURODEGENERATIVE DISEASES

Abnormally high levels of iron and oxidative stress have been demonstrated in many neurodegenerative disorders. The increased level of brain iron has been implicated as a major generator of reactive oxygen species which are capable of damaging biological molecules, including lipids, carbohydrates, proteins, and nuclei acids. Oxidative stress resulting from the increased iron levels, and possibly from defects in antioxidant defense mechanisms, is widely believed to be the cause of neuronal death in neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), and Hallervorden-Spatz Syndrome (HSS) (Drayer et al.

1987, Swaiman 1991, Connor 1992e, Olanow et al. 1992, Bonn 1966, Qian et al. 1997).

1.5.2.1 Parkinson's Disease

Parkinson's disease is characterized pathologically by progressive loss of catecholaminergic neurons in the brain stem. The neuropathological basis is the degeneration of melanin-pigmented dopaminergic neurons of the SN pars compacta and the resulting dopamine deficiency in the striatum. The cause of degeneration of nigrostriatal dopaminergic neurons in PD is unknown (Gerlach et al. 1994). Recent attention has focused on the involvement of iron in the degeneration, particularly as it relates to oxidative damage (Swaiman 1991, Gerlach et al. 1994, Gelman 1995, Gold and Lenox 1991). Iron has a high affinity for the melanin-containing lopaminergic neurons of the nigrostriatal region and its presence suggests a putative role for iron as a neurotoxin. Studies on the interaction of iron and melanin and its association with lipid peroxidation show that melanin selectively bound Fe^{3+} with high affinity. Iron initiates oxidative stress via the generation of cytotoxic hydroxy free radicals in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^+$) (Swaiman 1991). It has been reported in PD that the ratio of Fe^{2+} : Fe^{3+} decreased from 3:1 to 1:1 (Gold and Lenox 1995). This finding supports the view that Fe^{3+} is reduced to Fe^{2+} in the presence of iron chelators (melanin), initiating the Fenton reaction from which free radicals are generated. Riedener et al provided the evidence that support an association of a selective increase in iron content in the SN with accumulation in

the dopamine-rich cells of the zona sured the postmortem brain iron content of persons who has PD and found a 175% increase in total iron and a 225% increase in ferric iron in the SN. In addition to the direct measurement of brain iron content, there are two other lines of research that support the relationship between excess iron and PD (Gold and Lenox 1995). The first is that direct injection of iron into the SN of rats produces "behavioral and biochemical Parkinsonism". The second is that G-OH-dopamine induces Parkinsonism as well as lesions of the nigrostriatal dopaminergic neurons because G-OH-dopamine is thought to induce degeneration of the dopaminergic neurons via free radical formation catalyzed by transitional metals such as iron.

1.5.2.2 Alzheimer's Disease

Iron dysfunction has long been suspected in AD. This disease is characterized by structural changes in several cortical and subcortical areas, especially in the association centers, hippocampus, and the basal forebrain (Beard et al. 1993, Gerlach et al. 1994, Gelman 1995). The structural changes are mainly the progressive neuronal cell death and the presence of amyloid plaques and neurofibrillary tangles. In some specific brain regions such as the hippocampus and the motor cortex, there is an elevated iron levels relative to normal (Gerlach et al. 1994). However, a decreased iron content is found in the occipital cortex. In contrast to iron content, the levels of ferritin are differentially altered in the brain of the patients with this disease (Gerlach et al. 1994). Analyses of iron transport and storage proteins suggest that iron mobility in the brain is decrease in AD (Beard et al. 1993). The decreased

iron mobility would likely be associated with decreased metabolic activity and increased peroxidative damage in AD, without known cause. Abnormalities in oxidative metabolism in this disease are most relevant to AD with iron metabolism. A correlation between the cellular and regional distribution of TfRs and levels of cytochrome oxidase activity in the brain underscores the importance of iron and oxidative activity (Morris et al. 1992). A loss of TfR as well as a decreased mitochondrial enzymes and metabolic activity in specific brain regions in AD including the hippocampus have been reported. Recently, it is also suggested that iron regulatory proteins may also be involved in the development of AD (Beard et al. 1993). However, its role and functional mechanism need to be further investigated.

1.5.2.3 OTHER BRAIN DISORDERS

In addition to PD and AD, a number of brain disorders are associated with disruptions in iron homeostasis in the brain. In Hallervorden-Spatz syndrome (HSS) (Swaiman 1991, Gerlach et al. 1994) it has been reported that large amounts of iron are deposited in the globus pallidus and pars reticulata of the SN. At postmortem examination of the patients with this syndrome, the basal ganglia was found to be grossly rust-colored, suggesting large amount of iron storage in the areas. In Huntington's disease (Gerlach et al. 1994), there is an increase in iron content in the caudate nucleus but not in the substantia nigra and also there is no elevation in brain ferritin content in the SN or elsewhere. In contrast to Huntington's disease, the increased ferritin content of the SN was found in Steel-Richardson-Olszewski's disease (Gerlach et al. 1994). In addition, there are increases in the concentration of

iron in the caudate nucleus and putaman in this disease. The above studies reflect that an abnormal increase in iron content in some brain areas plays an important role in the pathogenesis and development of these disorders.

1.6 ASTROCYTES AND BRAIN IRON METABOLISM

1.6.1 ROLE OF ASTROCYTES IN THE BRAIN

Astrocytes make up a large percentage in the cellular compositions of the CNS and play an important role in brain function. Astrocytic processes contact the surface of nerve cell bodies and axons, in company with the processes of oligodendrocytes. Together they shield the neurons from direct contact with other neurons as well as from the surrounding interstitial fluid. Biological functions of astrocytes include:

(1) Maintaining the BBB: these cells have very restricted permeability characteristics, and so create a BBB that isolates the CNS from the general circulation;

(2) Creating a three-dimensional framework for the CNS: astrocytes are packed with microfilaments that extend from foot to foot across the breadth of the cell. This reinforcement assists them in providing a structural framework for the neurons of the brain and spinal cord;

(3) Performing repairs in damaged neural tissue: following damage to the CNS, astrocytes make structural repairs by producing scar tissue at the injury site;

(4) Guiding neuron development: astrocytes in the embryonic brain appear to be involved in directing the growth and interconnection of developing neurons; and;

(5) Controlling the interstitial environment (Martini 1992).

Cells corresponding to type 1 and type 2 astrocytes have been observed in the cultures of cerebellum, cerebral cortex and optic nerve. Type 1 astrocytes express an wide variety of neurotransmitter receptors and ion channels. The demonstration of ion channels, some of which are voltage dependent, surprises many investigators. Indeed, the distinction between neurons and astrocytes appears far less obvious. Similar to neuronal neurotransmitter receptors, astrocyte receptors activate a number of second messenger systems including cyclic AMP, cyclic GMP, phosphoinositide metabolism, intracellular calcium, and arachidonic acid. Activation of these systems may, in turn, affect a number of physiological processes, including enzyme induction, proliferation, membrane potential, glycogen metabolism, trophic factor release, neuromodulator secretion, cell morphology, and major histocompatibility complex antigen expression. In the future it will be important to determine whether the similar events are influenced by astrocytes receptors *in vivo.* (McCarthy and Levison 1990).

On the other hand, neuronal-astrocytic interaction is important in CNS development and function. It is obvious that any interruption in the event chain at a neuronal step or at an astrocytic step can lead to catastrophic dysfunction under pathological conditions. It is possible that astrocytes, dispersed over large brain areas, in unison respond to a neuronal signal in order to attend to neuronal demands as suppliers of

nutrients and precursors, and as removers of waste. It is also possible that astrocytes contribute actively to CNS output at a local level and play a role in deciding neuronal pathways. Such a situation may be compared with a neuronal network of routes and astrocytes whose routes are actually followed and maybe also with an astrocytes capability to make shortcuts between established routes. The astrocytes input maybe exert in at least two different manners. The first is that the astrocytes release glutamate and/or potassium that might lead to a direct excitation of adjacent neurons in an non-synaptic manner. The second is that astrocytes might promote some neuronal pathways by delivering transmitter precursors and paralyze other neuronal pathways by depriving neurons of transmitter precursors (Galambos 1961, Hertz 1991).

1.6.2 ROLE OF ASTROCYTES IN BRAIN IRON METABOLISM

Under normal conditions, iron is found mainly in oligodendrocytes. In the absence of a mature population of oligodendrocytes, iron accumulates in astrocytes rather than in the dysfunctional oligodendrocytes. The latter observation indicates the continued transport of iron into the brain in pathological states, and the altered iron uptake at the cellular level. Whether the uptake of iron into astrocytes is for sequestration and detoxification or for meeting the increased intracellular metabolic demands as the response of these cells to brain insult is unknown (Connor and Menzies 1990, Connor 1994). The presence of iron is associated with cell synthesis

of ferritin protein subunits. The astrocytes normally do not stain with the isoform of ferritin, except in the basal ganglia with numerous robustly labeled L-chain specific astrocytes. As we know, Tf is the iron transport protein that binds to its specific membrane receptor and enters the cell. An in vitro analysis has reported the presence of Tf mRNA in astrocytes (Espinosa de los Monteros et al. 1990). Astrocytes containing Tf have been found in AD, MS, PD and central pontine myelinolysis patients. In addition to the astrocytic Tf immunostaining in AD, Tf is also found in the neuritic plaques (Connor et al. 1990, Gocht and Lohler 1990). Thus, astrocyte maybe is responsible for the abnormal sequestration of brain iron in neurodegenerative disorders.

Iron transport protein (Tf) and iron storage protein (ferritin) are specially adapted to maintain metabolically available ferric iron concentration at negligible levels, however, this protective system is imperfect. Iron accumulation facilitates peroxidation of membranous debris with resultant lipofuscin formation; subsequently the formation of neuromelanin as well as spheroid bodies occurs. Astrocytes in striatum, hippocampus and perivertricular brain regions accumulate unique cytoplasmic inclusions with advancing age that are histochemically and morphologically distinct from lipofuscin. The gliosomes exhibit an affinity for Gomori stains, orange-red autofluorescence, and intense, nonenzymatic peroxidase activity likely mediated ferrous iron. Astrocyte hyertrophy accumulation of glial fibrillary acidic protein (GFAP)-positive intermediate filament, and possible hyperplasia (reactive gliosis) are characteristic pathological features of the major aging-related neurodegenerative disorders including AD, PD, Huntington's disease

and amyotrophic lateral sclerosis. Most significantly, the Gomori - positive gliosomes are rich in iron. The astrocytes may also exhibit specific cytopathological changes, suggesting that they may be the primary targets of the disease process, as in the certain rare neurodegenerative condition (Norenberg and Lapham 1974, Tomlinson and Corsellis 1984, Seielberger 1988, Schipper 1996). The role of astrocytes in iron regulation appears to increase when levels of iron increase. Thus, its ability responding to the fluctuation of iron levels which could occur with alterations of iron transport in disease or iron overload in trauma is significant in controlling or minimizing oxidative damage.

1.7 CONCLUSION AND PURPOSE OF THIS RESEARCH

1.7.1 CONCLUSION

Brain iron metabolism is an area of increasing interest in neurobiology and neuropathology. Studies reviewed in this chapter have made important contribution in this area. The findings obtained during the past two decades have provided key insights into understanding some fundamental aspects of brain iron metabolism. These aspects include cellular and tissue distribution of iron in the brain, mechanism of iron transport across BBB, and the possible cause of abnormally increased deposition of iron in some brain regions. Therefore they may help to clarify the pathogenesis of several neurodegenerative diseases.

However, it should be recognized that study on brain iron is still in its infancy and many important questions need to be answered. Firstly, detailed analysis on the distribution of transferrin receptor and other brain iron transport proteins in different types of brain cells and different brain regions in normal subjects is absolutely needed. Secondly, more work needs to be done on physiological function, mechanism, importance and relationship of transferrin receptor and other iron transport proteins in brain iron homeostasis. Thirdly, the studies should be conducted to understand how to control the expression of these proteins and what are the roles of dysregulation of these proteins' expression in the formation of excessive iron accumulation in the brain and in the development of neurodegenerative disorders. The full understanding of these questions will be fundamental and critical not only for elucidating the pathophysiological mechanisms responsible for excess iron accumulation in the brain but also for developing pharmacological interventions that can disrupt the chain of pathological events occurring is neurodegenerative diseases.

1.7.2 PURPOSE OF THIS RESEARCH

About 10 years ago, Swaiman and Machen (1984, 1985, 1986) investigated the mechanisms of iron uptake by cultured cortical glial and neurons. According to their results, iron transport across membrane of these cells is a transferrin receptor-mediated process. Their reports are the earliest studies on brain cell iron accumulation. Since then, very little study has been conducted in this important

aspect of brain iron homeostasis. However, it is well recognized today that the mechanism of iron uptake by and release from different types of brain cells is one of the most important questions on brain cellular iron balance. To my knowledge, no information is presented on mechanisms of iron transport across the membrane of astrocytes and the role of astrocytes in brain iron homeostasis. Therefore I select astrocytes as a model for the study of iron uptake mechanism. Another reason is that the cultured astrocytes, as mentioned before, can be routinely prepared with more than 95% homogeneity. The cells have been widely used in neurochemical and pharmaceutical studies, including receptor expression, neurotrophic factor secretion, ion fluxes, enzyme induction, protein synthesis and phosphorylation, neurotransmitter uptake, metabolic processes and lipid metabolism (McCarthy, 1990).

This research aims to investigate the following questions: (1) Effect of iron on the development of brain astrocytes in culture; (2) Expression of TfR on the membrane of the cultured brain astrocytes in rats; (3) Some aspects on mechanisms of transferrin-bound and Tf-free iron uptake by the cultured brain astrocytes. The findings obtained will add to knowledge about the physiology and biochemistry of brain iron metabolism and increase our understanding of several general aspects of brain iron transport and homeostasis. In addition, this study will provide key insights into the possible causes of brain iron imbalance occurring in neurodegenerative disorders.

Chapter 2

MATERIALS, APPARATUS AND METHODS

2.1 MATERIALS

2.1.1 SOURCE OF REAGENTS AND BIOCHEMICALS

Please refer the Appendix Section

2.1.2 RADIOCHEMICALS

2.1.2.1 Iodine^{125}

$Na^{125}I$, specific activity of 1mCi/ml, was obtained from Radiochemical Center, Amersham International Inc., England.

2.1.2.2 Iron^{59}

$^{59}FeCl_3$ with a specific activity of 0.1mCi/ml (21.6μg Fe/ml in 0.1M HCl) was obtained Radiochemical Center, Amersham International Inc., England.

2.1.3 ANIMAL

The astrocytes were cultured from the cerebral cortices of 2-postnatal day (PND) Sprague Dawley (SD) rats. The rats were supplied by the Animal House of Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University.

2.1.4 SOLUTIONS

2.1.4.1 Double Distilled Water

Water which had been distilled and deionized was further treated by passing it through a Fi-streem water purification system (England).

2.1.4.2 Phosphate Buffered Saline (PBS)

This was prepared by diluting one part of sodium phosphate buffer to 50 parts 0.15 M NaCl. The pH was adjusted to 7.4.

All solutions were prepared with double distilled water (ddH_2O). The solutions and media used in cell culture were sterilized by passing through a 0.22μm Millipore membrane.

2.2 APPARATUS

2.2.1 TOOLS FOR DISSECTION RAT

Surgical tools for dissection and tissue preparation including:

Large dissection scissors, small dissection scissors, small curved scissors, large forceps, pointed surgical forceps, small curved forceps, metal spatula and Swann-Morton knife.

2.2.2 TOOLS FOR CULTURE CELL

Cells were plated on $75cm^2$ flask or 35mm culture dishes purchased from Corning Ltd.

2.2.3 LAMINAR FLOW CABINET

All cell culture work was carried out in a Nuair Laminar flow cabinet, model Nu 425-400E (Nuair, Inc. USA) in the animal cell culture room at the Department of Applied Biology & Chemical Technology of the Hong Kong Polytechnic University. Cells were incubated in a CO_2 incubator, model TC2323 (Shel LAB).

2.2.4 AUTOCLAVE

All tools for culture cell were cleaned with ddH_2O, then were autoclaved at 121°C,

15PSI for 20min before use (Model HA-30, Japan).

2.2.5 MICROSCOPE AND PHOTOGRAPHY

Cultures were observed through a Nikon inverted microscope (Diaphot-TMD) and photographed by a Nikon 35mm camera, model FE2 (Japan).

2.2.6 INCUBATOR

A water bath (JB4, Grant instruments, UK) was used for all incubation during the iron uptake and cell culture experiment. These were generally performed at 37° ±0.5°C.

2.2.7 pH METER

pH value of all solutions were measured with an Orion research, Model 701 digital pH meter, in conjunction with Beckman Calibration buffers.

2.2.8 ELISA READER

A Micoplate Reader, model 450 (Bio-Rad Technology Ltd.) was used to measure

optical densities of the cells.

2.2.9 ELECTROPHORESIS CELL

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was ran with a Mini-protean II Electrophoresis Cell (Bio-Rad Technology Ltd.).

2.2.10 CENTRIFUGE

A centrifuge, model Jouan MR22 (Line Analytics Ltd.) was used for all spin and incubation respectively during the cell culture and iron uptake experiment.

2.2.11 RADIOACTIVITY MEASUREMENTS

The activity of the γ-emmiters, ^{59}Fe and ^{125}I, was measured using a three channel γ-scintillation counter (Packard 5003 COBRA Q, USA).

2.3 GENERAL METHODS

2.3.1 CELL CULTURE

2.3.1.1 Preparation of tissue separation buffer and culture medium

a) Tissue separation buffer:

Preparation of EBS:

Solution A: 50ml EBS+2mg DNase+0.15g BSA

Solution B: 50ml EBS + 2mg DNase+0.15g BSA + 12.5mg trypsin

Solution C: 80g NaCl + 29g $Na_2HPO_4.12H_2O$ + 2g KCl + 2g KH_2PO_4 + 1000ml ddH_2O

Solution D: 200ml solution C + 1g trypsin + 0.3946g EDTA + 1.7g NaCl (10X, 0.5%)

Solution A and **B** were freshly prepared, and sterilized with syringe filter of 0.22μm pore size, and heated to 37°C. **Solution C** and **D** were sterilized through of 0.22μm Millipore membrane and stored at -20°C.

b) Culture medium:

Preparation of DMEM:

1. 17.7g package of DMEM was first mixed with 50ml of ddH_2O into a 2-liter glass beaker containing a micromagnetic stirrer bar. The water temperature was 15-20°C. The original package was rinsed out to remove all traces of powder inside with a small amount of ddH_2O. The solution was gently stirring until dissolved.

2. 3.7g sodium hydrogen carbonate was added and stirred until dissolved. Additional ddH_2O was added to bring the solution to 1000ml.
3. The pH of solution was adjusted to 7.3. 1N HCl or 1N NaOH were used to adjust the pH.
4. The solution was sterilized immediately by filtration using a membrane with porosity of 0.22μm and stored at 4^0C. Small amount of the sample medium was transferred to a 35mm culture dish extracted and placed in the CO_2 at 37^0C incubator for 24 hours. The media were used when the sample was confirmed free of contamination through microscopic examination.

Medium A: DMEM without L-valine, with L-glutamine, supplemented with 187mg/litre D-valine

Medium B: Medium A supplement with 0.1mg/ml L-valine, 0.33mg/ml L-glutamine, 2.5% of penicillin/streptomycin solution (v/v) and 10% (v/v) FCS

Medium C: F12 combined 1:1 with Medium A supplemented with 2.5% (v/v) Penicillin/streptomycin and 1% of 100x G5

All medium were adjusted to pH 7.6, filtered sterilize and stored at 4°C.

2.3.1.2 Procedure of tissue separation and cell culture

All experiments were carried out with the cultured rat cortical astrocytes. The cells

were prepared from the brains of newborn rats by the modified method of Marriott (1995).

1. 5μg/ml of poly-L-lysine solution was covered on the surface of the flask for 30min, which was dried under UV light for about 2 hours.
2. Infant rats (2-PND, 3-4 rats per dissection) were anesthetized by hypothermia via placing them in an ice bath for 2-4min. This is also a serve to cool the brain tissue. The skin of the head and neck are cleaned and disinfected with 75% alcohol prior to sacrifice. Animals are sacrificed by rapid decapitation with large surgical scissors at the level of foramen magnum.
3. Three cerebral cortices were used, they were and placed into 1 x 50 tube with 15ml of **solution B**. After that, the tissue was chopped twice, then the mixture was pipetted into the 15ml **solution B** and incubated at 37^0C with gentle agitation for 15min.
4. Equal volume (15ml) of **medium B** was added to the mixture to terminate the trypsinization. After spinned at 250g for 1min, the supernatant was discarded and the pellet was triturated in 5ml of **solution A**.
5. A further 10ml of **solution A** was added. In the latter experiments, cell fragments were gently and repetitively pipetted without foaming until there was no visible cell debris. The tissue was allowed to settle for about 10min. The supernatant (10ml) was collected to a new tube, then the tissue was triturated with another 10ml of **solution A**. After 10min, the supernatant was removed again to the collecting tube.
6. Step 5 was repeated one more time.

