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**BRAIN IRON LEVELS IN CHILDREN WITH
ATTENTION-DEFICIT/HYPERACTIVITY
DISORDER (ADHD): MRI STUDY**

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SUMMARY

Background: Attention-Deficit/Hyperactivity Disorder (ADHD) is one of the most common childhood psychiatric disorders, estimated to affect about 5% of school-aged children worldwide. According to the Diagnostic and Statistical Manual of Mental Disorders-4th edition-Text Revision (DSM-IV-TR), ADHD is defined by a persistent and age-inappropriate pattern of inattention, hyperactivity-impulsivity or both. The exact etiopathogenesis underlying ADHD is not completely understood. It is likely that ADHD is a heterogeneous syndromic entity with a multifactorial etiopathogenesis, including genetic and environmental factors. Several lines of evidence, reviewed in detail in the first part of the thesis, suggest that iron deficiency (ID) might be involved in the etiopathogenesis of ADHD. First, iron is a co-factor of enzymes necessary for the synthesis and catabolism of the aminergic neurotransmitters (dopamine, serotonin, and noradrenaline), which have been shown to be involved in the pathophysiology of ADHD. Second, iron deficiency is associated with a decrease in dopamine D2 receptors, as well as of dopamine transporter in basal ganglia (in particular in the striatum), which have been implicated in ADHD pathogenesis. Third, there is a large body of research showing that ID with or without anemia in childhood is associated with cognitive and behavioral impairments, including poor attention and hyperactivity. Fourth, ID in basal ganglia is also increasingly recognized as a central factor in the pathophysiology of Restless Legs Syndrome, which may be co-morbid with ADHD, thus suggesting possible common pathophysiologic pathways in which iron deficiency may play a role. Finally, overweight, which is more common in children with ADHD than controls, has been associated with iron deficiency.

Rationale: To date, available studies on ID in ADHD are based on the measure of serum ferritin, a *peripheral* marker of ID. However, how well peripheral iron indices correlate with *central* (i.e. brain) iron content is still unclear. Since it is central iron that may impact on brain function, there is a need to assess brain iron levels in children with ADHD. No published study has assessed brain iron levels in children with ADHD by means of Magnetic Resonance Imaging (MRI).

Aims: The aims of the study, presented in the second part of the thesis, are the following: Primary: To compare brain iron levels, estimated by means of MRI, in a sample of children with ADHD, in a group of children with other psychiatric disorders (different from ADHD), and in a group of healthy controls. Iron levels were estimated in four regions which have been shown to contain iron: thalamus, putamen, pallidum, and caudate. Secondary: To assess the relationship between serum ferritin levels and estimated brain iron levels in the three study groups.

Methods: Subjects: Patients (6-14 years) with ADHD, as well as those with other psychiatric disorders, were recruited from the Child and Adolescent Psychopathology Unit of the Hospital Robert Debré in Paris (2006-2008). Healthy controls were recruited from relatives of hospital

employees. Non-inclusion criteria were the presence of one or more neurologic disorders, a previous or ongoing iron supplementation, anemia, and the current use of any drug that could significantly affect cognitive function. Procedures: *Psychiatric evaluation:* The diagnosis of ADHD, as well as of other psychiatric disorders, was made according to DSM-IV-TR criteria and was confirmed by the semi-structured interview Kiddie-SADS-PL. The Kiddie-SADS-PL confirmed the absence of any relevant psychiatric disorder in the control group. *Assessment of peripheral iron status:* A complete blood count and measurement of serum ferritin levels, as well as of serum iron and hemoglobin (Tinaquant and Ferrozine method) were obtained. *MRI measurements:* Estimation of brain iron was obtained on the basis of T2*. The inverse of T2* (R2*) are directly correlated with iron stores. MRI examinations were performed on a 1.5 T Philips Unit. Statistical analysis: T2* were compared in the three study groups using one-way ANOVA analysis with Bonferroni multiple comparison procedure. The correlation between serum ferritin levels and estimated brain iron levels in the four regions was assessed by means of the Spearman correlation. All statistical analyses were performed using SPSS v. 15.0 (SPSS, Inc., Chicago, IL, USA).

