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Brain Iron Deficiency and Excess; Cognitive Impairment and Neurodegenration with Involvement of Striatum and Hippocampus

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While iron deficiency is not perceived as a life threatening disorder, it is the most prevalent nutritional abnormality in the world, and a better understanding of modes and sites of action, can help devise better treatment programs for those who suffer from it. Nowhere is this more important than in infants and children that make up the bulk of iron deficiency in society. Although the effects of iron deficiency have been extensively studied in systemic organs, until very recently little attention was paid to its effects on brain function. The studies of Oski at Johns Hopkin Medical School in 1974, demonstrating the impairment of learning in young school children with iron deficiency, prompted us to study its relevance to brain biochemistry and function in an animal model of iron deficiency. Indeed, rats made iron deficient have lowered brain iron and impaired behaviours including learning. This can become irreversible especially in newborns, even after long-term iron supplementation. We have shown that in this condition it is the brain striatal dopaminergic-opiate system which becomes defective, resulting in alterations in circadian behaviours, cognitive impairment and neurochemical changes closely associated with them. More recently we have extended these studies and have established that cognitive impairment may be closely associated with neuroanatomical damage and zinc metabolism in the hippocampus due to iron deficiency, and which may result from abnormal cholinergic function. The hippocampus is the focus of many studies today, since this brain structure has high zinc concentration and is highly involved in many forms of cognitive deficits as a consequence of cholinergic deficiency and has achieved prominence because of dementia in ageing and Alzheimer's disease. Thus, it is now apparent that cognitive impairment may not be attributed to a single neurotransmitter, but rather, alterations and interactions of several systems in different brain regions. In animal models of iron deficiency it is apparent that dopaminergic interaction with the opiate system and cholinergic neurotransmission may be defective.

Keywords: Iron; Cognition; Dopamine; Hippocampus; Striatum; Neurodegeneration

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Neurochemical Consequences of NID

It is well recognized that nutritional iron deficiency (NID) is the most prevalent nutritional deficiency in the world. It can affect more than 400 million individuals (World Health Organization, WHO) and is indeed most prevalent in infants and young children. The importance of iron in systemic cellular biochemistry, where it is utilized in the synthesis of DNA and proteins and is involved as cofactor for numerous enzymes, structural protein and physiological responses is well recognized. The vast literature that has been published on its various function and dysfunction in systemic organs, as a consequence of its deficiency is too numerous to summarize in this short article. However, what is not known and not readily recognized is that early iron deficiency can have a profound long-term effect on brain function, with possible irreversible brain damage at the cellular and neuronal level. Even so, until 1974 little or no attention was paid to brain iron metabolism and brain function. Since then there has been an active interest in brain iron metabolism, not only as a consequence of its deficiency with an effect on learning and cognitive processes, but also the role of excess brain iron accumulation and its involvement in neurodegeneration and progressive neurodegenerative diseases (Parkinson's disease, Alzheimer's disease, Huntington chorea, Haller Voren Spatz disease, etc.) (Youdim and Riederer, 1997; 1999; Dobbing, 1990a; Youdim, 1990). To appreciate the effect of NID on brain one must consider its distribution.

The first studys of iron in human brain were those of Spatz (Pollitt *et al.*, 1989) and Hallgren and Sourander (1958) who showed that adult brain iron was unevenly distributed and some brain regions, namely the extrapyramidal regions (globus pallidus, substantia nigra, thalamus, ventral thalamus, red nucleus, intrapeduncular nucleus, dentate gyrus, cingulate nucleus) had the highest concentrations and in some cases there was more iron per gm weight wet in these regions than in the liver. Ferritin (FER) is similarly distributed. These data have been consistently confirmed for human, monkey, dog and rat brains.