7. The final mixture was spun at 250g for 5min. The supernatant was aspirated and 10ml of **medium B** was added to the pellet. The pellet was gently dispersed and then resuspended completely by trituration.
8. The number of cells was counted with cell counting chamber and cell viability was determined with 0.2% trypan blue in PBS. The cells were plated in $75cm^2$-flasks and the average density was 2×10^5 cells/cm^3.
9. The plated cells were incubated in a 5% CO_2 incubator at 37°C. The culture medium was changed after three days. On the 4^{th} day the **medium B** was replaced with **medium C** and changed at 3 days intervals thereafter.
10 After 9-10 days of culture, the cells were prepared to subculture according to Bernhard et al (1992). Each $75cm^2$-flask was added 10ml of 0.05% **Solution D** and incubated for 7min at 37°C. And then the solution was decanted and 10ml of **medium B** was added to terminate the trypsinization. The cells were gently triturated, then counted and seeded at approximately $4x10^4$ cells/cm^2.in 35mm dish and cultured in CO_2 incubator at 37°C until the day of the use.

2.3.2 DETERMINATION OF GFAP IN ASTROCYTES

2.3.2.1 Materials

Astrocytes were cultured in 35mm dishes after 15 days.

Monoclonal antibody to GFAP (primary antibody):

The concentration of 5μg/ml was used.

Goat serum:

The serum with initial of protein concentration of 71.9mg/ml was diluted to 1:10.

Biotin conjugate goat anti-mouse IgG (secondary antibody):

The working dilution was 1:1000.

Avidin-peroxidase conjugate:

The working dilution was 1:1000, it was diluted in 0.01 PBS, pH 7.4, containing 0.05% Tween 20 and 0.5%BSA.

OPD:

0.04% OPD and 0.012% Hydrogen Peroxide (H_2O_2) were diluted in phosphate-citrate buffer, pH 5.0.

PBS (0.01 mol/L, pH 7.4) and 0.75% H_2O_2-PBS (30% H_2O_2 5ml + PBS 200ml)

2.3.2.2 Procedure

Immunohistochemical method determination of GFAP in culture astrocytes by Labeled Avidin-biotin.

1. The samples in culture dishes were fixed with acetone at -20°C for 10min. Excess acetone was removed by washing with PBS for 5min. The samples were fixed again in formalmaldehyde for 10min at room temperature (RT) then washed two times with PBS for 3min.
2. The dishes were dipped into 0.75%H_2O_2-PBS at 37°C for 30min then washed two times in PBS for 5min in each washing.

3. The dishes were added with normal goat serum at 37°C for 30min to reduce non-specific binding.
4. 0.5ml anti-GFAP solution was added to the dish and incubated in a humid chamber at 37°C for 1 hour. After that, the dishes were washed three times in PBS for 3min in each washing.
5. 0.5ml anti-mouse IgG-peroxidase solution dipped the dish and incubated for 1 hour at 37°C in a humid chamber then washed out as step4 described above.
6. The dishes were exposed to Avidin solution for 1 hour at 37°C and washed again as in step 4 described.
7. For staining, OPD was overlaid in the dish at RT until clearly visible redbrown coloration develops. Then, the dish was washed in ddH_2O for 5mim to stop the color of development.

8 Serum without secondary antibody was applied to one of dishes as a negative control.

9 Finally, the dishes were observed through microscope and photographed, then, embedded in 50% glycerin for store.

2.3.3 MTT ASSAY

2.3.3.1 Materials

MTT: 5mg/ml MTT was prepared by dissolving 50mg MTT in 10ml of 0.9% NaCl. It was incubated at 60°C until completely dissolved.

Triple solution: 5ml iso-Butyl Alcohol + 120μl HCl (36-38%) + 10g SDS+ 100ml ddH_2O

2.3.3.2 Procedure

1. After cultured for 10 days, astrocytes were subcultured and plated at all density of $2x10^4$ cells/cm^2 into a 96-well tray. The total volume of cell and medium was 90μl in each well. The medium was replaced with **medium C** after 12 hours.
2. 10μl per well of Fe-NTA at different concentration was added to each well after culture 15 days. The average of 3-wells in each of concentration (including controls - no cells and Fe-NTA) were utilized. The plate was incubated in 5%CO_2 incubator at 37^0C for 48 hours.
3. After that, 20μl MTT solution was added to each well and incubated for 4 hours.
4. 100μl Triple solution was added to each well and incubated at 37°C for 12-24 hours.
5. The absorbance was read at 578nm with a microplate Reader.

2.3.4 SDS-PAGE

2.3.4.1 Materials

Rat, human, duck and camel transferrins (rTf, hTf, dTf and cTf)

Standard protein marker

0.1% coamassie blue R-250

10% acetic acid

40% methanol

2.3.4.2 Procedure

1. SDS-PAGE (0.75 mm thick) was prepared according to the method of Laemmli (1970).
2. The samples were analyzed under reducing conditions. The power condition was 200 volts and the run time was approximately 45 min.
3. For staining, gel was fixed and stained with 0.1% coamassie blue R-250 in 40% (v/v) methanol and 10% acetic acid at RT for 30-40 min. The stained gel was destained with 40% methanol in 10% acetic acid for 1 to 3 hours until the background become clear.

2.3.5 PREPARATION OF RADIO-LABLING RAT TRANSFERRIN

2.3.5.1 Material

Rat apo-Tf solution:

4.9mg rat apo-Transferrin (rTf) solution in 0.245ml of 0.1M $NaHCO_3$ was saturated with 0.005ml of Fe-NTA at 4°C. Excess ions were removed by dialysis against ddH_2O overnight at 4^0C and the final concentration of iron-saturated rat rTf was measured by absorbance at 280nm. It was stored at -20°C.

Hanks' balanced salt solution (HBSS):

1.26mM $CaCl_2$+5.36mM KCl+0.44mM KH_2PO_4+0.49mM $MgCl_2.6H_20$+ 0.41mM $MgSO_4$ + 0.137M NaCl + 0.34mM $Na_2HPO_4.7H_2O$+15mM Hepes +1000ml ddH_2O

Iodo-gen:

0.5mg Iodo-gen was dissolved in 100ml dichloromethane. 0.5ml solution was added into a dry glass tube. The tube was dried with gas of nitrogen oxide gas. It was covered with black paper and stored at 4°C.

Sephadex G-50:

5g Sephadex G-50 was soaked in 50ml ddH_2O overnight. The gas babbles were removed by a vacuum pump.

0.5mCi[^{125}I]Na

2.3.5.2 Procedure

Modified Iodo-gen Method labeled iron-saturated Tf by Markwell MAK (1978) was used.

1. The column was pre-equilibrated with 20ml PBS (0.5M, pH7.4) buffer containing 1% BSA.
2. 50μg of Tf $(Fe)_2$ was dissolved in 0.5ml PBS buffer and it was incubated with 0.5mCi[^{125}I]Na in an Iodo-gen precoated tube for 9min at 4°C with gentle stirring.

3. The iodinated Tf was separated from the free iodine on a Sephadex G-50 column (1x50cm), which was equilibrated with PBS buffer containing 1% BSA.
4. The iodine-labeled rTf was eluted in the same buffer at a flow rate of 0.2-0.4ml/min. 70 fractions of each 0.7 ml were collected with Eppendorfs.
5. 2µl of aliquot was taken from each tube and the ^{125}I-radioactivity was measured with a γ-scintillation counter.
6. ^{125}I-Tf activity was precipitable with TCA. 1ml TCA (20%) and 1ml PBS containing 1%BSA were added into each tube (step 5) after counting. They were placed at 4°C for 30min. After that, they were centrifuged at 4100rcf for 30min. The supernatant and pellet were separated into new tubes, and the ^{125}I-radioactivities were measured.
7. An elution profile was drawn. The first peak of the labeled protein was collected. They were stored immediately, by first separating them into small aliquots and rapidly freeze them at -20°C.

2.3.6 DOUBLE LABELING OF TRANSFERRIN

2.3.6.1 ^{59}Fe radiolabeling of apo-rTf

a) Materials:

apo-rTf :

30mg apo-rTf was dissolved in 50µl PBS buffer (0.2M, pH7.6)

^{59}Fe-NTA complex:

100μCi $^{59}FeCl_3$ was transferred into a small glass tube containing a micromagnetic bar. Then, 10mM NTA was added. The mixture was mixed and with the cap screwed. The solution was stirred slowly at RT for 2 hours.

b). Procedure:

Modified Chloramine-T Method double labeling of Tf by Morgan E.H (1981) was used.

1. 50μl apo-rTf was added to the ^{59}Fe-NTA complex, excess the ^{59}Fe-NTA was leave at 4°C with constant stirring for 2 hours.
2. The solution was warmed to RT, then 0.12M HEPES was added to it.
3. The pH was adjusted to 7.8 by the addition of 5-10μl NaOH (0.5N).
4. 2mg $NaHCO_3$ was added to the mixture and incubated overnight with a constant stirring at 4°C.
5. The mixture was passed through a column of Sephades G-50 column the next day and eluted with 0.2M PBS.
6. Samples were collected at 0.2-0.4ml/min, for 70 tubes (1ml/tubes).
7. 2μl aliquot was taken from each tube and the ^{59}Fe-radioactivity was measured with a γ-scintillation counter and an elution profile was plotted. The first peak was collected and total volume was measured. Protein concentration was determined by TCA preciptate method.

2.3.6.2 Radioiodination of ^{59}Fe-Tf

a). Materials:

Iodine-125: 1mCi

Buffer A: 0.25M Na Phosphate Buffer pH 7.4

Buffer B: 0.05M Na Phosphate Buffer pH 7.4

Buffer C: 0.01M Na Phosphate Buffer containing 1M NaCl, 0.1%BSA and 1%KI, pH 7.4 (KI may not be added)

Buffer D: 0.05M Na Phosphate Buffer containing 0.15M NaCl & 0.1% BSA, pH 7.4.

Chloramine-T solution:

1mg Chloramine-T was dissolved in 10ml **buffer B**

Na metabisulphite solution:

2mg Na Metabisulphite was dissolved in 10ml **buffer C.**

b). Procedure:

1. Tf-Fe and 0.5mCi Iodine-125 were pipetted into a tube. After that, 50µl **buffer A** was added.
2. Then 20µl chloramine-T was added, mixed and took time. The mix solution was allowed to stand at RT for 1min for radioiodination.
3. 2µl Na Metabisulphite solution to stop the radoiodination.

4. The mixture was applied to a small column (10 x 25 mm) of Sephadax G-50 and eluted with **buffer D**.
5. Fractions of 0.7 ml were collected with Eppendorfs until 70 tubes.
6. 2μl of aliquot was taken from each tube and the radioactivity was measured with a γ-scintillation counter. The results were used to draw an elution profile. The first peak of the labeled protein was collected. Protein was determined by TCA precipitate method.
7. The sample was separated into small aliquots and rapidly freezesd at -20°C.

2.3.7 PREPARATION OF TRANSFERRIN-FREE IRON ($^{59}Fe^{2+}$) SOLUTION

2.3.7.1 Materials

$^{59}FeCl_3$: 0.366mM in 0.1M HCl

$^{56}FeSO_4 .7H_2O$: 15mg + 0.1N HCl 25ml

0.32M Sucrose: 21.907g socrose +0.27704g pipes + ddH_2O 200ml

2-mercaptoethanol

2.3.7.2 Procedure

The method was used according to refereed to Morgan's study on reticulocytes in 1988.

1. The stock iron solution was prepared by mixing with $^{59}FeCl_3$ (21.6μg/ml in 0.1 M HCl) with $^{56}FeSO_4$ (2mM in 0.1M HCl) a molar ratio of 1:10.
2. 50-fold molar excess of 2-mercaptoethanol and 0.32M sucrose was added to give an iron concentration of 62.5μM.
3. Varying amounts of this solution were then added to cell culture dishes to give iron concentrations in the incubation mixture of 0.05 to 5.0μM.
4. A concentration of 1.0μM was used except where indicated.
5. The iron in the above solution was called Fe^{2+} throughout this paper. It was prepared immediately before use and was used within 1 week.

2.3.8 CHARACTERIZATION OF TRANSFERRIN RECEPTOR

TfRs of the cultured astrocytes were identified by a modified method of I^{125}-Tf binding assay according to Karin and Minta (1981).

1. After 15 days cultures, the cells were checked for confluence and only the completely confluent plates were subsequently used for the assay. Sample plates were examined to determine the cell density using a haemocytometer and 0.2% trypan blue exclusion.
2. Astrocytes were carefully washed in media free of FCS two times, then incubated with this medium (DMEM/F12) for 3 hours at 37^0C. After that washed

three times with cold HBSS (pH7.4). This treatment aimed to deplete endogenous protein.

3. The ^{125}I-rTf was diluted to the required concentration with 1% BSA in HBSS, pH7.4.
4. In 35mm^2-dish was added 1ml solution, it contained 0.2ml of HBSS (containing 0.5%BSA), 0.4ml of HBSS, 0.2ml of different concentrations of unlabeled-Tf and 0.2ml of ^{125}I-rTf (10^5cpm).
5. The mixture was incubated at 4°C for 10hours.
6. After incubation, the cells were washed three times with 2ml of cold 0.1% BSA-HBSS, the time for washing being usually less than 40 sec.
7. The cells were detached with 1N NaOH and transferred into new γ-counting tubes. The radioactivity of each tube was measured with γ-scintillation counter.

2.3.9 MEASUREMENT OF TRANSFERRIN-BOUND IRON UPTAKE

1. Astrocyte cultures of 15-days age was used for the Fe uptake assay. Sample plates were examined for the determination of cell density by using a haemocytometer and trypan blue exclusion.
2. The culture medium was decanted and the cells were carefully washed with HBSS (pH 7.4) two times. 2 ml of new medium without serum (DMEM/F12) was added to each dish. The cells were incubated with this medium for 3 hours at 37°C, and then washed three times with cold HBSS (pH7.4). This treatment

aimed to deplete endogenous protein. (Chemicals used for achieving the objectives of this project was added at this stage and pre-incubated with the cells for a certain time).

3. Tf-^{59}Fe was added to the dishes, which were incubated in water bath at 37°C for the required time.
4. The dishes were placed on ice. The medium was decanted and the dishes were washed with ice-cold HBSS for 3 times.
5. 2ml of pronase (1mg/ml in HBSS) was added in each dish. The dishes were incubated on ice for 60 min.
6. After that, the solution of each dish was transferred to a new counting tube. And the mixture was centrifuged at 250 x g for 10 min at 4°C.
7. The supernatant was transferred to a new counting tube and for the measurement of the radioactivity on the cell membrane. While the pellet was used for the measurement of the radioactivity within the cells. The γ-scintillation counter was used to measure the radioactivity in each case.

2.3.10 MEASUREMENT OF TRANSFERRIN-FREE IRON UPTAKE

The uptake of transferrin-free iron $^{59}Fe^{2+}$ was determined in most of the experiments that were carried out in this study for comparison with the uptake of transferrin-bound iron.

1. Astrocyte cultures of 15-days age was used for the iron uptake assay. Sample plates were examined for the determination of cell density by using a haemocytometer and trypan blue exclusion.
2. The culture medium was decanted and the cells were carefully washed with HBSS (pH 7.4) two times. 2 ml of new medium without serum (DMEM/F12) was added to each dish. The cells were incubated with this medium for 3 hours at 37°C, and then washed three times with cold HBSS (pH7.4). This treatment aimed to deplete endogenous protein.
3. $^{59}Fe^{2+}$ (0.32M sucrose buffered at pH 6.5 with 20mM Hepes) was added to the dishes, which were incubated in water bath at 37°C for the required time.
4. The dishes were placed on ice. The medium was decanted and the dishes were washed with ice-cold HBSS for 3 times.
5. 2ml of pronase (1mg/ml in HBSS) was added in each dish. The dishes were incubated on ice for 60 min.
6. After that, the solution of each dish was transferred to a new counting tube. And the mixture was centrifuged at 250 x g for 10 min at 4°C.
7. The supernatant was transferred to a new counting tube and for the measurement of the radioactivity on the cell membrane. While the pellet was used for the measurement of the radioactivity within the cells. The γ-scintillation counter was used to measure the radioactivity in each case

2.4 ANALYTICAL METHODS

2.4.1 ASTROCYTES COUNT

Astrocytes in DMEM/F12 medium were stain with 0.2% trypan blue. 20μl of stain were added to 20μl medium, then mixed it. The Survival cells have not containing blue granules of cellular cytoplasm when examined by microscopy was counted.

2.4.2 SCATCHARD AND HILL ANALYSIS

The curve by unlabeled rat-Tf specifically inhibition was transformed to Scatcard plot by the method of Rosenthal (1967). This plot under equilibrium conditions calculated the number of receptor sites per cell and the average receptor affinity of Tf. The number of binding sites computed from the abscissa intercept and the affinity of the receptors for labeled ligand is determined from the slope of lines (Ka). The dissociation constant (Kd) of the receptors is a reciproal of Ka.

In order to reveal whether there is a cooperation of receptor for the binding of ligand. Hill plot was used in providing evidence for the presence of multiple biding sites. The date of rat-Tf competition curve was transformed to Hill plot by the method of Hill (1910). The Hill coefficient was determined from the slop of the line.

2.4.3 STATISTICS

Statistical analyses were performed using an Excel computer and associated software. The means, standard errors, linear regressions and slops were calculated according to standard equations. The student's t-test was used to compare the results obtained with different groups of experimental data. Differences were considered statistically significant when the probability, p, was less than 0.05.

Chapter 3

The Effect of Iron on Rat Cortical Astrocytes in Culture

3.1 Abstract

This study was designed to investigate the effects of iron on the development of brain astrocytes in culture by the addition of ferric nitrilotriacetate (Fe-NTA) at varying concentrations to culture medium. Apart from morphologic studies, MTT assay was performed to determine the cell number. The result demonstrated that high concentration of chelated ferric iron had an adverse effect on astrocytes. Significant decrease of astrocyte population was found at concentration of 20μM to 5000μM, p values being smaller than 0.05, 0.01, or 0.001 respectively. The effects of Fe-NTA were both concentration and duration dependent. However, no significant difference of morphology was found between 5μM and 10μM groups and the controls. In contrast, when a low iron concentration (5μM) was added to dishes, the culture could be kept in normal condition for three weeks. Results also showed that the cultured astrocytes were more resistant to high concentration of chelated iron exposure than the cultured cerebellar granule cells. Therefore, these cultured brain astrocytes could be utilized as an experimental model for investigating the mechanisms of iron transport across the membrane of brain glial cells. Where the

concentration and incubation time are less that 5μM and 3 weeks respectively.

Key Word: Fe-NTA (ferric nitrilotriacetate); MTT assay
Concentration and incubation time; Morphologic observation
Experimental model

3.2 INTRODUCTION

Iron is the most abundant trace metal in the brain. The concentration of iron in the brain is region dependent (Hill et al. 1984). The amount of iron in the globus pallidus and SN was found to be enriched, with a concentration equivalent to that in the liver (Koeppen 1995). The importance of iron for normal neurological function has been well established. As in all cells, neurons require iron for many aspects of their physiology, including electron transport, myelination of axons and as a cofactor for many enzymes involved in neurotransmitter synthesis (Connor and Benkovic 1992a, Loeffler et al.1995). On the other hand, iron overload is toxic to brain tissues. During the past years, studies have demonstrated that there are abnormally high levels of iron and oxidative stress in neurodegenerative disorders such as PD, AD, HSS, etc (Swaiman 1991, Connor et al. 1995, Gorell et al. 1995, van Rensburg et al.1995). Oxidative stress resulting from an increased iron level, and possibly also from defects in antioxidant defense mechanisms, is widely believed to be the cause of neuronal death in some neurodegenerative diseases (Gerlach et al. 1994, Schipper

1996, Owen et al. 1996, Qian et al.1997). However, it is not yet understood why abnormal iron levels are found in some regions of the brain in these diseases (Qian and Wang 1998).

To date, very little is known about the regulation of iron concentration in the brain and mechanisms of iron uptake by different types of brain cells. The understanding of these aspects is critical for elucidating the causes of excess iron accumulation in the brain in neurodegenerative diseases. We intended to devote our efforts to these aspects, to utilize cultured astrocytes as an experimental model to study the mechanism of brain cellular iron uptake, but little information is available about the relationship between iron and this type of glia in culture.

It is well known that astrocytes are the most numerous cellular element of brain. They outnumber neurons by ten to one and occupy about one-third of the volume in the cerebral cortex. As their name implies, astrocytes are generally process-bearing cells. A characteristic feature of all astrocytes is the enormous surface area owing to the extensive branching of the cellular processes; this process formation results in astrocytes being structurally highly complex. Astrocytic processes contact the surfaces of nerve cell bodies and axons. It has become apparent that astrocytes are involved in many activities that are critical to brain function including neuronal migration, neurite outgrowth, synaptogenesis and synaptic plasticity; maintenance of the BBB; regulation of water, ion and amino acid of neurons; nodulation of immune/inflammatory responses; and phagocytic functions (Michael and Norenberg 1994, Schipper 1996). In addition, specific enzyme systems enable astrocytes to

metabolize ammonia, glutamate, free radicals, xenobiotics, and metals, contribute in protecting the brain from the toxicity of these agents (Fedoroff and Vernadakis 1986, Norenberg et al. 1988).