Results: Data from eighteen children with ADHD, nine patients with other psychopathologies (Anxiety Disorders, Mood Disorders, Obsessive Compulsive Disorder, and Early Schizophrenia), and nine healthy controls were used for the statistical analysis. According to the power analysis, this sample size allowed for a detection of a difference of 2 points in T2* with a power of about 85%. It was found that T2* were significantly higher (meaning that iron levels were significantly lower) in thalamus, both in right ($p=0.015$) and in left thalamus ($p=0.010$) in children with ADHD compared to healthy controls. No other significant differences were found for the other regions of interest. Children with ADHD had serum ferritin levels significantly lower than children with other psychiatric disorders ($p=0.006$) and healthy controls ($p=0.001$). Serum ferritin levels were inversely correlated with T2*, but the correlations were not significant in any regions of interest ($p>0.005$).

Discussion: This is the first study to assess brain iron levels in children with ADHD. MRI data suggest that low iron levels in thalamus might impair its functioning in children with ADHD. Although the thalamus has been scarcely investigated in ADHD, it is presumed to be a very critical brain region subserving normal attention processes. Iron deficiency might negatively impact thalamic functioning also in other psychiatric disorders, but further studies are needed to assess to what extent iron deficiency is specific of ADHD or can be found in other psychiatric disorders. Serum ferritin levels inversely increased with T2*, but the correlation was not significant. Although low peripheral iron levels may negatively impact on brain iron, our results suggest that serum ferritin levels might be only a proxy for brain iron but can not estimate it accurately.

Conclusions and future perspectives: This study provides a significant contribution to our understanding of the pathophysiology of ADHD, suggesting that brain iron deficiency might contribute to the pathophysiology of ADHD (and perhaps of other childhood psychiatric disorders) via its impact on thalamic functioning, which is part of neuronal circuits serving attention and alertness. Further studies, with novel MRI approaches to better estimate brain iron, are needed to confirm our results. This body of research might contribute to advance our knowledge on the etiopathogenesis and pathophysiologic pathways underlying the cluster of ADHD symptoms. The approach which underlies the rationale of these studies is an innovative one in the field of ADHD, and, in general, in child psychiatry, moving from the description of syndromes to pathophysiology-based disorders. This approach would also lay the groundwork for interesting treatment applications. In fact, it has been reported that the effect of ADHD drugs acting on the dopaminergic and noradrenergic systems is reduced in the presence of iron deficiency. Therefore, if further research confirms low brain iron levels in ADHD, it would provide a strong rationale for trials assessing the effects of ADHD medications plus iron supplementation for ADHD. This might contribute to improve our current clinical practice, thus allowing for a better quality of life of children with ADHD and their families.

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1. THEORETICAL BACKGROUND

1.1. Attention-Deficit/Hyperactivity Disorder (ADHD)

1.1.1 Definition and clinical features

Problems of inattention, hyperactivity, and/or impulsivity represent one of the most common reasons for referral to child neuropsychiatric services. According to the Diagnostic and Statistical Manual of Mental Disorders-4th edition (DSM-IV)¹ and its updated version (Text Revision, TR)², Attention-Deficit/Hyperactivity Disorder (ADHD) is defined by a persistent and age-inappropriate pattern of inattention, hyperactivity-impulsivity or both. Onset before the age of seven and impaired functioning in two or more settings are currently required for the diagnosis. The DSM-IV¹ and IV-TR² define four types of ADHD: “predominantly inattentive”, “predominantly hyperactive-impulsive”, “combined”, and “not otherwise specified”.