However, there is no correlation between distributions of iron-FER concentration and those of transferrin-transferrin receptors. Yet, the highest concentrations of the latter are found in the hippocampal and cortical regions, which have relatively low iron and FER contents (Connor *et al.*, 1987; Dwork *et al.*, 1988; Hill, 1988; Riederer *et al.*, 1989). By contrast, in rat brain at birth most of the iron is found in cortical and hippocampal areas. Studies involving brain ^{59}Fe uptake in newborn rats with limited blood-brain barrier (BBB), have shown two crucial aspects of brain iron metabolism. 1) The major portion of ^{59}Fe is found in the globus pallidus, substantia nigra, intrapeduncullar nucleus, dentate gyrus, etc. 2) More than 90% of the iron (^{59}Fe) present in newborn rats 24 hours after its injection is still in the brain in adulthood. This clearly indicates that iron is highly conserved in the brain and has a very slow turnover (Ben-Shachar *et al.*, 1986; Dwork *et al.*, 1990). Although iron transport into the brain is via transferrin receptors residing in capillarty endothelial cells, the mechanism(s) by which iron is transported from one brain sight to another is not known. The most puzzling question, which cannot be discussed at length in this review (due to lack of space), is why should such brain regions as globus pallidus, substantia nigra, dentate gyrus, caudate nucleus etc. accumulate the highest concentration of iron in the brain. More recent studies point to iron induced oxidative stress in the neurodegenerative processes in these regions and the consequences of it are some of the most devastating neurodegenerative diseases (Parkinson's Disease, Alzheimer's Disease, Huntinton Chorea, Haller-Voreden Spatz Disease). There have been several reviews dealing with brain iron metabolism and brain function in iron deficiency (Pollitt and Leibel, 1982; Dobbing, 1990b; Spatz, 1992) and iron in neurodegenerative diseases (Youdim and Riederer, 1997; 1999). This review will concentrate in updating the newer aspect of iron deficiency on brain function.

NID and Brain Iron

The first studies that dealt with the effects of NID on the brain were those of Werkman *et al.* (1964), Webb and Oski (1973) and Dallman *et al.* (1975; Dallman and Spirito, 1977). These investigators reported respectively that NID in children induced behavioral abnormalities that included a reduction of learning abilities (cognitive impairments), and

that NID in rats fed an iron deficient diet results in the reduction of brain iron, albeit not as much as that in the liver. These reports together with the earlier studies of Spatz (Hallgren and Sourander, 1958; Pollitt *et al.*, 1989) on the uneven distribution of iron in human brain prompted us to investigate the brain biochemical, pharmacological, physiological and behavioral effects of NID in rats as a model for the human condition (see Youdim and Green, 1977; Youdim *et al.*, 1989). Our original hypothesis was based on the notion that the behavioral changes noted in iron deficient children by Webb and Oski (1973) were related to changes in the metabolism of CNS aminergic neurotransmitters, dopamine, serotonin and noradrenaline. Abnormality in the metabolism and function of these neurotransmitters was implicated in behavior and learning processes and several neuropsychiatric diseases . There was good reason for this, since, the rate limiting enzymes for the synthesis (tyrosine and tryptophan hydroxylases) and catabolizing (monoamine oxidase) the aminergic neurotransmitters were shown to be dependent on iron for their full enzymatic activity. Thus, any changes in the activities of these enzymes may alter the brain level of these neurotransmitters at specific sites (hypothalamus, striatum, raphe nucleus, frontal cortex and hippocampus) in the brain and alter the physiology of their respective neurons as a consequence of over- or under-production of the neurotransmitters. Indeed we demonstrated that while NID in rats, as induced by feeding them a diet low in iron (5 ppm) results in the reduction of iron dependent enzymes (monoamine oxidase, phenylalanine hydroxylase, succinic dehydrogenase, cytochrome oxidase, to mention a few) in peripheral tissues, by contrast none of the brain enzymes containing iron, including tyrosine hydroxylase, tryptophan hydroxylase and monoamine oxidase) as a cofactor were changed (Youdim and Green, 1977; Youdim *et al.*, 1989) (Table I). This was in spite of the fact that rat brain iron was reduced by 30-40% in adult (48 days old) in the striatum, hippocampus, cortex and raphe nucleus. The effect is region dependent. It is now apparent that the effect of NID on tissue (brain and liver) iron is also dependent on the age of the animal and time on the nutritionally iron deficient diet. While rat (47 days old) liver iron and FER stores can be reduced relatively fast (within two weeks by 80-90%) brain iron is hardly changed until 3-5 weeks on the ID diet. Indeed newborn pups (10 days old) are more readily made ID than young (21 days old) animals (Dallman *et al.*, 1975; Dallman and Spirito, 1977; Youdim and Green, 1977; Ben-Shachar *et al.*, 1986; Youdim *et al.*, 1989).

Table I Effect of ID on brain enzymes*

Phenylalanine hydroxylase	Unchanged
Tyrosine hydroxylase	Unchanged
Tryptophan hydroxylase	Unchanged
Monoamine oxidase	Unchanged
Aldehyde dehydrogenase	Unchanged
Cytochrome oxidase C	Unchanged
Succinic dehydrogenase	Unchanged
Aminobutyric acid transaminase	Decreased
Glutamate decarboxylase	Decreased

*Adult (aged 48 d) male rats were made nutritionally iron-deficient by feeding them a semisynthetic diet deficient in iron. Control animals received the same diet, to which iron sulphate had been added. In both groups the animals were pair-fed to maintain similar weight.
Decrease, in liver, heart, and adrenal glands.