Cultured astrocytes are easily accessible for experimental manipulation and can now be routinely prepared with greater than 98% homogeneity. It has been widely used in neurochemical and pharmaceutical studies. The advantage of using this type of primary culture to study the iron uptake mechanism is its homogeneity. In the present experiment, we observed the effect of iron (in chelated form) on development of the astrocytes in vitro. The information obtained will be useful for determining whether the experimental system, containing the cultured astrocytes and iron, is valid for investigating mechanisms of iron uptake by glial cell.

3.3 MATERIALS AND METHODS

3.3.1 MATERIALS

The animal house at the Hong Kong Polytechnic University supplied SD rats 2-PND.Three rats were used in each preparation. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company, St. Louis, MO. USA. Heat inactivated FCS, DMEM and G-5 were from Gibco BRL. All solutions were prepared with ddH_2O, which was sterilized by passing through a 0.22μm Millipore. Fe-NTA was prepared freshly on the day of use.

3.3.2 CELL CULTURE OF ASTROCYTES

Please refer chapter 2

3.3.3 DETERMINATION OF GFAP IN ASTROCYTES

Please refer chapter 2

3.3.4 EFFECT OF Fe-NTA ON ASTROCYTES DEVELOPMENT

Astrocytes cultures of 10 days

↓

Subculture and seed cells (4 x 10^4/ml) in 200μl DMEM/FCS with 96-well plate

(culture 24hours)

↓

Change the medium with DMEM/G5

(culture 4 days)

↓

Add 90μl DMEM/G5 and 10μl Fe-NTA at different concentration in each well

(culture 2 days)

↓ (observe and photograph the cells)

↓

Add 20μl MTT solution

(culture 4 hours)

↓

Add triple solution

(overnight)

↓

Measure absorbance at 578nm

3.4 RESULTS

3.4.1 Development of Morphology on Astrocytes under Normal Conditions

The developmental characteristics of astrocyte morphology of astrocytes were observed (Fig.3). At the time plating, both phase-bright and phase-dark cells, about 6μm in diameter were seen as well separated spheres spread about the dishes (Fig.3A). Although floating cells and debris were still seen, most cells attached to the culture dish within 24 hours. Some cells appeared relatively flattened. The population of phase-bright cells was disproportionately decreased. On the 3rd day of culture (Fig.3B), the medium was changed with DMEM/G5. This medium was

developed for optimal growth and differentiation of astrocytes. By 5th day, cells of stellate, boomerang-shaped, or spindle-shaped were predominating. The cultures were still heterogeneous at this stage (Fig.3C). By 9th day (Fig.3D), the cells had very rapid proliferation and were attached to an underlying layer of confluent, flat, background cells. At this time, the cells were sub-cultured, no neuronal cells were observed. Fig.3E and Fig.3F illustrate the expansive process formation of rat cortical astrocytes in G-5 medium, which is significantly greater than in serum-containing medium where they have a fibroblast-like morphology. Astrocytes of characteristic morphology in G-5 medium were prominent; they exhibit multiple highly branched processes, which are formation of neural network. If packed cells in dish were not sub-cultured, it would begin to congregate and network begun to become sparse.

3.4.2 CHARACTERIZING ASTROCYTES IN CULTURE

Astrocytes after cultured for 15 days were immunostained with glial fibrillary acid protein (GFAP) antibody. In general, the presence of GFAP and the absence of other neural cell type-specific antigens best identify astrocytes. Purified astrocytes were stained with GFAP antibody after 15 days in vitro (DIV) (Fig.4). It is observed that GFAP staining (red-brown) was located in cytoplasm of cells. Fig.4A showed that about 95% cells were GFAP positive in a non-specific background, which suggests they were astrocytes. The same staining pattern remains unchanged in the control cells (Fig.4B), which were stained in absence of GFAP antibody.

3.4.3 Effect of Fe-NTA on Astrocytes Development in Vitro

Different concentrations of Fe-NTA were added to the cultures on the 15th days of plating. The dishes were incubated in 5% CO_2 incubator at 37°C for another 7 days. The morphological criteria considered are: outgrowth and spreading of cell processes, formation of processes network, formation of cell clusters, necrosis and detachment of the cells. All these parameters were followed for the evaluation of the effect of Fe-NTA concentration in time, using as controls cultures kept in DMEM/G5. There were no significant morphological changes in a short period (<5 days). However, cultures had apparent morphological changes according to Fe-NTA concentrations with time, while the cells displayed congregation and the network became sparse. When Fe-NTA is present in medium on 7th day, the cells began to show a tendency to form small clusters at Fe-NTA concentration of 20μM (Fig.5D). Apparent congregation and formation of cell clusters were seen at Fe-NTA concentration of 40μM (Fig.5E). Significant morphological change was observed at Fe-NTA concentration of 100μM (Fig.5F). The cells could not survive at Fe-NTA concentration of 200μM at this time point and at concentration of 100μM after 10 days incubation. There was no significant deviation of morphology from control dish in concentration of 5μM and 10μM. In contrast, with a low iron concentration (5μM), the cultures could be kept in normal condition for three weeks. Fig.6, 7 show that chronic exposure of the cultured cells to Fe-NTA (5μM and 40μM) may accelerate the procedure of somata migration and congregation, but toxic effect only

visible at a later date. After 20 days, the cells began to congregate and gradually decreased in number at the concentration of 5μM, almost all of them died when cultured more than 30 days. At 40μM Fe-NTA, some of the cells detached from walls and a few of the cells can survive up to the 20^{th} days.

3.4.4 MTT ASSAY DURING Fe-NTA INCUBATION

It can be seen from Figure 5E, 5F, 6c, 7a, 7b, 7c, 8a, 8b and 8c that the processes of the astrocytes are damaged mostly by the addition of Fe-NTA. MTT method was used for the determination of the number of cells. Fig.9-1 showed that in low iron concentration (<5μM), the cultures could be kept in normal condition. On the second day of culture, the MTT content of cultures with addition of 5μM Fe-NTA was increased to 113.7%. Although the growth promotion was tend to increase with increase of iron concentration, but reveals no statistical differences ($y=0.0094x + 1.0653$, $r=0.368375$). In 4 days culture, the degree of promotion was clearly concentration-related ($p<0.05$). The plot was drawn by use of linear regression ($y=0.0313x + 1.1608$), $r=0.949$, $p<0.001$) (Fig.9-1). Astrocyte population of cultures with higher iron concentration (20μM) was clearly decreased compared with controls (Fig.9-2). The MTT content of cultures with addition of 20μM was 0.94 ± 0.029 (mean $\pm$ SD), and in control cultures was 1.001 ± 0.012 ($p<0.05$). Significant decrease of astrocyte population was found at concentration of 20μM to 5000μM ($p<0.05$, 0.01, 0.001). Fig.9-2 showed an S-shape curve of relationship between the degree of inhibition and log iron concentration.

3.5 DISCUSSION

Myocardial and liver cells are among the most susceptible cells to iron (Cox et al. 1981, Williams et al. 1982). Chang cells (cultured human hepatocytes) loaded with Fe-NTA (161μM) became iron-loaded with an increase in ferritin content and total iron content (Jacobs et al. 1978). It is noteworthy that the exposure of Chang cells to high Fe-NTA concentrations caused rapid cell death. When these cells were in a slowly proliferating stage they did not survive longer than 5.5 weeks and often manifested toxic lesions within 3 weeks in medium containing 161μM Fe-NTA (Williams et al. 1982). Previous studies by our group have demonstrated that the addition of Fe-NTA at high concentrations (≥10μM) had an adverse effect on cerebellar granule cell cultures. The cerebellar granule cells cannot survive in the medium containing only 40μM Fe-NTA (60% neuronal loss) after 7 days of exposure (Pu and Qian 1998). Result obtained from this study show that astrocytes cannot survive longer than 5 days in the medium containing 200μM Fe-NTA and only a few of cells can survive up to 20 days in 40μM. After 20 days, culture containing 5μM Fe-NTA concentration appeared to have toxic lesions. It is suggested that astrocytes were more resistant to the Fe-NTA than the general population of neuronal cells. On the 4^{th} day cultures with 5μM Fe-NTA, MTT is significantly increased when compared to control ($P<0.001$). It shows that some of the cells may have proliferated. The mean age of the cells in the relatively static cultures of control is higher than that of the actively growing cultures. The cell death

in this culture occurs within 30 days. It seems likely that the newly proliferated cells are subjected to an irreversible toxic lesion. Thus, the toxic effect mainly affects cell growth at the early stage. The addition of iron changed the cellular behavior of astrocytes in vitro. The cells congregate much earlier than normal cells. It is suggested that the cell death could be accelerated by iron.

Most likely the differences in toxic iron effect are directly related to the presence of the chelating agent which served to reduce the concentration of free ferric iron and promote the formation of ferritin as was found in Chang cell, thus averting some of the oxidizing activity of the ferric iron (Jacobs et al. 1978, Swaiman and Machen 1989). Ferric iron is toxic to cells because of its oxidative potential. Swaiman et al.(1985) and Foucaud et al.(1987) had used non-chelated iron to study the effects of ferric iron concentration on cultured glial cells. They had demonstrated that TfRs are found on oligodendrocytes and that there are toxic effects after 7 days in 2.5μM concentrations of iron in oligodendrocytes. In contrast, relatively little toxic effect is found in this study, unless Fe-NTA concentration increase above 20μM. It may be suggested that the susceptibility of astrocytes to iron toxicity depends on chelated factors of iron.

Although the general pathological features of iron overload had been well described and the tissue changes occurring in brain had been well documented, there are still questions regarding the precise mechanism of iron toxicity remain unanswered. Most accounts of iron toxicity point directly or indirectly to the formation of free radical with increased lipid peroxidation and consequent membrane damage. A commonly

accepted explanation for the oxyradical-mediated neurotoxicity is the iron-catalysed Haber-Weiss reaction in which $O^{-}_{2}\bullet$ in the presence of reduced ferric iron attacks H_2O_2 to form $^{\bullet}OH$:

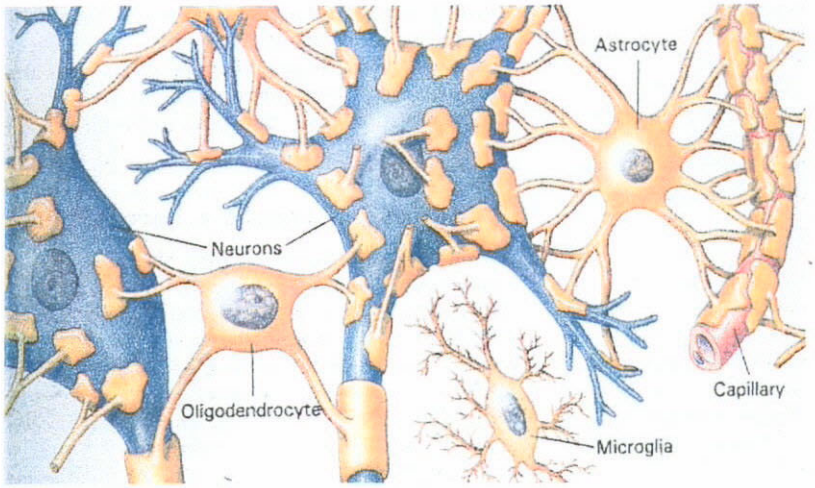
$$Fe^{3+} + O^{-}_{2}\bullet \Rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

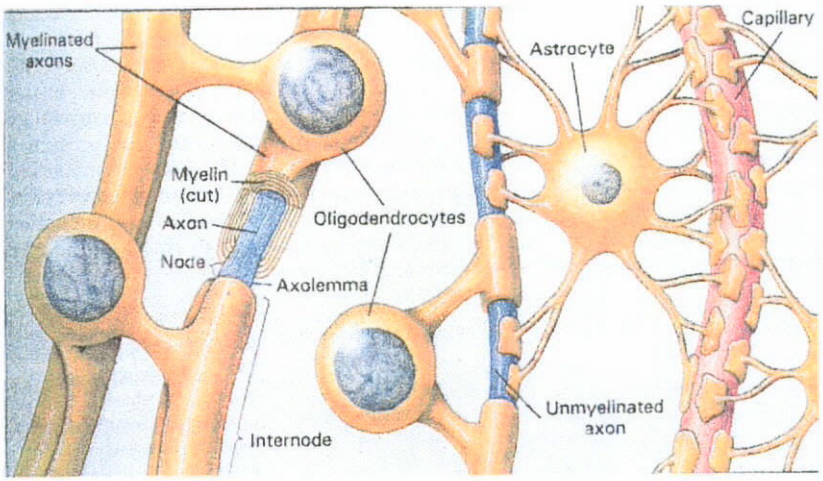
Peroxidation causes destruction of sulphydryl groups in a variety of compounds, mitochondrial membrane damage and the disruption of metabolic pathways. However, it is generally agreed that most brain iron is bound to proteins such as ferritin so that it cannot participate in redox reactions. Ferritin seems to be one of the most important components protecting the brain from iron-induced oxidative damage. A number of studies have demonstrated iron release from ferritin through several reductive mechanisms. The reducing agents including superoxide, glutathion, cysteine, ascorbate and quinones that have the ability to reductively mobilize iron stored in ferritin (Boyer et al. 1988, Monteiro et al. 1989). Consequently, understanding the effect of non-chelated iron on development of astrocytes and the relationship between the iron-loaded and free radicals are of great value for further study.

In summary, the data obtained from this study show that the addition of chelated ferric ion at high concentrations (≥20μM) has an adverse effect on the cultured astrocytes, while a lower concentration of iron (<5 μM) maintains the cultured cells

in a normal condition. Therefore this cultured astrocytes could be used to study the mechanisms of iron transport across the membrane of brain astrocytes when the concentration of chelated ferric iron used is less than 5μM.



(a) Gray matter



(b) White matter

Fig. 2 Histology of Neural Tissue in the CNS. (a) A diagrammatic view of gray matter, showing relationship between major glial elements and nerve cell bodies. (b) A comparable view of view of white matter, showing relationships between glial cell and axons.

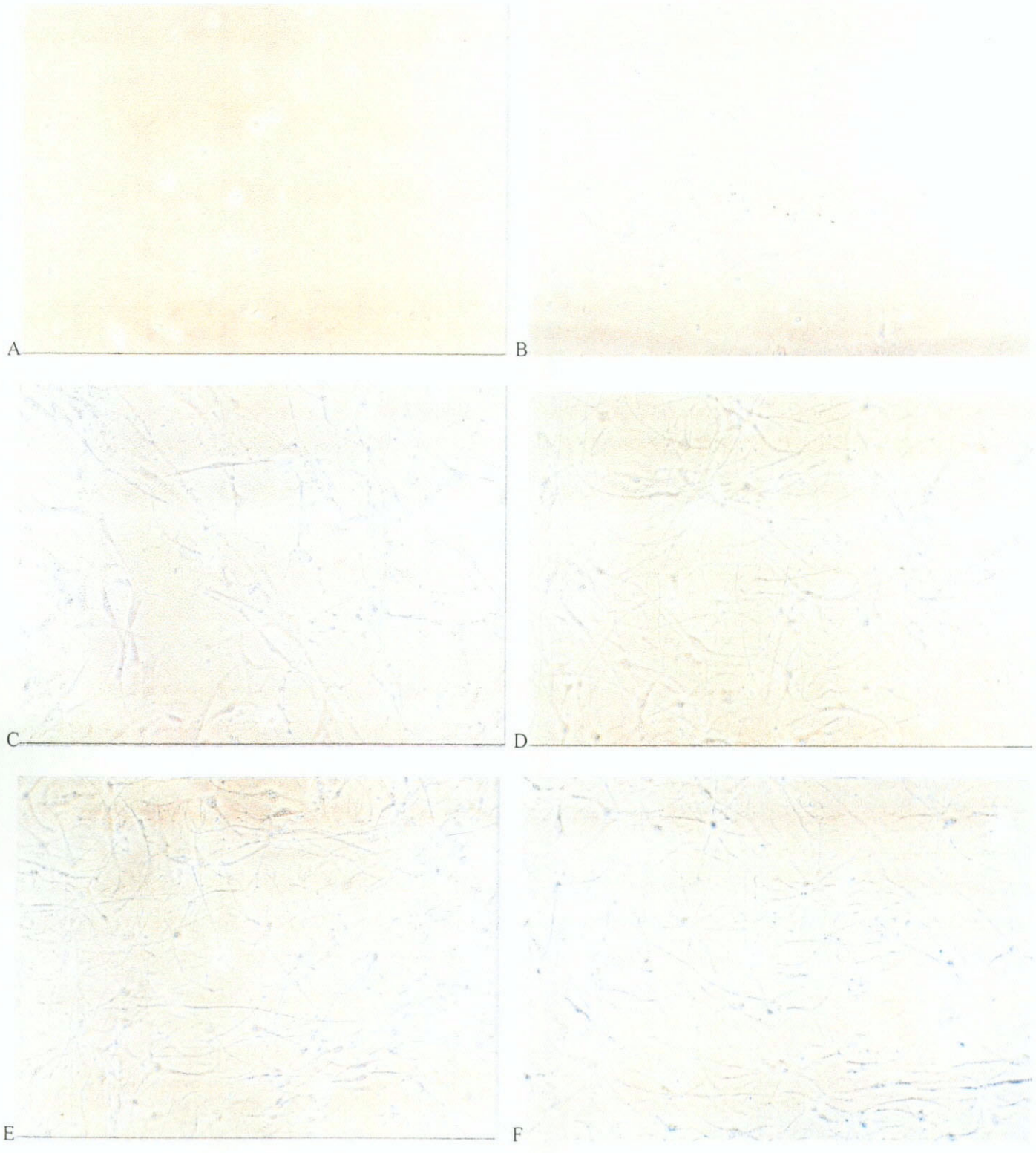


Fig. 3 Typical stages of astrocytes growing in CO_2 incubator at 37°C. The cells were observed through a phase-contrast microscope. (A) 0 DIV cells; (B) 3 DIV cells; (C) 5 DIV cells; (D) 9 DIV cells; (E) 11 DIV cells and (F) 15 DIV cells (DIV: days in vitro).

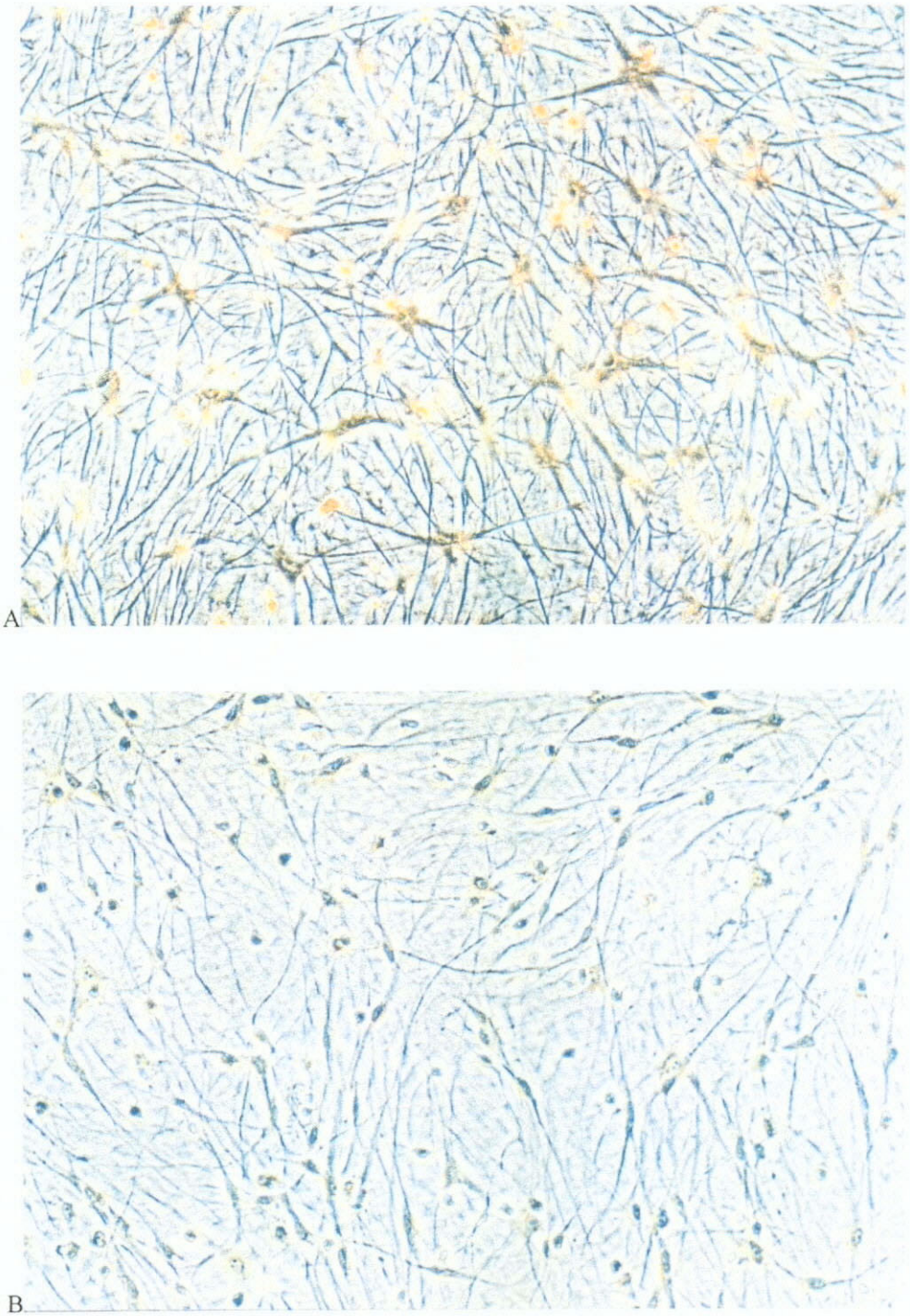


Fig. 4 Immunocytochemical labeling of astrocytes enriched cultures with a monoclonal antibody to GFAP after 15th days culture. (A) GFAP-positive cells (red-brown); (B) Control cells (stained in absence of antibody GFAP).