TABLE II Striatal Neurotransmitters and their precursor levels in brains of ID rats

	Concentration	**Turnover**
Serotonin	Decreased	Unchanged
Dopamine	Unchanged	Increased
Noradrenaline	Unchanged	Unchanged
Tryptophan	Unchanged	-
Tyrosine	Unchanged	-
5-Hydroxyindole acetic acid	Decreased	-
Met-enkephalin	Increased	
Dynorphin B	Increased	

The unchanged activities of brain neurotransmitter enzymes in ID rats are complimented with unaltered brain (striatum, caudate nucleus and raphe nucleus) levels and turnover of dopamine, noradrenaline and serotonin (Youdim and Green, 1977; Youdim *et al.*, 1989) (Table II). Nevertheless our animal behavioral studies with functional activities of neurotransmitters dopamine and serotonin indicated a highly significant degree of deficit, which for the first time complimented the "behavioral" deficits reported by Webb and Oski (Webb and Oski, 1973) in children with NID. The neurochemical explanation for the behavioral deficits was not

easily forthcoming and we suggested that brain ID may result in alteration in receptor number and function for any of these neurotransmitters (Youdim and Green, 1977). However, the abilities to measure various brain neurotransmitter receptor B_{max} (receptor number) and K_a, employing radioligands did not become available until 1976. As shown in Table III, the only neurotransmitter receptors that were affected were those of dopamine (Youdim *et al.*, 1989). Indeed, employing radioligand analysis of dopamine D_1 and D_2 receptors we observed an increase in K_a of dopamine D_1 and a decrease of dopamine D_2 B_{max} in the striatum of NID rats, which complimented the significant diminution of dopamine dependent behaviors, as elicited by the treatments of rats with the dopamine agonist, apomorphine. This was the first time that a neurochemical change could be associated with the behavioral effects observed in NID. Indeed our numerous behavioral and neurochemical investigations related to reduction of dopamine D_2 receptor B_{max} clearly indicates a subsensitivity of this receptor via the initiation of NID (see Table IV) (Youdim *et al.*, 1989). There was direct parallelism between time dependent reduction of brain (striatum) iron, dopamine D_2 receptor B_{max}, and apomorphine elicited dopamine dependent behavior (Ben-Shachar *et al.*, 1986; Youdim *et al.*, 1989). This effect is related to

TABLE III Effect of ID on brain neurotransmitter receptors as identified by specific radioligands

Receptors	K_a*	B_{max}
γ-Adrenoceptor (^{3}H-WBA101)	Unchanged	Unchanged
β-Adrenoceptor (^{3}H-DHA)	Unchanged	Unchanged
Muscrinic-cholinergic receptor (^{3}H-ONB)	Unchanged	Unchanged
Dopamine D_2 receptor (^{3}H-spiperone)	Unchanged	Decreased
Dopamine D_1 receptor	Decreased	Unchanged
5-HT_2 receptor (^{3}H-serotonin)	Unchanged	Unchanged
GABA receptor (^{3}H-musimol)	Unchanged	Increased
Benzodiazepine	?	?

*Affinity constant for the receptor.

TABLE IV Biochemical and behavioral consequences of reduced dopamine D_2 receptor in the brains of ID rats

	Response
Monoamine oxidase inhibitor plus tryptophan (serotonin behavioural syndrome)	Decreased
Monoamine oxidase inhibitor plus L-dopa (dopamine behavioural syndrome)	Decreased
Monoamine oxidase inhibitor plus 5-hydroxy-tryptophan (serotonin behavioral syndrome)	Decreased
5-methoxy-*N,N*-dimethyltryptamine (behavioural syndrome)	Decreased
D-amphetamine (dopamine release induced stereotypy)	Decreased
Apomorphine (dopamine receptor agonism stereotypy)	Decreased
Learning processes	Decreased
Thyrotropin-releasing hormone (TRH) and its analogue	Unchanged
Phenobarbitone sleeping time	Increased
Serum prolactin	Increased
Serum testosterone	Increased
Liver prolactin receptor	Increased
Antinociceptive response to β-endorphin and enkephalins	Increased
Dynorphin and m et- and leuenkephalins concentrations in globus pallidus, substantia nigra, caudate nucleus, and central grey and antinociception increased.	Increased
Response to neurotoxin (kainite , 6-hydroxydoapmine) induced neurodegeneration	Decreased

iron deficiency and not the anaemia resulting from it, since hemolytic anaemia induced by chronic treatment of rats with phenylhydrazine does not alter brain dopamine D_2 receptor number nor their behavioural responses (Ben-Shachar *et al.*, 1986). Furthermore, supplementation of ID rats with iron plus diet (control) can result in restoration of brain iron dopamine receptor B_{max} and behavioral responses. Again this is an age dependent phenomenon and has clear clinical implications in children with NID. One obvious but extremely important finding in our studies was the handling of iron by brain versus liver during iron supplementation. While rat liver iron could be restored with one or two weeks, brain iron increased very gradually, reaching its pre iron deficiency level within 3-4 weeks. Continuing iron supplementation for some 6 months resulted in liver iron being increased by some 20-fold whereas brain iron remained constant. This discrepancy between iron handing of liver and brain indicates that iron transport in the brain is handled differently and may be related to the BBB since in adult rats serum iron has no access to the brain. Furthermore the turnover of brain iron is significantly much slower than in the liver and almost all the iron that is present in brain is conserved throughout life.

Examination of the BBB in NID rats indicated selective alteration and we suggested this could be at the level of gap or tight junctions of capillary endothelial cells that constitute the BBB (Ben-Shachar *et al.*, 1988). However, Taylor and coworkers (Taylor *et al.*, 1991) have also provided evidence for up regulation of transferrin and transferrin receptors during NID and their down regulation when brain iron is restored. It is possible that both mechanisms are involved and clearly more work needs to be done to clarify the differences between liver and brain handing of iron. Certainly the roles of the recently described iron regulatory proteins IRP1 and IRP2, during iron deficiency and repletion in the brain need clarification (Eisenstein and Blemings, 1998; Haile, 1999).

Consequences of Early Iron Deficiency on Brain Function and Behavior.

It is in the first decade of a child's life (first 4 years) that the bulk of iron deficiency is seen (Dobbing, 1990b). This is apparently the most crucial period of brain development, where DNA and protein synthesis, neuronal growth and differentiation take place and maturation of enzymes occurs. In this period myelination of neurons is at its fastest, and there is an essential role for iron in myelin deposition by oligodendrocytes (Erikson *et al.*, 1997). Indeed iron deficiency significantly interferes with myelination of the neurons (Connor and Menzies, 1996). Numerous studies on rat brain development confirm this. The obvious question would be, what are the long-term consequences of iron deficiency in this period on brain development and function. Dallamn (Dallman *et al.*, 1975; Dallman and Spirito, 1977) reported persistent deficiency of brain iron during short-term deprivation in young rats, followed by iron supplementation. He has attributed this to the very slow turnover of iron in the brain as compared to liver.

We re-examined this in rats of different ages (newborn, young and adult) made nutritionally iron deficient. Not only did we confirm Dallamn's findings in newborn rats, but we also showed this feature to be age dependent (Ben-Shachar *et al.*, 1986). While young and adult rats could recover their brain iron, newborns could not after iron repletion. Furthermore, newborn rats had an unrecoverable behavioral deficit, which was related to the deficiency of striatal dopamine D_2 receptor and this and brain iron could not be restored even after 6 months of iron therapy, in spite of the normalization of their haematological indices. These findings point to long-term irreversible consequences of early iron deficiency on brain function. This may go some ways to support what Lozzof and co-workers and others (Lozoff and Brittenham, 1986; Lozoff, 1988; Parks and Wharton, 1989; Felt and Lozoff, 1996) have consistently found in iron deficient infants and young children with impaired attentional problems where long-term iron therapy was ineffective. Whether our animal models reflect the human condition may be a matter of debate and all animal models do have their drawbacks. Nevertheless early iron deficiency does impair brain biochemistry and function and its consequences need to be appreciated, considering the susceptibility of human brain to under nourishment in the first decade of life (Georgieff *et al.*, 1996; Guesry, 1998; Rao *et al.*, 1999), when the major portion (80%) of iron