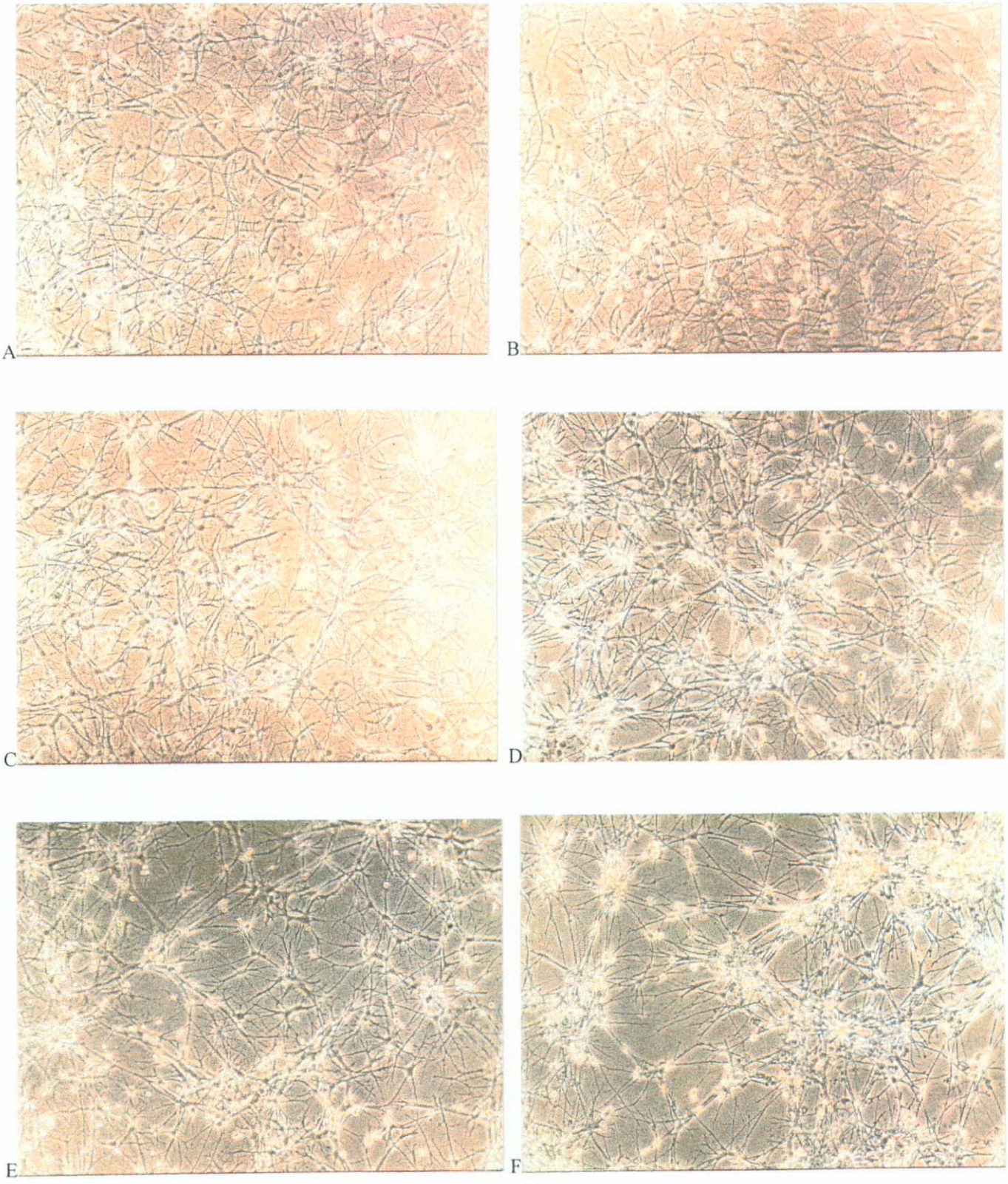


Fig. 5 Effect of Fe-NTA at different concentrations on astrocytes development. Fe-NTA was added to 15-DIV cultures. Then the cultures were incubated with Fe-NTA for 7 days in CO_2 incubator. Cells were observed through a phase-contrast microscope. (A) Control; (B) 5μM Fe-NTA; (C) 10μM Fe-NTA (D) 20μM Fe-NTA; (E) 40μM Fe-NTA; (F) 100μM Fe-NTA

Fig. 6 Time course of Fe-NTA (5μM) on development of astrocytes in vitro. The cells were incubated with or without Fe-NTA for certain days and then were observed through a phase-contrast microscope. Control: (A) 25 DIV cells; (B) 30 DIV cells; (C) 35 DIV cells. Experimental (incubation with 5μM Fe-NTA): (a) 10^{th} days; (b) 15^{th} days; (c) 20^{th} days.

Fig. 7 Time course of Fe-NTA (40μM) on development of astrocytes in vitro. The cells were incubated with or without Fe-NTA for certain days and then were observed through a phase-contrast microscope. Control: (A) 25 DIV cells; (B) 30 DIV cells; (C) 35 DIV cells. Experimental (incubation with 40μM Fe-NTA): (a) 10^{th} days; (b) 15^{th} days; (c) 20^{th} days.

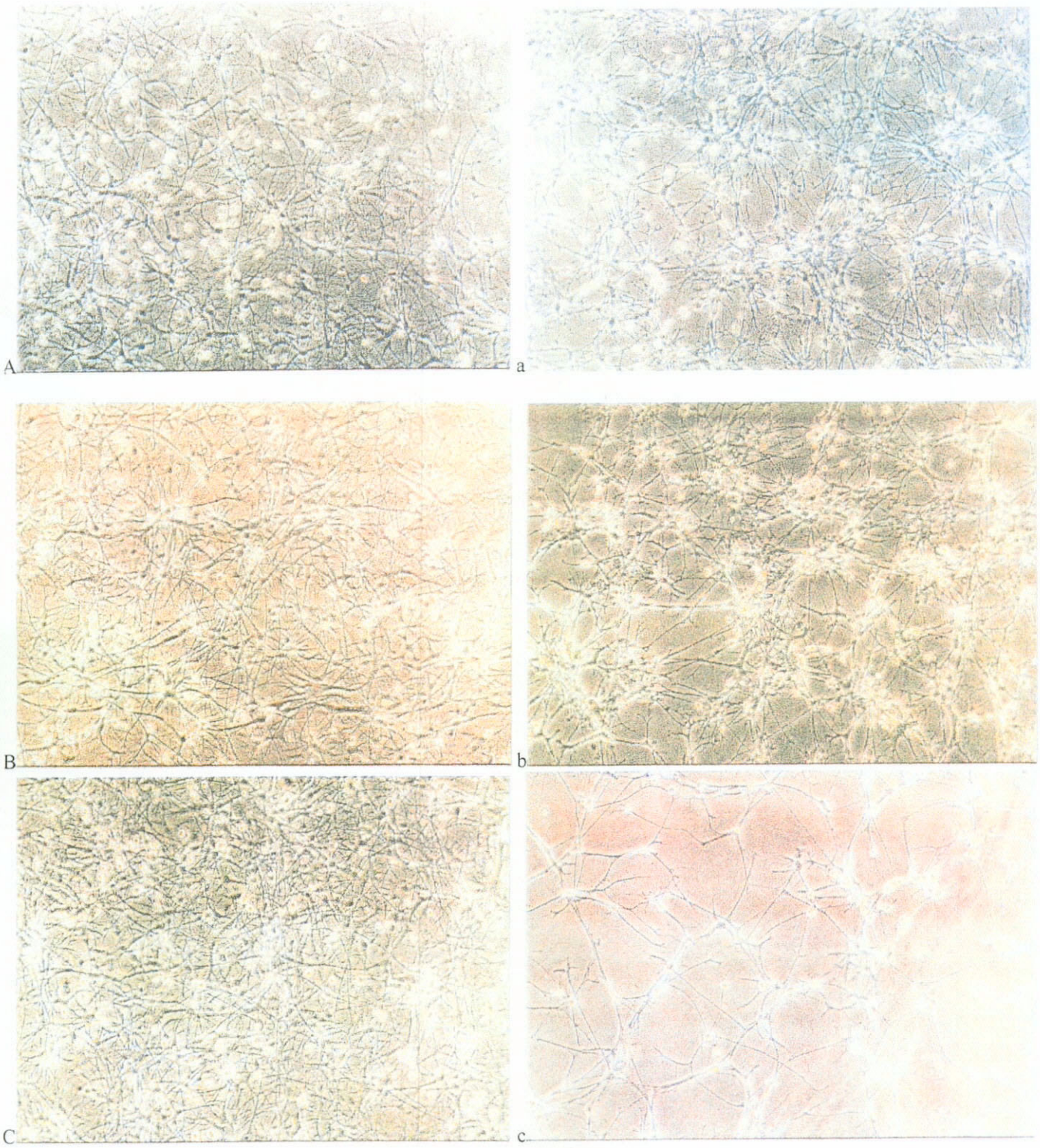


Fig.8 Time course of Fe-NTA (100μM) on development of astrocytes in vitro. The cells were incubated with or without Fe-NTA for certain days and then were observed through a phase-contrast microscope. Control: (A) 18 DIV cells; (B) 20 DIV cells; (C) 23 DIV cells. Experimental: (a) 18 DIV cells after 3 days of incubation with Fe-NTA; (b) 20 DIV cells after 5 days of incubation with Fe-NTA; (c) 23 DIV cells after 8 days of incubation with Fe-NTA.

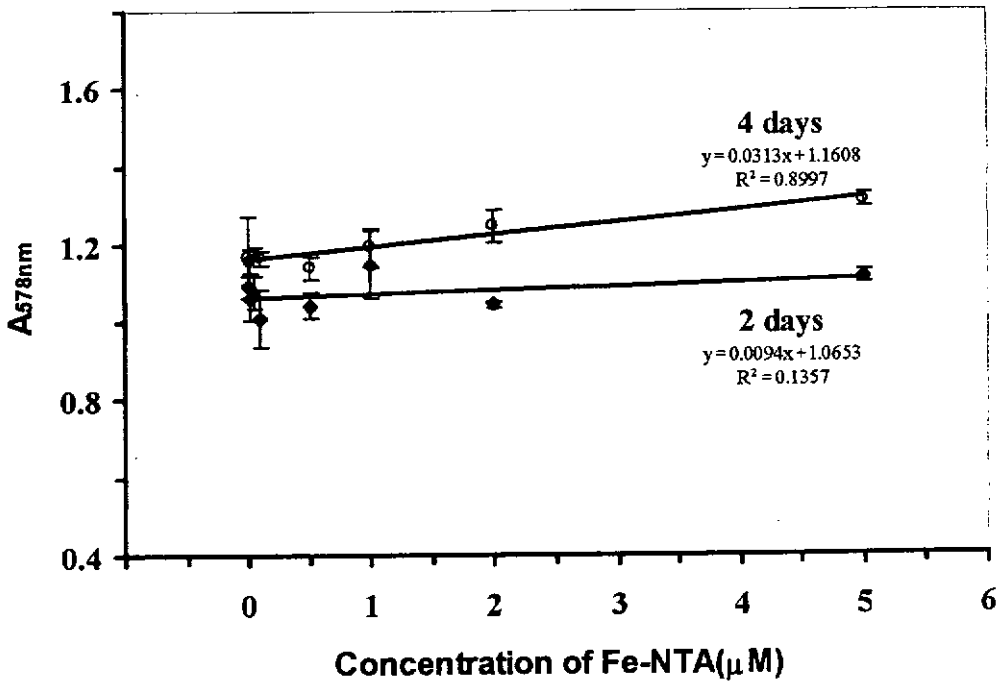


Fig.9-1 Effect of Fe-NTA on astrocytes in vitro. Add Fe-NTA at different concentrations (<5μM) to 15DIV astrocytes cultures. Incubate the cultures with the iron for 2 days and 4 days, then determine the count of cell with MTT assays.

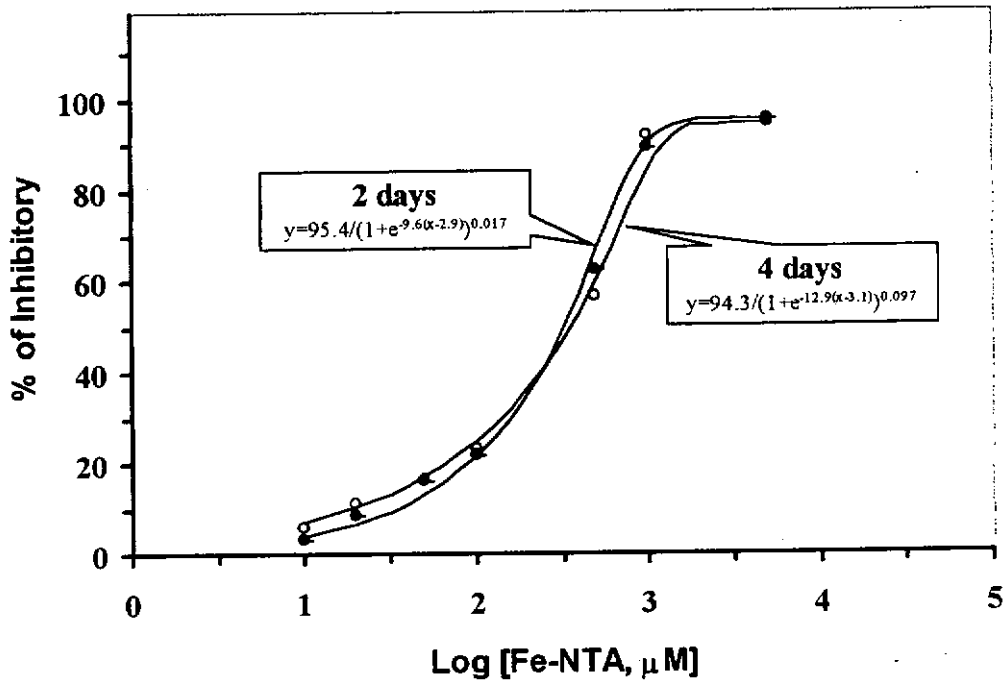


Fig.9-2 Effect of Fe-NTA on astrocytes in vitro. Add Fe-NTA at different concentrations (>5μM) to 15DIV astrocytes cultures. Incubate the cultures with the iron for 2 days and 4 days, then determine the count of cell with MTT assays.

Chapter 4

TRANSFERRIN RECEPTOR ON THE MEMBRANE OF THE CULTURED ASTROCYTES OF RATS

4.1 ABSTRACT

TfR is a transmembrane glycoprotein that consists of two disulfide-linked subunits of 95 kDa. This protein has been found on a variety of mammalian cells. In the brain, it is generally accepted that this receptor is located in endothelial cells, whereas its existence in other brain cell types is poorly established. In this study, the binding of iron saturated ^{125}I-Tf to the cultured rat brain astrocytes was investigated at pH 7.4 and 4°C. The results demonstrated that cortical astrocytes in suspension bound ^{125}I-Tf by a saturable and specific manner, indicating the existence of receptors for Tf on the membrane of the cells. Scatchard and Hill plot analysis showed that the dissociation constant (Kd) of the binding was about 3.45×10^{-8} M and the number of receptor were about 7.09×10^{4}/ cell. The receptor was specific for rat and human Tf. The binding of ^{125}I-rat Tf could be competitively and specifically inhibited by unlabeled iron-saturated rat and human Tf and no difference was found between interaction of rat and human Tf with this

receptor. Whereas the interaction of duck or camel Tf with this receptor was very weak. The Hill coefficient was 0.9877, almost no different from 1, indicating the absence of cooperativity. It suggested that ^{125}I-Tf bound to a single class site on the membrane of astrocytes.

Key Word: Cultured cortical astrocytes; SD rats; Transferrin; Transferrin Receptor; Specific and saturable binding

4.2 INTRODUCTION

TfR is a transmembrane glucoprotein whose expression can determine intracellular iron level. This glycoprotein has been found on a variety of mammalian cells. In these cells, the TfR-mediated endocytosis has been widely considered to be the main route for mammalian cell iron accumulation under physiological conditions. Tf-bound iron, a major transport form of iron in blood and extracellular fluid, binds to this glycoprotein on the cellular membrane, subsequently, the complex of iron-Tf-TfR is internalized to form endosome. Acidification within the endosome leads to dissociation of iron from Tf. Then iron crosses the endosomal membrane to enter into cytosol probably by a membrane iron carrier-mediated process, while the endosome containing apo-Tf and TfR is recycled to the cell surface (Qian et al. 1995, De Silva et al. 1996, Qian 1997).

In the brain, autoradiographic and immunohistochemical studies on the distribution of the TfR have demonstrated much regional variation of this receptor (Hill et al. 1985, Connor and Fine 1986, Lin and Connor 1989, Giometto et al 1990, Mash et al. 1990, Connor et al. 1990, Connor et al. 1995). It is now generally accepted that TfR is located in endothelial cells of brain capillaries (Jeffries et al. 1984, Connor and Menzies 1995) where it is involved in mediating iron delivery to the CNS (Fishman et al. 1987, Pardridge et al. 1987). Whereas the existence of this receptor in the other types of brain cells is less well established. Whether there is TfR in the brain astrocytes is still not well determined. The reported results of relevant studies are inconsistent. Lin and Connor (1989) investigated that the development of the Tf-TfR system in relation to astrocytes in normal and myelin-deficient rat optic nerves. They did not find the expression of TfR on the astrocytes and microglia. In an analysis of the cellular distribution of the TfR (and iron, Tf and the ferritin subunits) in selected regions of the normal human brain and human brain from AD patients obtained at autopsy, Connor and Menzies (1995) observed that TfR is abundantly expressed on blood vessels, large neurons in the cortex, striatum, hippocampus, and oligodendrocytes and is also present on astrocytes. However, recently Moos (1996) reported that TfR immunoreactivity was not detected in astrocytes and other two types of glial cells, oligodendrocytes and microglial cells in the adult mouse (male, the NMRI strain aged 8 weeks) CNS by immunohistochemistry using a monoclonal antibody raised against the TfR. Factors leading to the inconsistency of findings may due to

the difference in species, age and experimental techniques and conditions used. It is because of this reason that it is very difficult to reach a final conclusion on the presence of TfR on brain astrocytes based on the studies mentioned above. Therefore further investigation is needed. In present study, the cultured rat cortical astrocytes and ^{125}I-Tf binding assay were used to determine whether there is receptor for Tf on the membrane of this type of brain cell.

4.3 MATERIALS AND METHODS

4.3.1 MATERIALS

The animal house of The Hong Kong Polytechnic University supplied SD rats of two postnatal days. Chemicals were all purchased form Sigma chemical Co. Heat inactivated FCS, DMEM/F12 and G5 were purchased form Gibco BRL. ^{125}I-Na was purchased from Amersham International, Amersham, UK. Duck and camel Tf were kindly provided by National Key Laboratory of Molecular Biology, Shanghai Institute of Biochemistry, Academia Sinica.

4.3.2 ASTROCYTES PREPARATION

Please refer Chapter 2.

4.3.3 IRON SATURATION AND IODINATION OF TRANSFERRIN

Please refer Chapter 2.

4.3.4 ^{125}I-TRANSFERRIN BINDING ASSAY

The following procedures were used to identify TfR of astrocytes:

Astrocytes cultures of 15 days

(Determine the count of astrocytes by staining with 0.2% trypan blue)

↓

Decant the culture medium

Wash the cells with HBSS (pH 7.4) for 2 times

↓

Incubated with serum-free culture medium (DMEM/F12) for 3hours

(This treatment aimed to deplete endogenous protein)

↓

Decant the culture medium and wash the cells with HBSS again

↓

Add 1ml solution

(0.2ml unlabeled Tf + 0.2ml 0.5% BSA-HBSS + 0.4ml HBSS + 0.2ml ^{125}I-Tf)

↓

Incubate the dishes at 4°C for 10hours

↓

Decant the solution and wash the cells with 0.1% cold BSA-HBSS for 3 times

↓

Add 1ml NaOH (1N) to each dish at RT for 15min

↓

Collect the solution containing the cells of each dish into new γ-counting tubes

↓

Measure the radioactivity

4.4 RESULTS

4.4.1 SDS-PAGE PATTERN OF DIFFERENT TRANSFERRIN

Various animals Tf (rat, human, duck and camel) were used to competita ^{125}I-Tf in the receptor-binding assay. To confirm the purity of the Tf from the commercial and self-prepared sources, SDS-PAGE was carried out under reducing condition (Laemmli 1970). Fig.10 shows each type of Tf gave a single band of apparent molecular weight about 80 kDa. Thus, the Tf are pure.

4.4.2 SPECIFICITY OF ^{125}I-TRANSFERRIN BINDING

To assess the binding of ^{125}I-Tf to astrocytes, the cultured astrocytes (2.35×10^6/dish) were incubated with ^{125}I-rat Tf (10^5 cpm) at 4°C for 10 hours. The various amounts of unlabeled iron saturated rat, human, duck and camel Tf were added respectively. The binding (cpm) and % of binding were calculated as follows:

Binding (cpm) = cpm (sample) - cpm(non-specific adsorption)

$$\% \text{ of binding} = \frac{\text{cpm (sample) - cpm(non-specific adsorption)}}{\text{cpm (blank) - cpm (non-specific adsorption)}} 100\%$$

Fig. 11 showed the different interaction of these proteins with astrocytes. Binding of ^{125}I labeled rat Tf to the receptor could be specifically and competitively inhibited by unlabeled iron saturated rat and human Tf, but not by unlabeled duck and camel Tf that showed a very small inhibitory effect on this binding. There was no difference between IC_{50} (the concentration of inhibitor, unlabeled Tf, that gives 50% inhibition of the labeled rat Tf binding) of rat and human Tf. The IC_{50} of unlabeled camel and duck Tf was approximately 1.83% and 0.17% of that of rat Tf. It suggested that the binding of I^{125}-Tf to its receptor is specific and saturable.

4.4.3 QUANTITY OF THE TRANSFERRIN RECEPTOR ON ASTROCYTES

The number of TfRs on the membrane of the cultured astrocytes and their affinity for ^{125}I-Tf were determined by using the method of Scatchard and Hill analysis. The incubation was carried out at 4° C, a temperature at which only receptor binding occurs. The inhibitory curve of ^{125}I-Tf binding by unlabeled rat Tf was transformed to Scatchard plot according to Rosenthal (1967). The number of TfRs per cell and the receptor affinity were calculated by the plot under equilibrium conditions. Scatchard plot analysis (Fig. 12) showed that the number of binding sites on the membrane of astrocytes, computed from the abscissa intercept (2.35×10^6 cells in assay), is 7.09×10^4/cells with 280 fM bound (B_{max}). The affinity of the receptors for labeled Tf is determined from the slope of line (Ka). The dissociation constant (Kd) of the receptors is a reciprocal of Ka. The Kd value is 3.45×10^{-8} M. To determine whether there was a cooperation of the receptor for the binding of Tf, the data of rat Tf competition curve were transformed to Hill plot (Fig. 13) by the method described previously (Hill 1910). The Hill coefficient is 0.9877, approximately equal 1, indicating the absence of cooperativity and suggesting that Tf bound to a single class site on the membrane of astrocytes.

4.5 DISCUSSION

TfR density is high in rapidly growing cells and the expression of TfR is regulated by intracellular iron in some cell systems (Testa et al. 1989, Theil 1990). The TfR has been well characterized on a number of peripheral cell types in direct binding studies using radiolabeled Tf (Steiner 1980, Newman et al. 1982). The present study provides the first evidence for the presence of TfR in the cultured rat brain astrocytes (SD rats, 2-PND and 15 days culture) using cell culture method and ^{125}I-Tf binding assay.

In the initial attempts to use astrocytes to study and characterize TfR, the medium containing FCS were decanted and the cells were washed three times with HBSS buffer. After this, the competitive inhibition experiment was performed. No specific ^{125}I-transferrin binding was observed under this condition. This was considered that endogenous Tf presents and binds with TfR, the procedure inhibited the binding experiment. Thus, in order to deplete the endogenous Tf, the cells were incubated for periods up 3-4 hr at 37^{O}C in DMEM serum-free medium before the binding experiment. Our experimental system employed in this study would not contain endogenous Tf, it was valid in studying the competitive inhibition of the binding of ^{125}I-labeled Tf on rat astrocyte. In order to understand the specificity of the receptor, various animal sources of Tf including rat, human, duck and camel were used as the ligands in these binding studies. Fig.11 shows the different interactions of various animal sources of Tf with astrocytes. There

was no difference between IC_{50} of rat and human Tf. The IC_{50} of unlabeled camel and duck Tf was approximately 1.83% and 0.17% of that of rat-Tf. A possible explanation for this observation is that the differences between the species merely reflect heterogeneity in Tf. Then, there is no species difference between rTf and hTf as we had observed here and the results are consistent with previous studies on culture cell. Since it was shown that Tf was essential to cell growth, the role of Tf and its receptor in cell function and proliferation has been thoroughly investigated (Bomford and Munro 1985). In most of the rat cell cultures, hTf was an available supplement in the serum-free medium. It suggested that the binding of ^{125}I-Tf to its receptor is specific and. The Scatchard curve gives a straight line, which is fitted to a single class binding site model. This is consistent with the Hill plot that the Hill coefficient was 0.9877, approximately equal 1, indicating the absence of cooperativity. This suggests the Tf is attaching to a single class site on the astrocyte membrane, which is also consistent with other studies of Tf in different types of cell.

This result suggested that brain astrocytes might have the ability to acquire iron from extracellular fluid by a TfR-mediated process as found in other mammalian cells. However, the contribution of this receptor in astrocytes for maintaining the intracerebral iron homeostasis is unknown. The expression of TfR in other cells has been demonstrated to be affected by age, animal strain and many other factors. Therefore it is necessary to have further investigation on the changes in expression of this protein in this type of brain cells in different culture stage and different age in rat and other animals. This approach would be more useful in

determining the biological significance and physiological importance of this receptor expression on the membrane of astrocytes in brain metabolism. It is well known that astrocytes comprise a major class of neuroglia and perform a wide range of adaptive functions in the mammalian nervous system. It is also generally accepted that these cells are important in the maintenance of the BBB as well as the iron homeostasis (Schipper 1996). It has been reported that astrocytes are able to accumulate iron under abnormal circumstances (the mylelin deficient rats) (Connor and Menzies 1990). Although the mechanism of iron accumulation they suggested is phagocytosis not TfR-mediated transport, it is possible for astrocytes to play a role in brain iron balances based on their results and findings reported. Accumulated evidences have shown that disruption in expression of brain iron transport proteins including TfR and other proteins such as LfR, P97 and CP is probably one of the causes of the altered brain iron metabolism (Qian and Wang 1998). Therefore, it is extremely important to make a detailed examination on the distribution of these proteins including TfR in different types of brain cells. The knowledge of this aspect is important for us to understand not only the mechanisms of brain iron homeostatic control but also the cause of altered brain iron found in neurodegenerative disorders.

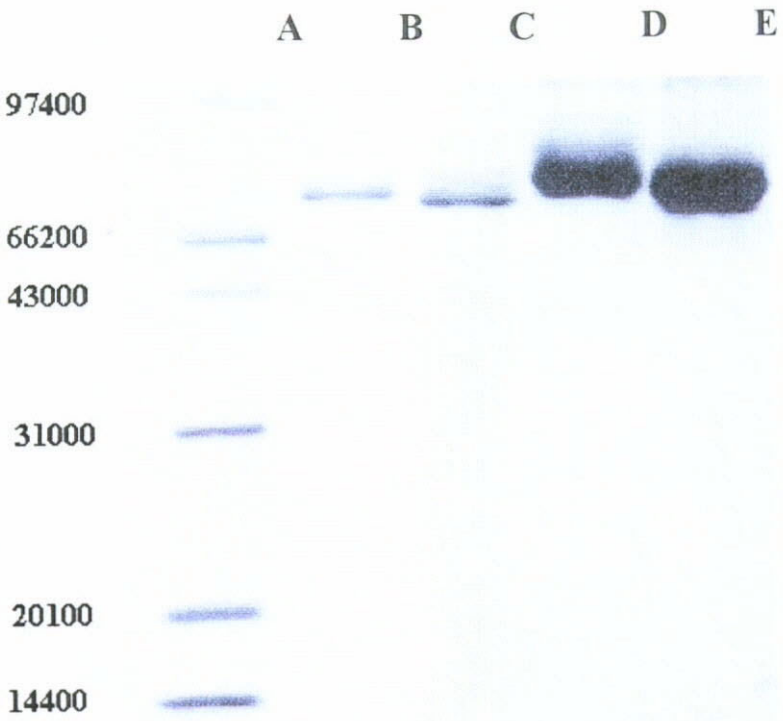


Fig. 10 SDS-PAGE pattern of different transferrins. Lane **A**, standard protein (pharmacia, Low Molecular Weight Calibration Kit), **B**, cTf, **C**, dTf, **D**, hTf and **E**, rTf. Lane A from top to bottom: Rabbit phosphorylaseb (97400), Bocine Serum Albumin (66200), Rabbit Actin (43000), Bovine Carbonic Anhydrase (31000), Trypsin Inhibitor (20100), Hen Egg white Lysozyme (14400). The gel was stained with coomassiebrilliant blue R-250.

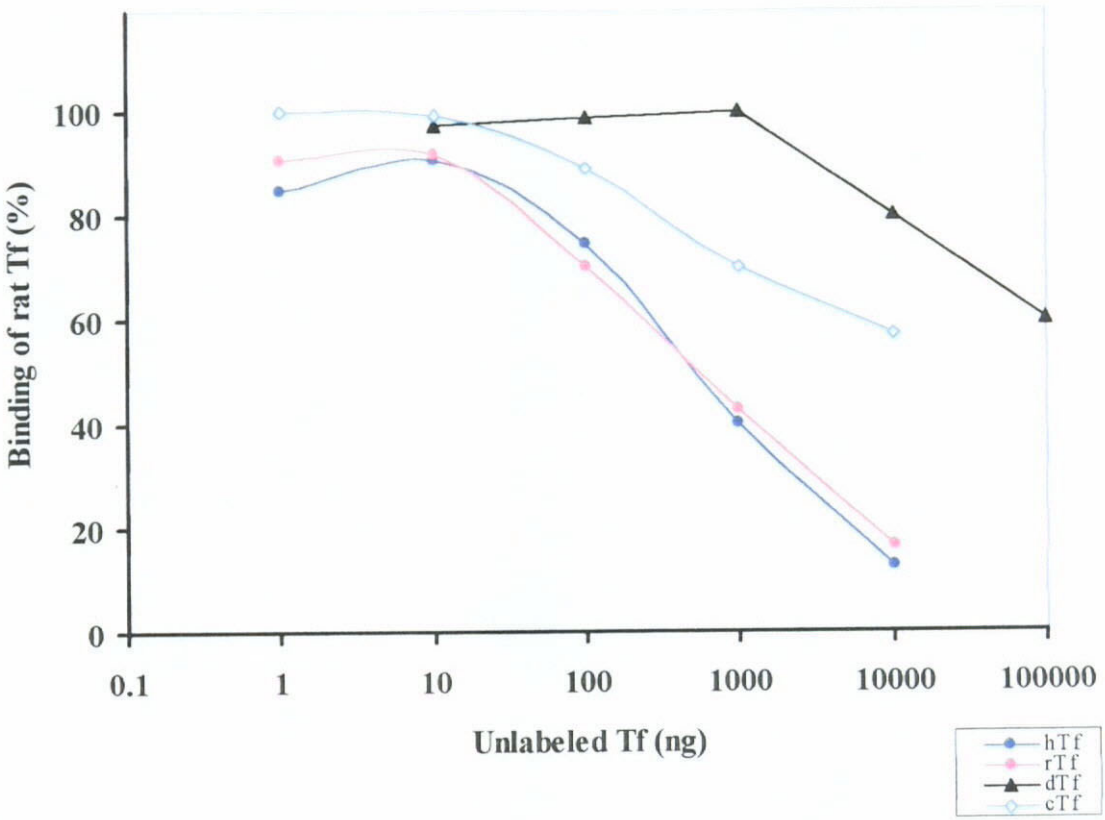


Fig. 11. Effect of different concentration of unlabeled iron saturated transferrin of rat (●), human (●), duck (▲) and camel ( ) on the binding of ^{125}I-Tf to the receptor on the membrane of cultured rat brain astrocytes. All data points are averages of five times deteminations.

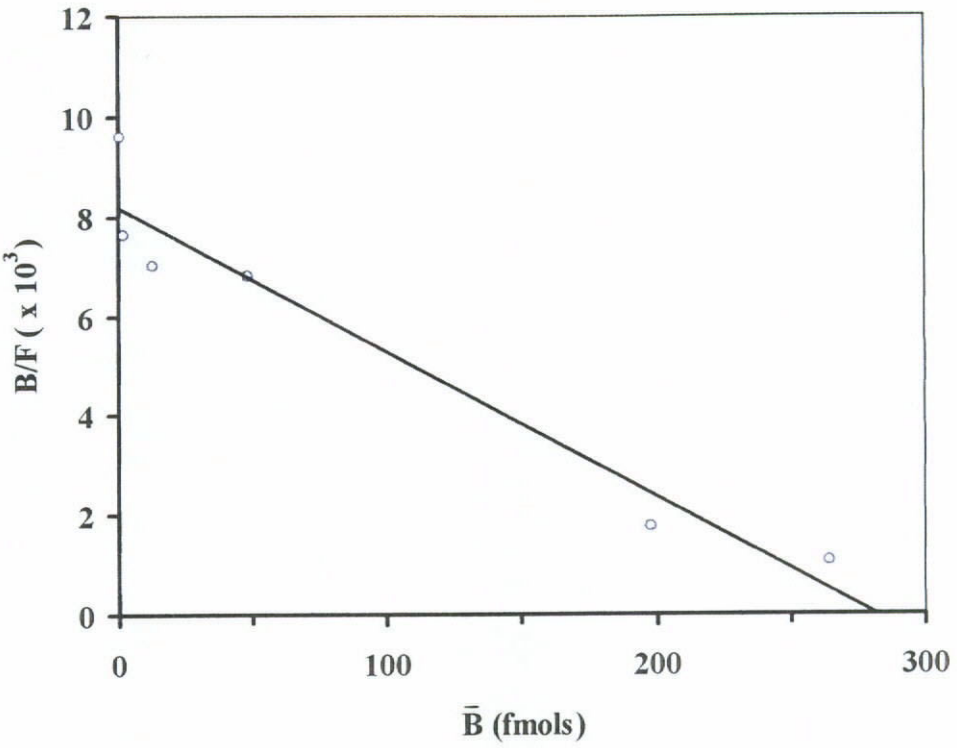


Fig. 12. The data of rat transferrin competition curve was transformed to Scatchard Plot

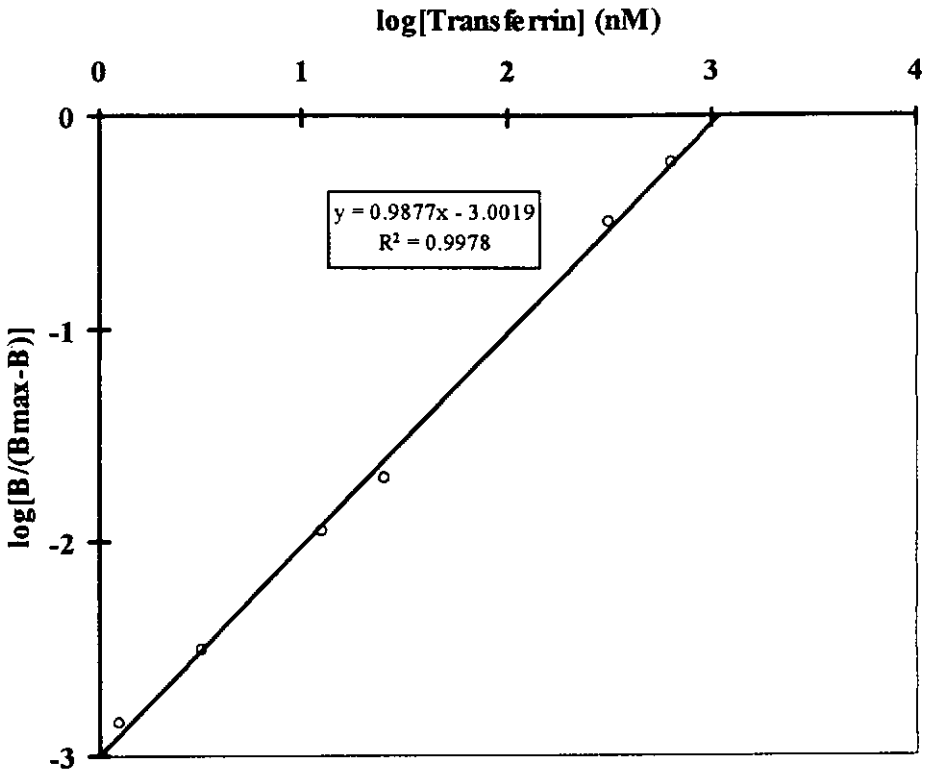


Fig. 13. The data of rat transferrin competition curve was transformed to Hill polt.

Chapter 5

TRANSFERRIN-BOUND IRON UPTAKE BY THE CULTURED BRAIN ASTROCYTES OF RATS

5.1 ABSTRACT

The aim of the experiments described in this chapter was to investigate some aspects of mechanisms of Tf-bound iron uptake by the cultured brain astrocytes in rats. After 15 days of culture, the astrocytes were exposed to 1μM of double-labeled Tf (^{125}I-Tf-^{59}Fe) at 37° C or 4° C for varying time. The cellular Tf-bound iron (Tf-Fe) and Tf uptake were analyzed by measuring the intracellular radioactivity with γ-counter. The results showed that Tf-Fe uptake kept increasing in a linear manner up to at least 30 min. In contrast to Tf-Fe uptake, the internalization of Tf into the cells was most rapid during the first 10 mins and then slowed to a plateau level, indicating that transferrin internalization into the cells is a saturable process. Both Tf and Tf-Fe uptake were temperature-dependent. The uptake in 37° C was significantly higher than that in 4° C. The addition of either methylamine (CH_3NH_2) or ammonium chloride (NH_4Cl), both are blockers of Tf-Fe uptake via inhibiting iron release from Tf within endosomes, significantly decreased the cellular Tf-Fe uptake but had

no significant effect on Tf internalization. The pretreatment of cells with trypsin significantly inhibited the cellular Tf-Fe and Tf uptake. These results suggested that transferrin-bound iron transport across the membrane of astrocytes was TfR-mediated process and the pattern generally parallels characteristics of uptake in other mammalian cells.

Key Words: Rat brain astrocytes; Tf-bound iron uptake; Transferrin internalization CH_3NH_2 and NH_4Cl

5.2 INTRODUCTION

In 1963, Jandl and Katz first reported the role of the membrane TfR in the Tf-bound iron uptake by reticulocytes. Since then, considerable research effort has been devoted to study the mechanisms of Tf and TfR-mediated iron uptake. To date, it is generally believed that the TfR-mediated endocytosis is the main route of iron uptake by mammalian cells under physiological conditions. This process can be distinguished into seven main steps. These include (1) Binding of transferrin (Tf and iron complex) to its receptor on the cellular membrane; (2) Internalization of Fe-Tf-TfR complex and the formation of endosome or vesicle; (3) Acidification of microvironment within endosome; (4) Dissociation of iron from Tf and reduction of Fe^{3+} to Fe^{2+}, (5) Translocation of iron from endosome to cytosol (transport across the membrane of endosome); (6) Cytosolic transfer of iron into intracellular

compounds such as heme and ferritin; and (7) Endosome containing the complex of Tf and TfR returns to cellular membrane.

Almost all of these steps of this process have been widely investigated in many types of non-brain cells such as reticulocytes, hepatocytes, intestine cells, Hela cells, and K562 cells, etc. (Morgan et al. 1981, 1986, 1988, Qian et al. 1990, 1991, 1992, 1995, Stranhan et al. 1992). However, only a few studies were conducted to investigate the role of Tf and TfR-mediated iron uptake system in brain cells during the past years. Information is very limited on mechanisms of iron transport across the membrane of different types of brain cells and the role of this pathway in iron transport between the brain cells. Although the existence of this receptor has been demonstrated in brain neuronal cells, oligodendrocytes and brain capillary endothelium, cellular distribution of TfR in the brain is still not fully examined. It is unknown whether this receptor exists on the membrane of other brain cell types such as microglia and astrocytes. It is also not well determined what are the effects of age and growth on the expression of this receptor in brain cells that have this receptor. Based on the current knowledge, it has been suggested that Tf-bound iron is endocytosed into brain capillary endothelial cells by TfR on luminal surface. Iron is separated from Tf within the cells presumably by the general mechanism of acidification within the endosome as mentioned above, and then might be transport alone, perhaps as Fe^{2+}, across the abluminal membrane of the endothelium into brain interstitial fluid. Within brain interstitial fluid, iron will bind with any unsaturated Tf synthesized

either in choroid plexuses or in oligodendrocytes. Then Tf-bound iron will bind to its receptor on the membrane of neuronal cell or other brain cell types that have TfR, and finally endocytosed. However, many fundamental questions concerning this process have not been answered. Firstly, there are uncertainties regarding to the role of endogenous Tf in brain extracellular iron transport as well as the transport form of iron (Fe^{2+}) across the abluminal membrane of the endothelium. Secondly, it is unknown of what are the transport forms of iron in CSF and brain interstitial fluid and why there is uneven distribution of iron in the different areas of the brain. Thirdly, almost all steps of this process need to be confirmed by further investigation. Obviously, there is a long way to go before we achieve total understanding of mechanism of iron transport in the brain.