found in the adult brain is deposited, and interaction of iron with other metals may be crucial (Adhami *et al.*, 1996; Chua and Morgan, 1996). Although our original behavioral studies were related to dopamine neurotransmission deficit in iron deficiency, we have extended our work to closely investigate learning parameters in Morris Water Maze. Indeed iron deficient young rats were poorer performers (longer time and more trials) in finding the platform in the Morris maze and had lower activity (Yehuda *et al.*, 1986; Yehuda and Youdim, 1989; Yehuda, 1990). Moreover, 4 weeks of iron therapy (repletion) did not alter the learning performance of these rats. Confirmation of this result has recently come from Felt and Lozzof (1996). These results raise the concern that iron deficiency during the course of early brain development, prenatally and postnatally, are damaging. Thus it is crucial to maintain normal brain iron concentration (Adhami *et al.*, 1996; Chua and Morgan, 1996; Georgieff *et al.*, 1996; Guesry, 1998; Rao *et al.*, 1999) where neuronal iron uptake is age dependent and susceptible to iron deficiency as a consequence of its dependence on transferrin receptor (Moos *et al.*, 1998). The one puzzling factor in comparing the effects of NID on newborn and adult rat brain iron is that adult rat brain recovers its iron but not the newborn. This would suggest some other underlying irreversible cause, which we have not identified as yet.

The Consequences of Iron Deficiency on Dopamine-Endogenous Opiate Interaction and Function

The role of dopamine in learning and cognitive processes has been discussed at length by Yehuda and Youdim (Yehuda and Youdim, 1989; Yehuda, 1990). Nevertheless consideration also has to be given to other mechanisms and changes that occur in the brain during ID, as a consequence of reduction in dopamine neurotransmission or other processes. For example, we showed that iron deficiency alters several brain proteins as identified by two-dimensional electrophoresis and among them a protein with a molecular weight identical to that of dopamine D_2 receptor decreased, while other proteins are increased (Youdim *et al.*, 1986). We were not able to identify many of the protein changes. However consideration has to be given to the alterations in these proteins. What roles they have in brain function clearly need thorough investigation.

The brain areas known to have the highest concentration of iron (globus pallidus, substantia nigra, dentate gyrus, caudate nucleus, thalamus, putamen, ventral tegmentum) are innervated with the densest population of opiate-peptides (enkephalins, endorphins and dynorphin B). The importance of endogenous opiate peptides alone and their interaction with dopamine in learning process has been investigated and it is now evident that they are closely involved in learning and cognition processes. On several occasions it has been reported that administration of the opiate antagonists (*e.g.*, naloxone, melanocyte inducing factor (MIF)) improves learning (Huidobro-Toro and Way, 1983; 1985) and this may be dependent on dopamine transmission functional activity and learning processes, as a consequence of dopamine D_2 receptor subsensitivity. Indeed, ID rats showed a highly significant antinociception, which was further exaggerated when treated intraperitoneally with the opiates, morphine, met-enkephalin, leu-enkephalin or endorphin. Naloxone and MIF (Yehuda and Youdim, 1984; Yehuda *et al.*, 1988; Youdim *et al.*, 2000) could block these effects. Normal rats do not show antinociception to opiate-peptides, since these peptides do not cross the BBB and are rapidly metabolized by zinc-dependent metalopeptidase in systemic organs. Thus, in newborns metabolism of opiate-peptides and their brain transport are affected. BBB studies have clearly shown uptake of the opiate peptide, β-endorphin, in ID rats but not control (Ben-Shachar *et al.*, 1988). The latter results may indicate the reason why ID but not control rats exhibit antinociception in response to opiate-peptide (met-enkephalin and dynorphin B) as measured in globus pallidus, caudate nucleus, substantia nigra, midbrain tegmentum, and central gray (Huidobro-Toro and Way, 1983; 1985; Yehuda and Youdim, 1984; Yehuda *et al.*, 1988; Youdim *et al.*, 2000). The mechanism whereby NID brings about increased brain levels of opiate peptides is not well understood. It is well established that dopamine is inhibitory to opiates (Huidobro-Toro and Way, 1983; 1985; Tang *et al.*, 1983), and antagonism of dopamine receptors with dopamine D_2 receptor antagonists (haloperidol, chlorpromazine)

results in brain elevation of opiate-peptides similar to those as shown by us in ID rats (Tang *et al.*, 1983; Morris *et al.*, 1988; Angulo, 1992; Durham *et al.*, 1996). The explanation for this may be found in studies where it has been demonstrated that dopamine D_2 receptor antagonists induce the proenkephalin mRNA (Tang *et al.*, 1983; Morris *et al.*, 1988; Angulo, 1992; Durham *et al.*, 1996). Whether NID brings about the same changes in opiate mRNAs, and opiate antagonists can reverse the diminished learning processes, remains to be investigated.