Because of the well-determined importance of TfR in iron accumulation and cellular iron balance in extra-brain cells, it is extremely important to pay special attention to investigating the role of this receptor in brain iron transport, especially in iron uptake by different brain cell types in addition to the role of TfR in the process of iron transport across the BBB. About ten years ago, Swaiman and Machen investigated the process of Tf-bound iron uptake by cultured mammalian cortical neurons and glial cells respectively (Swaiman and Machen 1984, 1985,1986). Their studies demonstrated that Tf-Fe uptake by these cells was rapid and linear over 4 hours, and could be inhibited by lysosomtropic agents, such as NH_4Cl, CH_3NH_2 and chloroquine. They suggested that the mechanism of Tf-bound iron uptake by brain neurons and glial cells is similar to that of other tissues and is a receptor-mediated

process. However, the glial cells they used have not been specified. Therefore, it is still not determined whether Tf-bound iron uptake or Tf internalization is mediated by TfR in brain astrocytes or other glial cell types. The experiments described in Chapter 4 provides the first time evidence for the presence of TfR in the cultured rat brain astrocytes. Therefore, it is possible for this receptor to play a role in Tf-bound iron uptake by astrocytes as well. The works described in this Chapter designed to investigate this possibility.

5.3 MATERIALS AND METHODS

5.3.1 MATERIALS

All chemicals, except specially mentioned, were purchased from Sigma Chemical Co., St. Louis, MO. USA. Heat inactivated FCS, DMEM/F12 and G-5 were obtained from Gibco BRL. ^{59}FeCl (in 0.1M HCl, 0.1mCi/ml) and Na^{125}I (1mCi/ml) were purchased from the Radiochemical Center, Amersham. The animal house at the Hong Kong Polytechnic University supplied SD rats of 2-PND.

5.3.2 CELL CULTURE OF ASTROCYTES

Please refer to Chapter 2.

5.3.3 DOUBLE LABELING OF TRANSFERRIN WITH ^{125}I AND ^{59}Fe

Please refer to Chapter 2

5.3.4 MEASUREMENT OF TRANSFERRIN-BOUND IRON UPTAKE

Tf-bound iron uptake by astrocytes was determined by the following procedure:

Astrocytes culture (15 days)

(Determine the cell count of astrocytes by staining with 0.2% trypan blue)

↓

Decant the culture medium

(Wash the cell with HBSS (pH 7.4) for 2 times)

↓

Incubate with 2ml serum-free culture medium (DMEM/F12) for 3 hours

(This treatment aimed to deplete endogenous protein)

↓

Decant the culture medium and wash the cell with HBSS again

↓ (Add chemicals required here

↓ and pre-incubated the cells for

↓ certain min)

↓

Add ^{125}I-Tf-^{59}Fe to each dish with final iron concentration of 1μM

Incubate the dish in water bath at 37° C for desired time

↓

Decant the medium and wash the cells with ice-cold HBSS for 3 times

↓

Add 2ml pronase (1mg/ml in HBSS) to each dish

(To digest the cells as well as remove the bounding ^{125}I-Tf-^{59}Fe

on from the membrane)

Incubate the dishes on ice for 60min

↓

Collect the solution containing the cells of each dish into new counting tubes

Centrifuge at 150xg for 10min at 4°C

↓

Transfer the supernatant into new counting tube

↓

Measure the radioactivity of the supernatant (^{125}I-Tf-^{59}Fe on the membrane)

and the pellet (^{125}I-Tf-^{59}Fe Fe within the cell)

5.4 RESULTS

5.4.1 EFFECT OF TEMPERATURE ON TRANSFERRIN AND TRANSFERRIN-BOUND IRON UPTAKE BY ASTROCYTES

The internalization of Tf and Tf-Fe uptake were determined by incubating the 15 DIV astrocytes with 1 μM of double labeled Tf at 37° C or 4° C. The samples were taken out to measure the radioactivity within the cells at certain minutes of intervals. The results showed that both Tf and Tf-bound iron were taken up by the cultured astrocytes in a temperature-dependent manner (Fig.14). The uptake of Tf and Tf-bound iron by the cultured cells was significantly higher at 37° C than at 4° C. Very little progressive uptake of either the protein or its binding iron occurred during incubation at 4° C for 30 min. However, at 37° C, Tf-Fe uptake by the cells increased progressively with the incubation time throughout 30 min of incubation period (Fig.14A). The rate of Tf-Fe uptake was approximately 9.1481 fmol/10^3cell/min ($y=9.1481x+9.8011$, $r=0.9957$). The internalization of Tf into the cells was rapid initially but slowed to a plateau level after 10 min (Fig.14B). The plateau level implied that the rate of Tf endocytosis and exocytosis were equal this time point. The rate of Tf internalization was 6.312 fmol/10^3 cells/min at the first 10 min.

5.4.2 EFFECT OF CH_3NH_2 AND NH_4Cl ON TRANSFERRIN-BOUND IRON UPTAKE BY ASTROCYTES

NH_4Cl and CH_3NH_2, known to block the process of Tf-dependent iron uptake via inhibiting the release of iron from Tf within the endosome, were used to study the role of Tf in iron accumulation by the cultured astrocytes. Astrocytes culture of 15th day were exposed to CH_3NH_2 (10μM) or NH_4Cl (10μM) at 37° C for 15 min before the addition of ^{125}I-Tf-^{59}Fe (final iron concentration of 1μM) into the incubation medium. The intracellular radioactivity was measured after certain time of incubation as described above. The result showed that both CH_3NH_2 and NH_4Cl could inhibit the total iron uptake by the cells over the 30 min-incubation period. After 10 min of incubation, the differences between the experimental and control values at all time points (10, 20, 30 min) were statistically significant ($p<0.05$ or 0.001). The degree of inhibition was clearly concentration-related and both substances were associated with similar degrees of inhibition. Fig.15 showed that the degree of inhibition of total iron uptake was linear ($y1 = 5.5771x + 13.772$, $r1 = 0.9968$; $y2 = 4.3706x + 17.162$, $r2 = 0.9891$). However, it is noteworthy that in the pre-treatment of the cells with CH_4Cl and CH_3NH_2 did not result in a significant change in Tf uptake by the cells. These results suggested that iron in this study internalized by the cultured astrocytes was Tf-bound iron form. The failure of iron

releasing from Tf, induced by the addition of these two chemicals, resulted in the decreased iron uptake

5.4.3 EFFECT OF TRYPSIN ON TRANSFERRIN-BOUND IRON UPTAKE BY ASTROCYTES

Trypsin can destroy the extracellar portion of TfR and hence affect the function of this receptor in Tf-bound iron uptake. Therefore it was used in this study to investigate the role of this receptor in Tf-bound iron uptake by the cultured astrocytes. The cells were pre-incubated with trypsin (5μg/ml) for 15 min at 37° C, then the cells were washed with HBSS (pH 7.4) for three times before the addition of ^{125}I-Tf-^{59}Fe. The intracellular radioactivity measurement showed that the iron ($^{59}Fe^{2+}$) uptake was inhibited by 38.1% after 30 min of incubation with the treatment of trypsin (Fig.16A). The same effect of pre-treatment of trypsin was found in Tf uptake (Fig.16B). The result demonstrated that both Tf-Fe and Tf uptake could be significantly inhibited by pre-incubation of trypsin ($p<0.05$). These results inferred that TfR participates in Tf-bound iron uptake by the cultured astrocytes.

5.5 DISCUSSION

The findings obtained from this study showed that the cultured brain astrocytes have

the ability to accumulate both Tf and its binding iron in vitro. Iron uptake by the cultured cells increased in a linear manner within the incubation period, while the internalization of Tf reached a plateau level after 10 min of incubation. Experimental results also showed that NH_4Cl and CH_3NH_2 could significantly inhibit Tf-bound iron but not Tf uptake by the cells. Furthermore, pre-treatment of the cells with trypsin resulted in a significant decrease in the uptake of both Tf and Tf-bound iron by the cultured astrocytes. The above findings suggested that TfR on the membrane played a role in the process of Tf and Tf-bound iron uptake by the cultured astrocytes and provide evidence for the existence of a Tf and TfR-mediated pathway in the cultured brain astrocytes in vitro.

The first step of Tf and TfR-mediated iron uptake by the mammalian cells is that Tf (and associated iron) combines with its specific receptor on the plasma membrane surface (Newman et al. 1982, Octave et al. 1983, Qian et al. 1995). Then the complex of TfR-Tf-iron will be internalized and the endosome formed. It is within the endosome that Tf-associated iron dissociates from Tf and transports across the membrane of the endosome into cytosol probably in the form of Fe^{2+}. The reduction in pH (from 7.4 to 5.5 – 6.5) in the endosome, induced by the activity of H^+-ATPase leads to the dissociation of iron. At pH 5.5 – 6.5 the affinity of Tf to iron becomes much lower than that at pH 7.4 (physiological pH). It is why iron can release from Tf within the low-pH endosome. The present experimental results showed that NH_4Cl and CH_3NH_2 inhibited iron uptake by astrocytes, but Tf internalization into

the cells was not significantly affected. It is most likely due to the effect of these two chemicals on pH within endosome. It has been reported that CH_3NH_2 and NH_4Cl have a role to increase intracellular pH in a study using reticulocytes. The pre-treatment of the cells with these two chemicals led to the pH within endosome keeping a higher level. Thus it becomes more difficult for iron to be released from Tf. Under this circumstance, iron will return to surface of the plasma membrane together with Tf and TfR (Morgan 1981).

As having been described before, TfR is a dimer of subunits of molecular mass 90 kDa linked by a single disulphide bond. Each subunit contains a Tf-binding site. Structurally, TfR is composed of three fragments: 70kDa extracellular fragment with one transferrin binding sites; 20 kDa intramembrane fragment with the single disulphide bond; 5 kDa cytoplasmic tail includes a phosphoryl serine and covalently bound fatty acid (Worwood 1989). Trypsin digestion releases the 70 kDa peptide and results in destruction of the molecule of TfR. It will block the first step of Tf-bound iron uptake process, that is, the binding of Tf (and associated iron) to TfR. It might explain why both Tf-bound iron uptake and the internalization of Tf decrease after the pre-treatment of the cultured brain astrocytes with this destructive agent.

This study clearly showed that Tf-bound iron uptake by the cultured astrocytes is mediated by TfR on the membrane under in vitro condition. Also, the existence of this receptor on the membrane of the cultured astrocytes was determined by the binding assay described in Chapter 4. However, these results do not mean that this

iron transport system on the membrane of astrocytes plays a role in iron accumulation by this type of cell and in brain iron homeostasis under physiological or in vivo conditions. At present, we know little about this aspect. More relevant studies are absolutely needed. The relevant knowledge will increase our understanding of mechanism of brain iron metabolism and astrocyte iron balance.

The importance of iron for CNS function has been well-documented (Pollitt and Leibel 1982). The high iron requirement of the brain coupled with the high susceptibility of the brain to iron-generated peroxidative damage requires stringent regulation of the iron availability (Connor et al. 1992). Iron must be presented to the brain and transported to brain cells in a timely and well-regulated manner. A disruption of brain iron metabolism and brain cell iron balance has been implicated in a number of disease, including PD, AD, Pick's disease, MS, HSS, Huntington's chorea etc. A possible link between the pathogenesis of these diseases and oxidative stress induced by brain iron imbalance has been suggested (Youdim and Ben-Shachar 1990). Many studies using the cells outside of the brain showed that the expression of TfR on the membrane is a key factor in determining the amount of intracellular iron. Thus it is reasonable to believe that the disruption of this receptor's expression in the brain might play a role in abnormally higher brain iron level found in neurodegenerative disorders. But this possibility is not well determined. Although extensive literature has generated in this regard. The results reported on the changes in the expression of TfR in the brain of patients with PD and AD are controversial, being increased, decreased or unchanged (Kalaria et al. 1992, Dexter et al. 1992,

Connor et al. 1992f, 1995, Faucheux et al. 1995, Jefferies et al. 1996). The reasons for the difference are not clear. From the physiological point of view, however, the increased iron concentrations in the brain, induced by any reason, should result in a decreased TfR expression in relevant cells. Therefore, the unaltered or increased density of TfR found in the brain of patients with PD and AD suggest that at least in some PD and AD patients there is a malregulation of TfR. This may play an important role in the development of excessive accumulation of iron in the brain in addition to the possible role of other iron transport proteins. On the other hand, it is also possible that normal regulation of TfR is not disrupted in those PD and AD patients with decreased TfR expression and in the brain. These possibilities need to be examined more thoroughly. The full understanding of physiology role of TfR in brain iron transport and balance may lead to a better understanding of these possibilities.

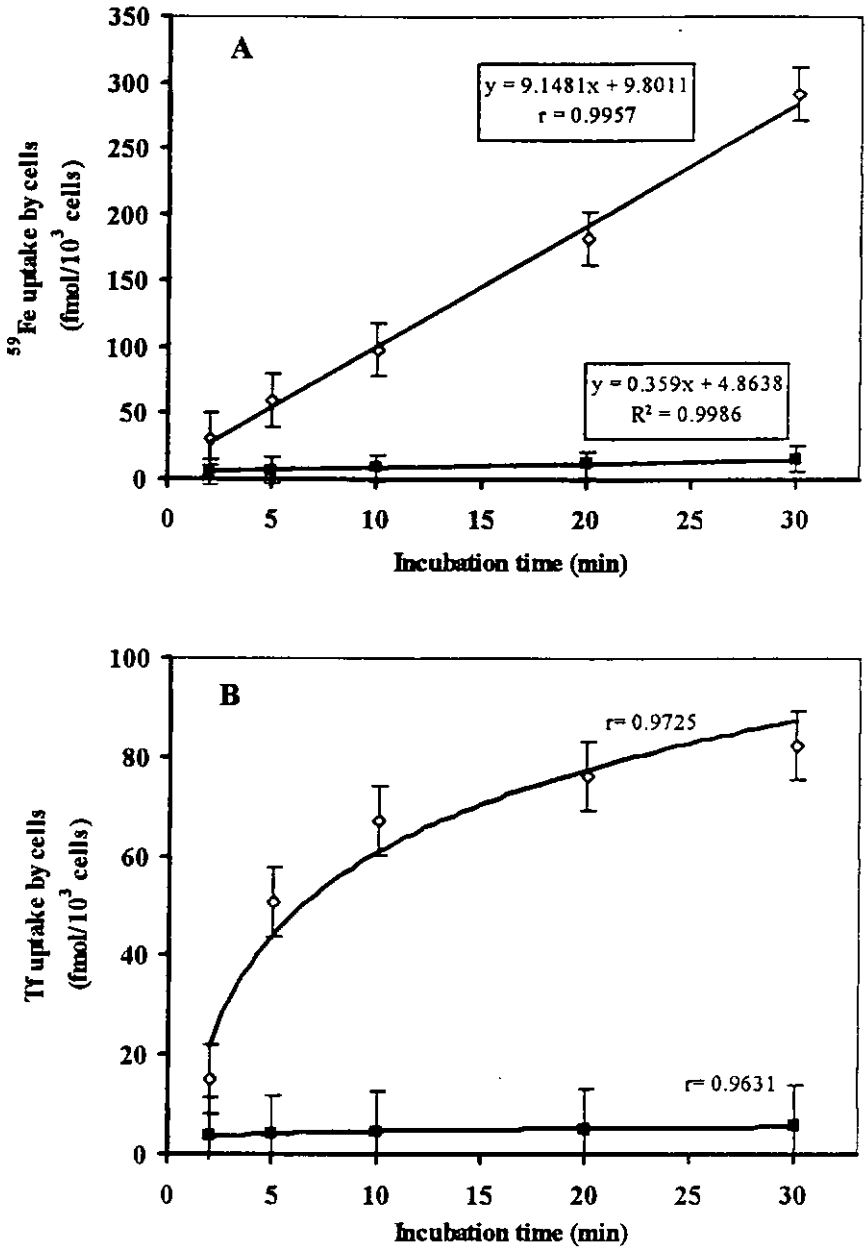


Fig. 14 **Time course of Tf-bound iron and Tf uptake by cultured astrocytes.** The cells were incubated with ^{125}I-Tf-^{59}Fe (1μM) for certain time at 37°C (□) or 4° C (■). Each point is the means of three experiment.

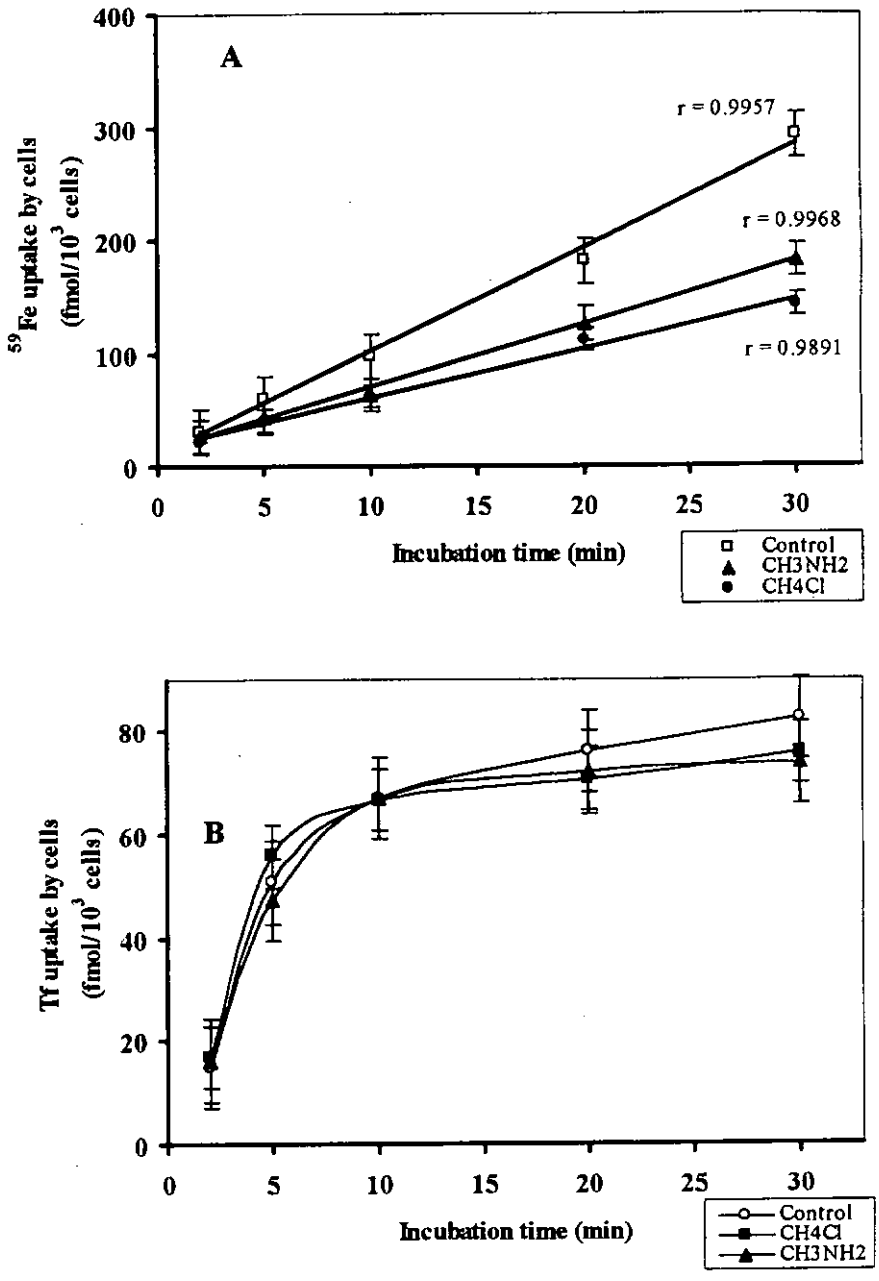


Fig. 15 **Effect of NH_4Cl or CH_3NH_2 on Tf-bound iron and Tf uptake by cultured astrocytes.** The cells were preincubated with NH_4Cl (10μM) or CH_3NH_2 (10μM) for 15 min at 37°C, then added isotope.

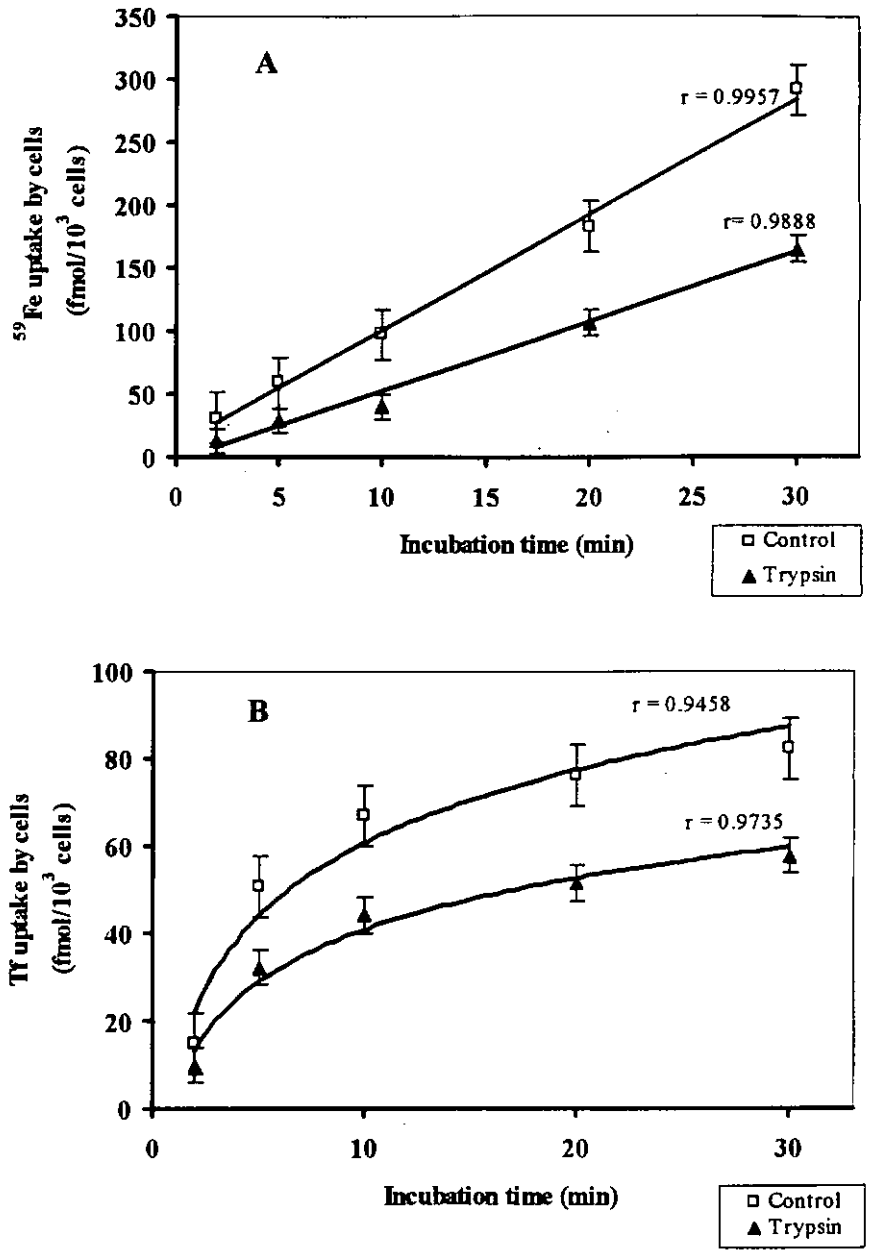


Fig. 16 **Effect of trypsin on Tf-bound iron and Tf uptake by cultured astrocytes.** The cells were perincubated with trypsin (500μg/ml) for 15 min at 37°C, then added isotope.

Chapter 6

TRANSFERRIN-FREE IRON UPTAKE BY THE ASTROCYTES

6.1 ABSTRACT

In this study, the mechanism of Fe^{2+} (Tf-free iron) uptake by the cultured brain astrocytes was investigated. Effects of incubation time, iron concentration, pH, temperature and some other divalent metals on the cellular iron uptake were determined. After 15 days of plating, the cells were incubated with Fe^{2+} in isotonic sucrose solution at different temperatures for certain time. The cellular Fe^{2+} uptake was analysed by measuring the cellular radioactivity with γ-counter. The result showed that the cultured astrocytes had the capacity to acquire Fe^{2+}. The iron uptake by cells increased with incubation time in a linear manner at a rate of 5.776 fmol/10^3 cell per min within the 20 min of incubation period ($y = 5.776x$, $r = 0.9703$). The uptake was time, pH and temperature dependent, iron concentration saturable, and inhibited by several divalent metal ions, including Mn^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Ca^{2+}. The uptake was also pH-sensitive and the pH optimum was 6.5. These characteristics of Fe^{2+} uptake by the cultured astrocytes observed in this study, similar to those obtained from cells outside of the brain and the cultured cerebellar granule cells,

implied that a carrier-mediated iron transport system might be present on the membrane of this type of brain glial cells.

Key word: Tf-free iron uptake; The cultured astrocytes
Divalent metal ions; Brain iron transport.

6.2 INTRODUCTION

The studies described in Chapter 5 showed that the cultured brain astrocytes could take up Tf-bound iron by TfR-mediated endocytosis. The results obtained also demonstrated that all blockers used, including NH_4Cl, CH_3NH_2 and trypsin, could not completely inhibit the Tf-bound iron uptake by this cultured cell. These results might imply that iron could enter this type of brain cells by pathways independent of Tf and TfR-mediated internalization.

In vivo and in vitro studies have suggested that there are other alternative pathways for the entry of iron into non-brain cells (Morgan 1988, Sturrock et al. 1990, Kaplan et al. 1991, Nunez 1992) which are independent of Tf and TfR. The first report on Tf-independent iron transport system in intestinal cells was published by Wheby and co-workers (1963) about 45 years ago. Since then, a number of studies have provided the evidences for that many types of somatic cells such as reticulocytes, liver cells, CHO cell lines, K562 cells, Hela cells, fibroblasts and L1210 cells, have the ability to acquire Tf-free iron.

An important study in this aspect was conducted by Morgan in 1988. He investigated the transport of non-Tf-bound iron into rabbit reticulocytes by incubating the cells in 0.27M sucrose with iron labeled with ^{59}Fe and reported that the rabbit reticulocytes can transport Tf-free iron into the cytosol and incorporate it into haem. The membrane transport process could be divided into two components: saturable or specific uptake and non-saturable or non-specific uptake. The two types of transport had different features. Saturable uptake displayed saturation kinetics with a Km value of approx. 0.2μM and was competitively inhibited by other divalent transition metal ions such as Co^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}, while the other process was non-saturable and increased linearly with increasing iron concentration (Morgan and Baker 1988). This uptake was also pH- and temperature-sensitive, the pH optimum being 6.5. The activation energy for iron transport into cytosol was about 80 KJ/mol. Fe^{2+} and Fe^{3+} both could be transported into the cytosol and utilized for haem synthesis. It was concluded from these results that the transport of non-Tf-bound iron into the cytosol occurs in part via a carrier-mediated process. Subsequent studies (Qian and Morgan 1990, 1991,1992) support the conclusion, and report that the uptake of Tf-free iron by reticulocytes is dependent on the cellular concentration of ATP and that Tf-free iron crosses the cell membrane by an active carrier-mediated process. A similar conclusion was reached by Egyed (1988). In the experimental system used by Morgan, no carrying or reducing agent was used other than sucrose, which served as the iron-stabilizing agent (Morgan 1988). Sucrose is unable to enter reticulocytes (Morgan 1988) and acts only as a weak iron chelator for maintaining the iron in solution in a form which can be assimilated by the cells. Hence, it is likely that the iron was transported into the cells in the ionic state, although the possibility

of participation of iron chelators derived from cells, such as ATP, cannot be entirely excluded.

In a recent study conducted in this laboratory, Qian et al. (Neuroscience, in press) investigated the Tf-free iron uptake by the cerebellar granule cells in culture. It is the first report on non-Tf-bound iron transport across the membrane of brain neuronal cells. The study showed that the cultured neuronal cells has the capacity to acquire Tf-free iron at a rate of 0.1076 pmol/μg protein per min, much higher than that of Tf-bound iron uptake (0.047 pmol Fe/μg protein per min). The iron uptake was time and temperature dependent, concentration saturable, and inhibited by other transitional metals such as Co^{2+}, Zn^{2+}, Ni^{2+} and Mn^{2+}. The Fe^{2+} uptake by the cultured cells occurred at pH 6.5. At this pH it was demonstrated that Tf binds iron very poorly and very little Tf can be internalized by reticulocytes (Morgan 1988) and Hela cells (Sturroch et al. 1990). Based on these findings it was suggested that Tf-free iron uptake by the cultured neuronal cells was Tf-independent. The characteristics of Fe^{2+} uptake, including temperature dependence, saturability of uptake and metal selectivity, suggested that the uptake of Fe^{2+} found in this study was probably a membrane iron carrier-mediated process. However, it is unknown whether there is a membrane iron transport pathway or system, that is Tf and TfR independent, in other types of brain cells including astrocytes. The experiments described in this chapter aim to investigate some aspects of Tf-free iron uptake by the cultured brain astrocytes to find out whether other mechanisms are involved in glial iron uptake in addition to TfR-mediated pathway.

6.3 MATERIALS AND METHODS

6.3.1 MATERIALS

All chemicals, except specially mentioned, were purchased from Sigma Chemical Co., St. Louis, MO. USA. Heat inactivated FCS, DMEM/F12 and G-5 were purchased from Gibco BRL. ^{59}FeCl (in 0.1M HCl, 0.1mCi/ml) was purchased from the Radiochemical Center, Amersham. The animal house at the Hong Kong Polytechnic University supplied SD rats of 2-PND.

6.3.2 CELL CULTURE OF ASTROCYTES

Please refer Chapter 2.

6.3.3 PREPARATION OF $^{59}Fe^{2+}$ SOLUTION

Please refer Chapter 2.

6.3.4 MEASUREMENT OF TRANSFERRIN-FREE IRON UPTAKE

Tf-free iron uptake by astrocytes was measured by following procedure:

Astrocytes culture of 15 days

(Determine the cell count of astrocytes by staining with 0.2% trypan blue)

↓

Decant the culture medium

(Wash the cell with HBSS (pH 7.4) for 2 times)

↓

Incubate with 2ml serum-free culture medium (DMEM/F12) for 3 hours

(This treatment aimed to deplete endogenous protein)

↓

Decant the culture medium and wash the cell with HBSS again

↓ (Add chemicals required here and

↓ pre-incubated the cells for certain min)

Add 2ml sucrose solution (0.32M with 4mM Pipes) and $^{59}Fe^{2+}$ to each dish

Incubate the dish in water bath at 37°C for desired time

↓

Decant the medium and wash the cells with ice-cold HBSS for 3 times

↓

Add 2ml pronase (1mg/ml in HBSS) to each dish

Incubate the dishes on ice for 60min

↓

Collect the solution containing the cells of each dish into new counting tubes

Centrifuge at 150 x g for 10min at 4°C

↓

↓

Transfer the supernatant into new counting tube

↓

Measure the radioactivity of the supernatant ($^{59}Fe^{2+}$ on the membrane)

and the pellet ($^{59}Fe^{2+}$ within the cell)

6.4 RESULTS

Three separate dissections and experiments were performed, which yielded essentially the same results. The data were expressed below as the mean iron uptake (femtomoles of iron per 10^3 cells) $\pm$ SD. Statistical calculations were performed using the student t-test.

6.4.1 TIME COURSE OF ($^{59}Fe^{2+}$) UPTAKE BY ASTROCYTES IN VITRO

Tf-free iron uptake was investigated by incubating the 15 DIV cells with 1μM of Fe^{2+} for different time periods in isotonic sucrose solution at 37^0C. The result showed that Fe^{2+} uptake by the cultured cells increased with time in a linear manner (y=5.776, r= 0.9703) at a rate of 5.776 fmol/10^3 cell per min with the 20 min of incubation period (Fig. 17). The effect of incubation time on iron uptake found in the

study was significantly different from that found in a previous study using the cultured cerebellar granule cells. In that study, a linear increase in Tf-free iron uptake be the cells with incubation time was found only at the first 10 min. The uptake values measured at 15 and 20 min of incubation time are lower than that measured at 10 min. The causes of the difference in " linear-time" were unknown and further investigation is needed.

6.4.2 EFFECT OF IRON CONCENTRATION AND INCUBATION TEMPERATURE ON TRANSFERRIN-FREE IRON ($^{59}Fe^{2+}$) UPTAKE BY THE CULTURED ASTROCYTES

Effect of iron concentration on $^{59}Fe^{2+}$ was studied by incubating the cells with different concentrations of iron at 37^0C for 15 min. As shown in Fig. 18, the cellular uptake of $^{59}Fe^{2+}$ reached saturation as the iron concentration in incubation medium was raised to about 3 μM. Hence, there was evidence of a saturable or specific uptake process. When the cells were incubated with iron at different temperature, it was found that iron uptake was higher at 37^0C than at 4^0C ($p<0.05$) (Fig. 18). This demonstrated that the Tf-free iron uptake by astrocytes was temperature-dependent as that found in other types of somatic cells.

6.4.3 EFFECT OF pH ON TRANSFERRIN-FREE IRON ($^{59}Fe^{2+}$) UPTAKE BY THE CULTURED ASTROCYTES

To determine the effect of different pH on Tf-free iron uptake by the cultured astrocytes, the 15 DIV astrocytes were incubated with $^{59}Fe^{2+}$ in sucrose solutions buffered by Pipes to different pH levels (from 5.0 to 8.0). The results (Fig. 19) showed that the uptake of $^{59}Fe^{2+}$ into the cell was influenced by the pH, optimum uptake occurring at pH 6.5. When the effect of iron uptake at 6.5 and 7.5 were compared, it was found that iron uptake into cell was much lesser at pH 7.5 than at 6.5. It indicated that there was a less efficient transport of extracellular $^{59}Fe^{2+}$ to the astrocytes at pH 7.5 than at 6.5. There is evidence suggesting that Tf-free iron uptake is associated with iron cross the membrane the endosomes after it is released from Tf or pathologic process at the pH 6.5.

6.4.4 INHIBITION OF IRON UPTAKE BY OTHER METALS ON TRANSFERRIN-FREE IRON ($^{59}Fe^{2+}$) UPTAKE BY THE CULTURED ASTROCYTES

Several divalent metal ions (Mn^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Ca^{2+}) were tested for their ability to affect Fe^{2+} uptake by astrocytes. The cells were incubated with 1μM $^{59}Fe^{2+}$ in 0.32 M sucrose (pH 6.5) in the absence or presence of these divalent metal ions at 37°C. The concentration of the divalent metals used are 50μM (50-times the molar

concentration of iron), referred to Morgan (1988). As shown in Fig. 20, all the ions used including Mn^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Ca^{2+} had an inhibitory effect on Tf-free iron uptake by the cultured cells of 84.66%, 77.25%, 81.17%, 78.36% and 75.02%, respectively. Among the metals used, Mn^{2+} demonstrated the greatest inhibitory effect on Fe^{2+} uptake which was similar to the finding in Hela cells by Sturrock et al. Ca^{2+} was shown to be the least effective in inhibiting iron uptake by this type of cell. The effect of Ca^{2+} on iron uptake by the cells was completely different from those found in the cultured cerebellar granule cell, liver cells and Hela cells. The addition of Ca^{2+} resulted in an increase in cellular iron uptake in those cells.

6.5 DISCUSSION

This study showed that the cultured astrocytes had the capacity to acquire Tf-free iron at a rate of 5.776 fmol/10^3 cell per min. The iron uptake was time and temperature dependent, concentration saturable, and inhibited by other transitional metals such as Co^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+} and Ca^{2+}. The Fe^{2+} uptake by the cells was also pH sensitive, the pH optimum being 6.5. At this pH it was demonstrated that Tf binds iron very poorly and very little Tf can be internalized by reticulocytes (Morgan 1998) and Hela cells (Sturrock et al. 1990). Based on the characteristics of the Fe^{2+} uptake which are temperature and pH dependent, the uptake is saturable and having metal selectivity, it is reasonable to consider that the Tf-free (Fe^{2+}) iron uptake by the cultured astrocytes was probably a membrane iron carrier-mediated process.

In a previous study conducted in this lab, it was found that addition of Ca^{2+} resulted in an increase in Fe^{2+} uptake (119.14% of control) by the cultured cerebellar granule cells. Similar results were also obtained in perfused rat liver by Wright et al. (1990) and in Hela cells by Sturrock et al. (1986). They observed a Ca^{2+}-dependence of Tf-free iron uptake by the cells they used. When Hela cells were incubated in Ca^{2+}-free solution iron uptake was reduced. Removal of Ca^{2+} by washing cells with EGTA-containing solutions was more effective at reducing cellular iron accumulation, and iron uptake was increased above control when Hela cells were incubated with higher concentration of Ca^{2+} (above 10μM) (Sturrock 1990). However in the present study, the results showed that the addition of Ca^{2+} to incubation medium led a decrease in iron uptake. The reason is unknown. Probably, it is related with the difference between the membrane structure and components of these cells. Results obtained suggested that there was Tf-independent iron transport pathway on the membrane of the cultured astrocytes. It is important to clarify the physiological functions of the suggested transport system in the cells. Studies in somatic cells pointed out that under physiological conditions, Tf-dependent iron transport pathway is the main route for cellular iron accumulation, while Tf-independent pathway has been suggested likely to participate the process of Tf-bound iron uptake, playing a key role in translocation iron across endosomal membrane after its release from Tf within endosome (Egyed 1988, Morgan 1988, Qian and Tang 1995, Qian et al. 1997). In the study described in Chapter 5, we demonstrated that there was Tf-dependent iron transport pathway on the membrane of the cultured astrocytes. Probably, the carrier-mediated Fe^{2+} transport or the Tf-independent pathway is also involved in the process of Tf-bound iron uptake, participating in translocation of iron

across endosomal membrane in the cells as found in cerebellar granule cells and in cells outside of the brain. This possibility needs to be determined further.

Other functions of Tf-independent pathway in somatic cells have been reported or suggested as well. These include (1) to remove non-Tf-bound iron (or Tf-free iron) from the plasma to protect cells and tissues from free radical reaction and lipid peroxidation-induced toxic effect (Kennard et al. 1995, Qian et al. 1996, Silva et al. 1996); (2) to serve as a mechanism for the other metals to enter some types of mammalian cells non-competitively with iron (Conrad et al. 1994); (3) to participate in the absorption of iron by intestinal cells that have no TfR on their luminal surfaces (Conrad and Umbreit 1993, Conrad et al. 1994); and (4) to involve in the redistribution of iron into tissues that are not accessible by Tf (Kennard et al. 1995). With respect to the first function, it should be noted that this pathway probably transports little iron because there is little Tf-free iron in the plasma under the physiological conditions. Doubtlessly, this mechanism is operative and plays a more important role when the plasma iron concentration exceeds the iron binding capacity of plasma Tf (Kennard et al. 1995). This occurs in acute iron poisoning, in dying cell-induced higher iron level, and in iron overloading disorders such as haemochromatosis (Smith 1990), thalassaemia and atransferrinanaemia (Kaplan et al. 1991). Although no information are available at present on the role of the possible transport system of Tf-free iron on brain cells, it is possible that the carrier-mediated Fe^{2+} uptake system in astrocytes might play the same role as that did by somatic cells. It may helps in protecting neuron and other brain cells from free iron-induced toxic effect by removing free iron from interstitial fluid in the brain when needed.

Also, it is worth investigating whether the dysfunction of this transport system is partly associated with the excessive cellular iron accumulation in the brain in neurodegenerative disorders.

What is the nature of the suggested iron carrier on the membrane of the cultured astrocytes? It is another important subject needed to investigate further. Some possible candidates for the membrane iron carrier in somatic cells have been reported or suggested, including p97 (Kennard et al. 1995, 1996, Rothenberger et al. 1996), K+-ATPase (Watkins et al. 1991, Li et al. 1994, 1995), integrin-mobilferrin (Conradnd et al.1992, Conradnd and Umbreit 1993, 1994), TfR (Qian and Morgan 1992, Qian et al. 1997) and some other molecules (Qian et al.1997). In addition, the possible role of p97 and LfR (Fancheheux et al. 1995, Bonn 1996) as well as DCT1 in brain iron transport was recently reported (Qian and Wang 1998). Further studies on the functions of these molecules in the membrane iron transport of brain will be helpful to elucidate the nature of the suggested membrane carries.

In conclusion, this investigation has provided some evidence for the presence of membrane iron carrier-mediated transport of Fe^{2+} in the cultured astrocytes in rats. Physiological role of the carrier-mediated Fe^{2+} transport in brain iron metabolism and the nature of the suggested iron carrier are two important aspects that require further investigation.