Iron and Zinc Interaction in the Hippocampus

The involvement of hippocampus in cognition has prompted our investigation into distribution and development of the iron storage protein FER in rat hippocampus (Shoham and Youdim, 2002). a) In normal rats, FER positive cells appeared first in lateral CA3 of hippocampus and hilus of dentate gyrus and then spread over the entire mossy fiber (MF) system. No such spread was observed in the hippocampal CA1 field. b) NID retarded development of FER in the MF system. No change in FER was observed in the CA1 field. c) Zinc distribution can be altered by iron deficiency. Thus, the effect of zinc added to iron supplementation was tested in iron-deficient rats. Significant FER recovery was observed only in hippocampal MF of rats receiving both zinc and iron. It is apparent that for accelerating recovery of hippocampal function in iron deficiency, both zinc and iron are required.

The background for our study (Shoham and Youdim, 2002) was the cognitive impairment and resistance to nutritional iron-therapy in young iron-deficient (ID) children (Parks and Wharton, 1989; Walter *et al.*, 1989; Lozoff *et al.*, 1991; 1996) and their confirmation in our animal studies (Youdim and Green, 1977; Ben-Shachar *et al.*, 1986; Yehuda and Youdim, 1989; Youdim *et al.*, 1989; Yehuda, 1990). The main objectives of the study were (a) to chart the normal development of FER distribution in the hippocampus, (b) examine the effects of ID on FER immunohistochemical distribution, and (c) study the consequences of nutritional therapy with iron alone compared to zinc alone and compared to zinc added to iron-therapy (Shoham and Youdim, 2002).

(a) The normal development of FER distribution in the hippocampus

The study revealed a spatial pattern of FER distribution in the hippocampal MF system and a spatiotemporal order in the development of FER cells in this hippocampal system. It should be noted that the sensitivity of the immunohistochemical methods used in the present study depend on the quality and affinity of the antibody. Thus, some cells that appeared to lack FER, in the present study, might have actually contained some FER. In other words, lack of FER cells in a given region of the hippocampus should not be taken to mean absence of FER. Nevertheless, assuming that the threshold for detection of FER was the same for all groups compared in the study, we have been able to detect a spatiotemporal trend in the development of FER cells in the hippocampal MF system. Several previous studies have touched the subject of iron and FER in the hippocampus (Dwork *et al.*, 1990; Connor *et al.*, 1994; 1995; Dwork, 1995). However, an association of FER distribution with the hippocampal MF system has not been noted in previous studies. Noting this association is important, since, it provides a potential clue to the effects of brain iron deficiency on cognitive function.

(b) Effects of iron-deficiency on distribution of FER in the hippocampus

Previous studies on the impact of iron deficiency on the hippocampus, have not reported any effect specific to the hippocampal MF system (Taylor *et al.*, 1991; Felt and Lozoff, 1996; Erikson *et al.*, 1997; Hansen *et al.*, 1999; de los Monteros *et al.*, 2000; Han *et al.*, 2000; Pinero *et al.*, 2000; Shoham and Youdim, 2000). Furthermore, in these studies, two weeks of iron-supplementation to iron-deficient rats was sufficient to restore iron and FER to the hippocampus as measured in a homogenate of the dissected hippocampus. Since the MF terminal zone comprises only a small fraction of hippocampal tissue, it is not surprising that only with the resolution power of immunohistochemistry as employed in the present study, it was possible to discover that ID had a specific effect on the hippocampal MF system, and that two weeks of iron supplementation did not restore FER distribution to this anatomical system.

It is also important to note that despite the impact

of ID, no change in MF neuroanatomy was detected by the sulfide/silver stain, or by immunohistochemical staining with markers of hippocampal innervation such as the potassium channel Kv1.4 or calbindin D28k. This suggests that the retarded development of FER distribution was not secondary to an effect of iron deprivation on MF development. On the other hand, the fact that FER distribution could be retarded without a gross change in MF development led to the search for subtle effects of ID on hippocampal MF composition and one candidate aspect was zinc. Although zinc accumulates in the hippocampus, MF zinc comprises only 8% of hippocampal zinc (Frederickson *et al.*, 1983). Nevertheless, MF zinc appears to have an important role in signal transduction in the MF system (Frederickson *et al.*, 1983). Thus, we examined the potential impact of zinc added to iron-therapy in iron deficient rats.

(c) Effects of zinc alone and zinc added to iron-therapy

The diet employed in the study was not zinc-deficient and there was no reason to consider the iron-deprived rats were zinc-deprived. However, based on previous studies, alterations in zinc levels can occur in some tissues secondary to iron deficiency (Howell *et al.*, 1984; Shukla *et al.*, 1989; Yokoi *et al.*, 1991). Thus, a subtle change in MF zinc under iron-deficiency was considered as a potential contributing factor in resistance of learning ability to iron therapy. In the present study, zinc-supplementation alone failed to increase the number of FER cells in the MF system of ID rats. But addition of zinc to iron-therapy significantly increased the number of FER cells in the hippocampal MF. The mechanisms underlying this effect remain to be investigated. One possible line of investigation may be interactions of iron and zinc with endogenous opioid peptide neurotransmission. There is evidence suggesting that zinc modulates enkephalinergic neurotransmission (Stengaard-Pedersen *et al.*, 1981). Met-enkephalin and dynorphin B levels were increased in several brain regions in ID rats including the striatum, substantia nigra, ventral tegmentum and pallidum (Yehuda *et al.*, 1986; Youdim *et al.*, 2000) and these animals exhibit highly significant antinociception, which could be blocked by naloxone and MIF (Youdim *et al.*, 2000). Normally rats to not exhibit antinociception to systemic administration of enkepalins. However, iron deficient rats do so to Met- and Leu-enkephalins (Youdim *et al.*, 2000; Georgieff *et al.*, 1996) because they are metabolized rapidly by zinc dependent metalopeptidases. This may be a result of the fact that in normal circumstances enkephalin-peptides are metabolized by zinc-dependent metaloproteases. Since iron deficiency affects zinc metabolism it is most likely that metabolism of the enkephalins by zinc-metaloproteases are reduced and they would have access to the brain. Intracerebral administration of enkephalin analogs causes partial depletion of zinc in several brain regions including hippocampus (Gulya *et al.*, 1991). Thus, brain zinc may be affected in ID through effects on enkephalinergic neurotransmission. In the hippocampal MF system there are opioid neurotransmitters. Both enkephalin and dynorphin are released from glutamatergic nerve terminals of the hippocampal MF system and modulate hippocampal pyramidal cell iron, zinc and FER in hippocampus of iron-deficient rats (Morris and Johnston, 1995). The present study may provide clues to further investigate alterations in opioid neurotransmission in the hippocampal MF in iron-deficiency. Another domain in which zinc may interact with opioid peptides in the phenomenology of iron deficiency may be the regulation of food intake and body weight. Iron deficiency is associated with a reduction in spontaneous food intake and reduction in body weight (Dhur *et al.*, 1990). Zinc deficiency also is associated with reduced food intake body weight (Essatara *et al.*, 1984). However, the present study is the first, to the best of our knowledge, to show that zinc supplementation to iron-deficient rats is sufficient to restore food intake and body weight. This finding suggests that the anorexia of iron-deficiency may be mediated by a secondary deficiency in zinc in some brain regions. Given the interaction of zinc with enkephalin (Stengaard-Pedersen *et al.*, 1981) it is possible that ID, indirectly affects zinc and hence enkephalinergic modulation of food intake (Gosnell *et al.*, 1986).

The neurobiological significance of the present findings on FER distribution remains to be elucidated. In the present study, the developmental spread of FER cells in the rat hippocampus follows the pattern of developmental synaptic plasticity in

this region (Bayer, 1985). Thus, in the present study, FER cells were mostly localized in the vicinity of the stems of apical dendrites of pyramidal cells in CA3. These apical dendrites receive multiple afferents, which arrive in the course of development with a temporal order. In the hippocampal dentate gyrus (DG), cell division continues into adult life (Bayer, 1985). Thus, according to the present findings, the formation of new MF synaptic contacts appears to be accompanied by the increase in FER, possibly reflecting increased accumulation of iron. Possibly, iron in glial cells, localized along the MF system, has a role in support of developmental synaptic plasticity. In the present study and in previous studies by others (Kaneko *et al.*, 1989; Connor *et al.*, 1994). FER immunohistochemistry revealed cells with microglial morphology in the hippocampus. Iron has been detected in amoeboid microglia in early development (Kaur and Ling, 1995). Possibly, iron is important in some microglial "macrophagic" functions in the hippocampal MF system. With normal development, synaptic reorganization in the MF system requires removal of old synaptic structures as well as formation of new structures. Microglia are involved in removal of synaptic structures in various paradigms of synaptic plasticity (Nakajima and Kohsaka, 1993). Possibly iron stored in microglia participates in this "macrophagic function" of microglia. Indeed, there is evidence for release of iron from FER stored in microglia (Yoshida *et al.*, 1995). In iron deficiency, lower availability of iron to microglia may slow down developmental plasticity in the MF system, which may be manifested in cognitive deficits.