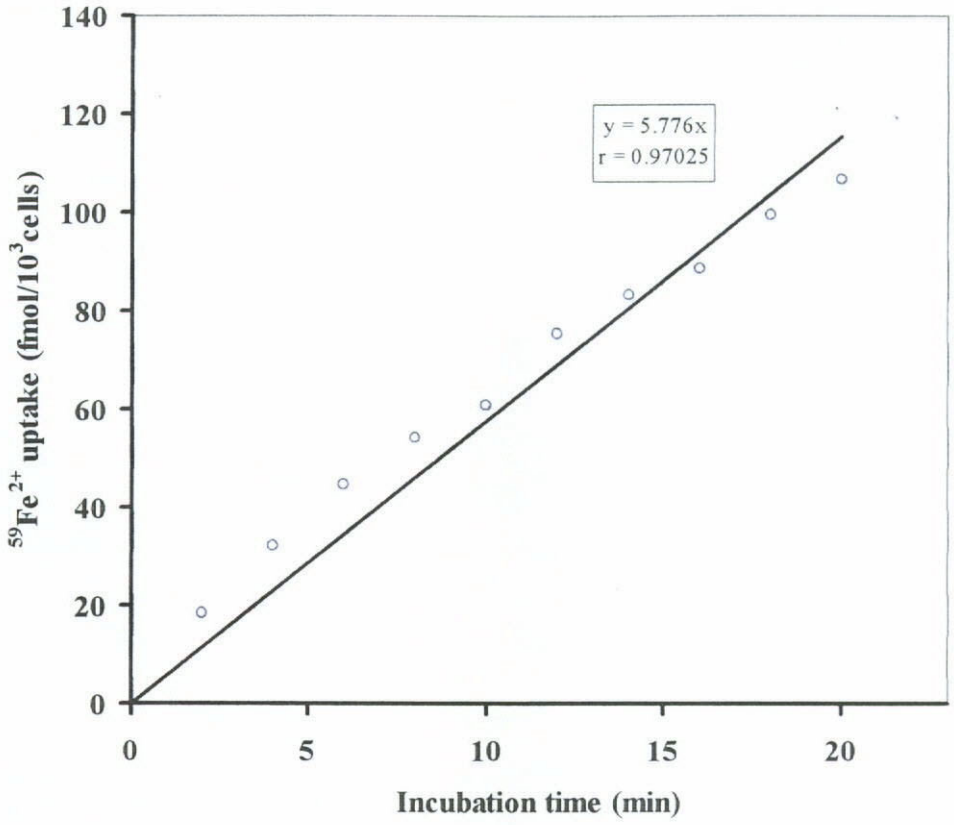


Fig. 17 **Time course of Tf-free iron ($^{59}Fe^{2+}$) uptake by cultured astrocytes.** The cells were incubated with ($^{59}Fe^{2+}$)(1μM) in sucrose (0.32 M, pH 6.5) for certain time at 37°C. Radioactivity in the cells was measured then with γ-counter. Each point is the means of three experiments.

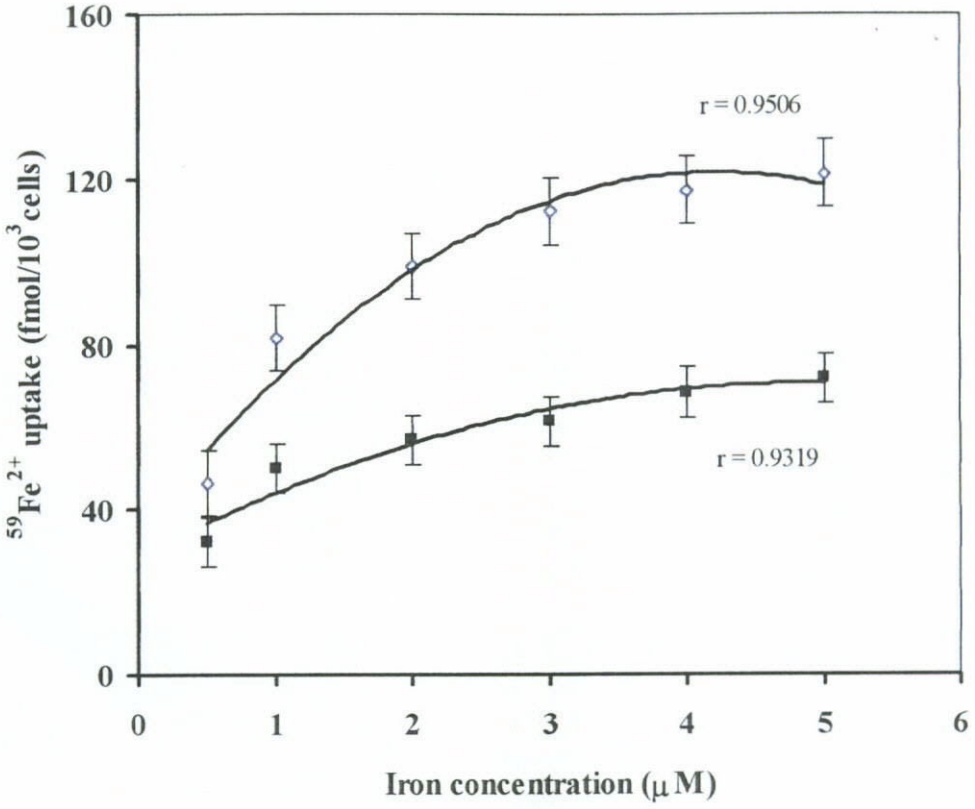


Fig. 18 Effect of iron concentration onTf-free iron uptake by cultured astrocytes at 37°C (□) or 4°C (■), respectively. Cells were incubated with $^{59}Fe^{2+}$ at different concentrations in 0.32 M sucrose (pH 6.5) for 15 min. Radioactivity in the cells was measured then by γ-counter. Each point is the mean of three experiments.

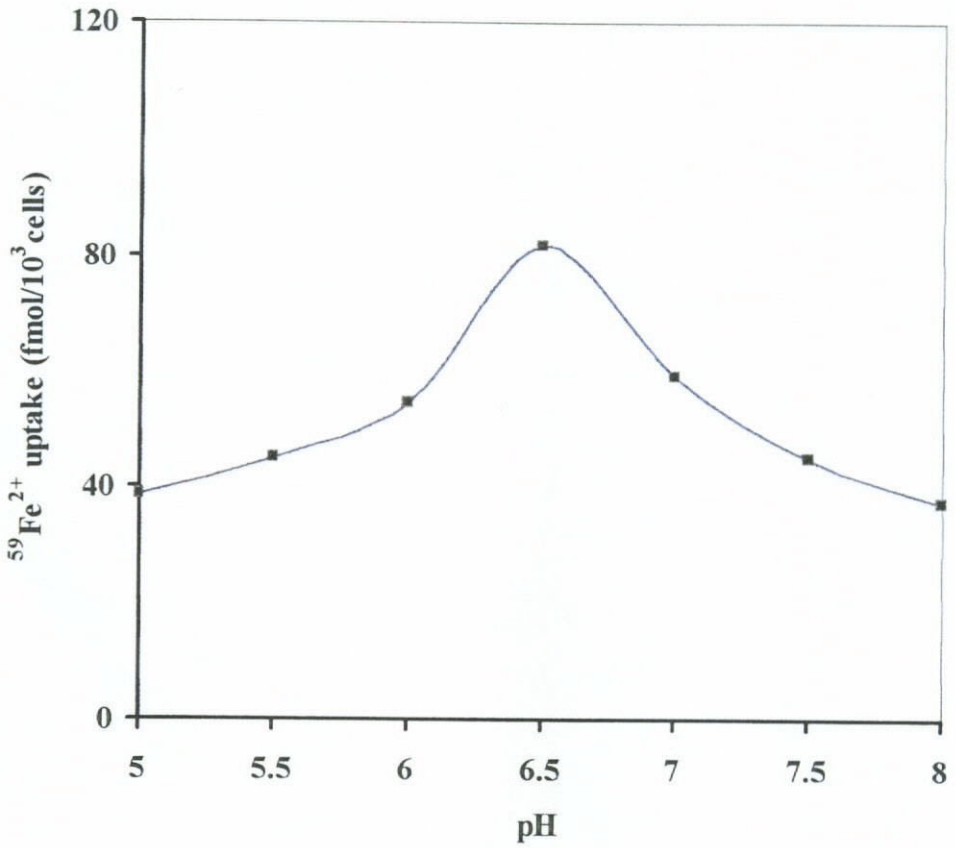


Fig. 19 **Effect of pH on Tf-free iron uptake by cultured astrocytes.** The cells were incubated with ($^{59}Fe^{2+}$)(1μM) at different pH (5.0-8.0) in sucrose (0.32 M) at 37°C for 15 min. Radioactivity in the cells was measured then by γ-counter. Each point is the mean of three experiments.

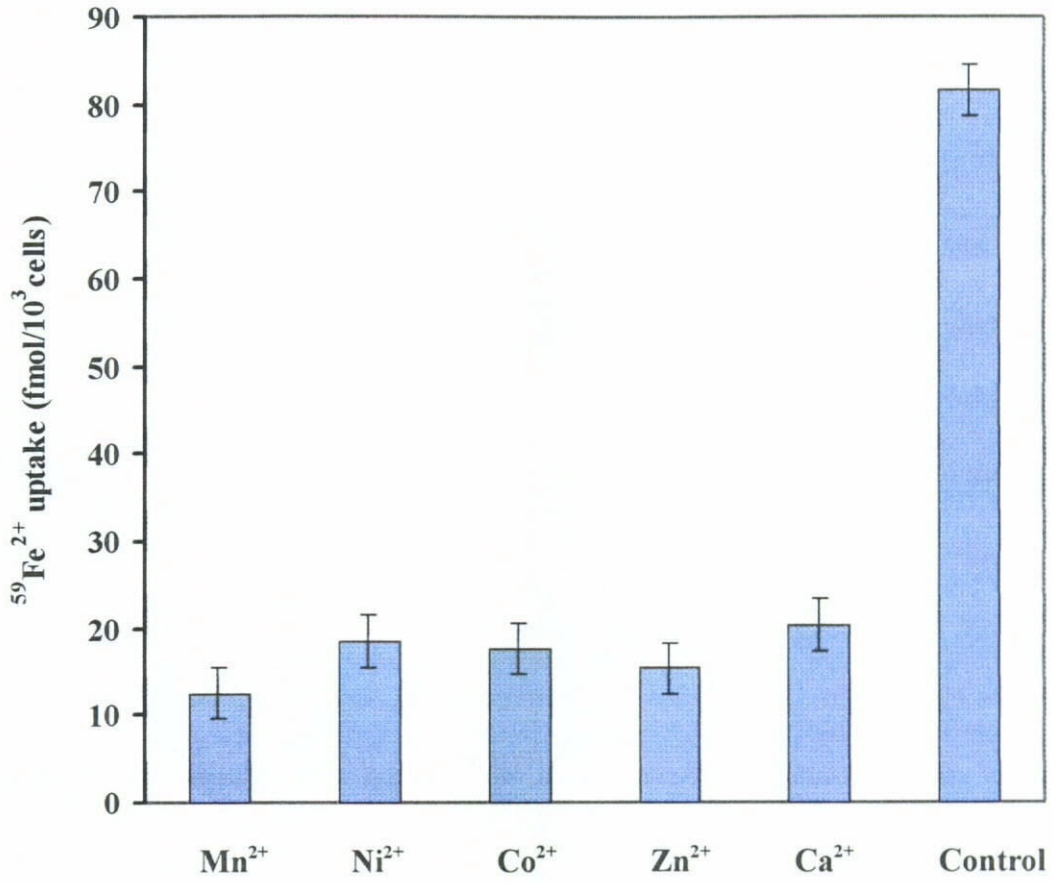


Fig. 20 **Effect of transitional metal ions on Tf-free iron uptake by cultured astrocytes.** Cells were incubated with $^{59}Fe^{2+}$(1μM) in the presence of transitional metal ions (50μM) at 37°C for 10 min. Then the radioactivity in the cells was measured by γ-counter.

Chapter 7

GENERAL DISCUSSION

Many biological functions of brain iron have been identified, in particular its role in many enzymatic processes, its effect on dopamine receptor function, its interaction with other neurotransmitters, and its catalytic role in the nonenzymatic mechanisms of oxidation, hydroxylation, and peroxidation reactions (Sachdev 1993). On the other hand, as having been described in Chapter 1, disorder of brain iron metabolism will result in many diseases. Abnormally increased iron accumulation in the brain has been associated with a number of neurodegenerative disorders, such as AD and PD. Therefore, the cellular iron homeostasis is critical for the brain to play normal function.

In humans, iron metabolism appears to be highly regulated. Studies using somatic cells show that the regulation and management of iron at the cellular level is primarily controlled by two proteins: transferrin receptor and ferritin. By controlling the level of expression of these two proteins, the cell can determine the amount of iron acquired (proportional to the number of membrane transferrin receptor) and sequestered (proportional to the cytoplasmic level of ferritin). However, the mechanism of cellular iron regulation and management in brain cells may be more

complicated than that in somatic cells. Although research interest in the area of brain iron metabolism is increasing in recent years, the knowledge is very limited about the physiological mechanism of iron uptake by different types of brain cells. The understanding of this aspect is critical for elucidating mechanism of brain cell iron balance and the pathophysiological mechanism of brain iron overload.

It is well known that astrocytes make up a large percentage in the cellular compositions of the CNS and play an important role in brain functions including brain iron homeostasis. It has also reported that astrocytes may responsible for the abnormal sequestration of brain iron in neurodegenerative diseases. In addition, more than 95% homogeneity of the culture can be easily prepared. It is due to the above-mentioned reasons that the cultured astrocyte is selected as a model to investigate some aspects of mechanism of brain glial cellular iron accumulation in the studies described in this thesis.

The results obtained were summarized in chapter 3, 4, 5, 6. Experiments described in chapter 3 demonstrated that high concentration of chelated ferric iron had an adverse effect on astrocytes. Significant decrease of astrocyte population was found at concentrations of 20μM to 5000μM. However, no significant deviation of morphology was found between 5μM and 10μM groups and the controls. In contrast, when a low iron concentration (5μM) was added, the cultures could be kept in normal condition for three weeks. These findings showed that the experimental system used is valid for the studies of mechanism of iron uptake by astrocytes.

Results obtained from studies described in chapter 4, 5, 6 demonstrated (1) the cultured astrocytes had the ability to express transferrin receptor on the membrane (Chapter 4), (2) the Transferrin-bound iron transport across the membrane of astrocytes was a transferrin receptor-mediated process and the pattern generally paralleled to the characteristics of uptake in other mammalian cells (Chapter 5), and (3) the transferrin-free iron uptake was time, pH and temperature dependent, iron concentration saturable, and inhibited by some divalent metal ions, including Co^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+} and Ca^{2+}, suggesting that a carrier-mediated iron transport system might be present on the membrane of this type of brain cells (Chapter 6).

Cultured astrocyte is an ideal model to study brain iron uptake mechanism. The result of this study provided us the information that we have expected, as described in Chapter 3, 4, 5 and 6. Iron overload may cause astrocytes death in vitro through lipid peroxidation. Astrocytes are especially resistant to oxidative stress. It is associated with antioxidant defenses that may augment the ability of astrocytes to protect neurons from free radicals in some neurodegenerative diseases.

These finding provide important insights into the understanding of the mechanism of iron uptake by brain astrocytes. However, many relevant questions need to be answered. These question include:

(1) What is the physiological role of these two systems (transferrin receptor-mediated iron transport and transferrin-free iron uptake system) on the membrane in astrocyte iron balance and brain iron homeostasis?

(2) What is the functional relationship between these two systems of iron transport?

(3) What is the suggested membrane iron carrier in astrocytes, p97, DCT1, LfR, or others?

(4) Does the dysfunction of these two systems play a role in brain iron overload?

All of these questions need to be investigated further.

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APPENDIX

SOURCS OF REAGENTS AND BIOCHEMICALS

Acrylamide	Sigma Chemical Co., MO, USA
Anti-glial fibrillary acidic protein (GFAP)	Boehringer Mannheim Biochemica Ltd.
Apo-transferrin	Sigma Chemical Co., MO, USA
Avidin-peroxidase conjugate	Sigma Chemical Co., MO, USA
2-b-mercaptoethanol	Sigma Chemical Co., MO, USA
Biotin conjugate goat anti-mouse IgG	Sigma Chemical Co., MO, USA
Bovine serum albumin (BSA)	Sigma Chemical Co., MO, USA
Bromophenol blue	Sigma Chemical Co., MO, USA
Coamassie blue R-250	Sigma Chemical Co., MO, USA
3-(4,5-dimethylthylthiazole-2-yl) -2,5-diphenyl tetrazolium bromide (MTT)	Sigma Chemical Co., MO, USA
DNase	Sigma Chemical Co., MO, USA
Dulbecco's modified Eagles medium (DMEM)	Gibco BRL, USA
Earle's balanced salt solution (EBS)	Sigma Chemical Co., MO, USA
Ethylenediaminotetraacetic acid (EDTA)	Sigma Chemical Co., MO, USA
Ghycine	Sigma Chemical Co., MO, USA
L-glutamine	Sigma Chemical Co., MO, USA
Glycerol	Sigma Chemical Co., MO, USA
Goat serum	Sigma Chemical Co., MO, USA
Ham's nutrient mixture F-12 (F12)	Gibco BRL, USA

Heat inactivated fetal calf serum (FCS)	Gibco BRL, USA
N-2-hydroxyethylpiperazine	Sigma Chemical Co., MO, USA
-2-ethanesulfonic acid (Hepes)	
Iodo-gen	Sigma Chemical Co., MO, USA
2-mercaptoethanol	Gibco BRL, USA
N'N'-bis-methylene-acrylamide	Sigma Chemical Co., MO, USA
0-Phenylenediamine dihydrochloride (OPD)	Sigma Chemical Co., MO, USA
Penicillin-streptomycin	Sigma Chemical Co., MO, USA
Piperazine-N'-N'-bis (2-ethanesulfonic acid) (Pipes)	Sigma Chemical Co., MO, USA
Poly-L-lysine	Sigma Chemical Co., MO, USA
Pronase	Sigma Chemical Co., MO, USA
Sephadex G-50 median	Sigma Chemical Co., MO, USA
Sodioum dodecyl sulfate	Sigma Chemical Co., MO, USA
Standard protein marker	Pharmacia Biotech, Sweden
Supplemented G-5 (G-5)	Gibco BRL, USA
Temed	Gibco BRL, USA
Trichloroacetic acid (TCA)	Sigma Chemical Co., MO, USA
Tris (hydroxymethyl)-aminoethane (TRIS)	Sigma Chemical Co., MO, USA
Trypan blue	Sigma Chemical Co., MO, USA
Trypsin	Sigma Chemical Co., MO, USA
Tween	Sigma Chemical Co., MO, USA
D-valine	Sigma Chemical Co., MO, USA
L-valine	Sigma Chemical Co., MO, USA

SYNOPSIS OF THE THESIS

MECHANISMS OF IRON UPTAKE BY THE CULTURED BRAIN ASTROCYTES IN RATS

Submitted by TO YU

Department of Applied Biology and Chemical Technology

For the Degree of Master of Philosophy at Hong Kong Polytechnic University

October 21, 1998

As in all cells, brain cells require iron for many aspects of their physiology, including electron transport, NADPH reductase activity, myelination of axons, and as a co-factor for several enzymes involved in neurotransmitter synthesis. On the other hand, iron is also a potent toxin. It has been reported that abnormally increased iron accumulation in the brain may be involved in neuronal death and the development of neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, Hallervorden-Spatz Syndrome and Multiple Sclerosis. However, to date, little information is available about the regulation of iron concentration in the brain and mechanisms of iron uptake by different types of brain cells. It is for this reason that we are not able to answer a key question: why do iron levels increase abnormally in some regions of the brain in these neurodegenerative disorders

The studies described on this thesis are performed to investigate some fundamental aspects on mechanisms of iron uptake by brain glial cells using the cultured rat astrocytes. These aspects include the effect of iron on the development of brain astrocytes in vitro and mechanisms of transferrin-bound and transferrin-free iron uptake by the cultured brain astrocytes in rats. Techniques including primary cell culture, microphotography, immunohistochemistry, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay, sodium dodecyl sulfate-polyacrylamide gel

electrophoresis analysis, protein radiolabeling, and radioactivity measurement, etc. are used in this study.

The results obtained show that: (1) The toxic effect of iron on brain astrocyte in culture is directly related to concentration and duration of exposure. (2) The cortical astrocyte of rat in suspension binds to transferrin by a saturable and specific binding manner, inducting the presence of transferrin receptor on the membrane of the cells. The number of receptor is about 7.09×10^4/cell and the dissociation constand (Kd) of the binding is about 3.45×10^{-8} M. The receptor is specific for rat and human transferrin. The Hill coefficient is 0.988, indicating the absence of cooperativity. (3) Iron transport across the membrane of astrocytes, much like other mammalian cells, is mediated by transferrin-transferrin receptor endocytosis. The uptake rate of transferrin receptor-mediated endocytosis is much higher than that of transferrin-free iron uptake system. (4) The cultured astrocytes have the capacity to acquire iron at pH 6.5, in which very little transferrin-bound iron can be acquired by cells. This iron uptake system is time dependent, temperature and pH sensitive, concentration saturable, and competitively inhibited by other transitional metals. It suggests that brain astrocyte can acquire non-transferrin-bound iron probably by a carrier-mediated process. The findings I obtained provide for the first time evidence for the presence of transferrin receptor in the cultured astrocytes. The results also demonstrate that transferrin receptor-mediated mechanism may play a role in iron uptake by astrocytes and that there is the transferrin receptor-independent pathway for iron uptake on the membrane of the type of brain cells.

This thesis consists of 7 chapters, a general introduction, followed by general methods, then 4 chapters describing the experimental work (Chapter 3 – 6), concluding with a general discussion.