More research is required to understand how the addition of zinc to iron therapy may accelerate the recovery of iron-storage in glial cells along the MF system and how this may affect cognitive deficits in ID. In another study, our finding that zinc added to iron-therapy potentiates kainate neurotoxicity in ID (Shoham and Youdim, 2000) suggests that zinc added to iron-therapy enhances glutamatergic neurotransmission in the hippocampus. Glutamatergic neurotransmission in hippocampus is important for learning processes (Bliss and Collingridge, 1993). Since microglia are sensitive to glutamate via *N*-methyl-D-aspartate receptors (Tikka and Koistinaho, 2001), it is possible that the addition of zinc modulates functional states of microglia via zinc modulation of NMDA receptor function (Xie and Smart, 1994). Furthermore, there is evidence that microglia secrete factors that enhance neuronal excitability in the hippocampus (Hegg and Thayer, 1999). It remains to be further explored whether this function is modulated by iron in microglia and whether this process is subject to modulation by zinc levels. Furthermore studies are now under way to assess the impact of zinc added to iron-therapy on learning ability in ID rats.

Conclusion

The effect of iron deficiency on brain function is a relatively new subject. Nevertheless, it is clear from animal studies that ID can profoundly affect the CNS structural components, neurons, its neurotransmitter metabolism, and function. The most obvious systems that are affected are the dopaminegric and enkephalenrgic systems which interact with each other. This may not be unexpected since iron plays a crucial role in many physiological processes. Furthermore, iron metabolism is one of the most tightly regulated events with in the cells, since both its deficiency or excess can affect many enzymatic and structural proteins. Thus, iron homeostasis is crucial for brain function. What is obvious is that the abnormality of iron in brain metabolism and function has not received as much attention as in the studies in systemic organs; this may be related to the notion that brain was impervious to such changes. For example, it is now well recognized that there are a number of important neurodegenerative diseases (Friedreich ataxia, Parkinson's disease, Alzheimer's disease, Haller Vorden Spatz disease, Wilson's disease) where iron has access to the brain by accumulating in specific neurons and may be involved in the neurodegenerative processes in these diseases (Youdim and Riederer, 1999).

This short review has dealt with the effects if ID on the dopamine-opiate system in the striatum and its interaction with hippocampal zinc metabolism and function, mainly because little attention has been paid to other brain neurotransmitter systems (*e.g.*, GABA, glutamate, nitric oxide) which could also be affected (Li, 1998). Because dopamine-opiate interaction plays such a crucial central role in brain neurotransmission and affects other neuronal systems, it is most likely that the effects of

nutritional ID on brain are more complex than so far studied (Youdim and Yehuda, 2000).

Finally, more recent work from our own laboratory has indicated that brain iron deficiency is a two edged sword. Although throughout this review we have demonstrated that deficiency of iron can profoundly affect brain biochemistry and function, we now have evidence that in certain circumstances iron deficiency can be protective to the adult brain. Thus, rats made iron deficient, where brain iron falls by about 30%, are less susceptible to brain neurodegeneration in response to neurotoxins (such as MPTP [1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine], kainate and 6-hydroxydopamine), which are used as tools for animal models of epilepsy and Parkinson's disease (Li, 1998; Shoham *et al.*, 1996), where a significant increase in iron occurs in the substantia nigra, similar to what has been observed in parkinsonian substantia nigra (Zecca *et al.*, 2004). These neurotoxins induce neurodegeneration by a mechanism that initiates oxidative stress and proliferation of reactive microglia that generate the reactive oxygen species from interaction of iron with hydrogen peroxide (Fenton reaction). The mechanism of neurotoxin-dependent neurodegeneration has been attributed to the ability of these neurotoxins to release FER-dependent chelatable iron and the participation of the metal in redox generation of oxygen radical species, with the consequential onset of oxidative stress, which includes activation and proliferation of reactive microglia (Shoham and Youdim, 2000; Zecca *et al.*, 2004). Under the condition of reduce brain iron these neurotoxins do not induce proliferation of reactive microglia or neurodegenration. Thus, it is suspected that generation of reactive oxygen species is limited.

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