The International Classification of Diseases–10th Edition (ICD-10)³ defines a more narrow and severe syndrome, i.e. the Hyperkinetic Disorder (HKD), including a more rigorous criterion for pervasiveness than does DSM-IV-TR, requiring all the three types of symptoms (inattention, hyperactivity, and impulsivity) and discouraging the use of multiple diagnoses (but allowing for a diagnosis of hyperkinetic conduct disorder). Thus, HKD can be approximately assimilated to the combined type of ADHD.

DSM-IV-TR criteria for the diagnosis of ADHD and ADHD types and ICD-10 criteria for the diagnosis of HKD are reported in Table 1 and Table 2, respectively.

Tab. 1. DSM-IV-TR criteria for the diagnosis of Attention-Deficit/Hyperactivity Disorder (ADHD).

A. Either (1) or (2)

(1). 6 (or more) of the following symptoms of inattention have persisted for at least 6 months to a degree that is maladaptive and inconsistent with developmental level:

Inattention

- (a) often fails to give close attention to details or makes careless mistakes in schoolwork, work, or other activities
 - (b) often has difficulty sustaining attention in tasks or play activities
 - (c) often does not seem to listen when spoken to directly
 - (d) often does not follow through on instructions and fails to finish schoolwork, chores, or duties in the workplace (not due to oppositional behaviour or failure to understand instructions)
 - (e) often has difficulty organising tasks and activities
 - (f) often avoids, dislikes, or is reluctant to engage in tasks that require sustained mental effort (such as schoolwork or homework).
 - (g) often loses things necessary for tasks or activities (e.g. toys, school assignments, pencils, books, or tools)
 - (h) is often easily distracted by extraneous stimuli
 - (i) is often forgetful in daily activities
- (2) 6 (or more) of the following symptoms of hyperactivity-impulsivity have persisted for at least 6 months to a degree that is maladaptive and inconsistent with developmental level

Hyperactivity

- (a) often fidgets with hands or feet or squirms in seat
- (b) often leaves seat in classroom or in other situations in which remaining seated is expected
- (c) often runs about or climbs excessively in situations in which it is inappropriate (in adolescents or adults, may be limited to subjective feelings of restlessness)
- (d) often has difficulty playing or engaging in leisure activities quietly
- (e) is often "on the go" or often acts as if "driven by a motor"
- (f) often talks excessively

Impulsivity

- (g) often blurts out answers before questions have been completed
- (h) often has difficulty awaiting turn
- (i) often interrupts or intrudes on others (e.g. butts into conversations or games)

B. Some hyperactive-impulsive or inattentive symptoms that caused impairment were present before age 7 years.

C. Some impairment from the symptoms is present in two or more settings (e.g. at school [or work] and at home).

D. There must be clear evidence of clinically significant impairment in social, academic, or occupational functioning.

E. The symptoms do not occur exclusively during the course of a Pervasive Developmental Disorder, Schizophrenia, or other Psychotic Disorder and are not better accounted for by another mental disorder (e.g. Mood Disorder, Anxiety Disorder, Dissociative Disorder, or a Personality Disorder)

314.01 ADHD, Combined Type - if both A1 and A2 for at least 6 months

314.00 ADHD, Predominantly Inattentive Type

314.01 ADHD, Predominantly Hyperactive-Impulsive Type

Tab. 2. ICD-10 criteria for the diagnosis of Hyperkinetic Disorders

G1 At least six of the following symptoms of attention have persisted for at least six months, to a degree that is maladaptive and inconsistent with the developmental level of the child:

- (1) often fails to give close attention to details, or makes careless errors in school work, work or other activities;
- (2) often fails to sustain attention in tasks or play activities;
- (3) often appears not to listen to what is being said to him or her;
- (4) often fails to follow through on instructions or to finish school work, chores, or duties in the workplace (not because of oppositional behaviour or failure to understand instructions);
- (5) is often impaired in organising tasks and activities;
- (6) often avoids or strongly dislikes tasks, such as homework, that require sustained mental effort;
- (7) often loses things necessary for certain tasks and activities, such as school assignments, pencils, books, toys or tools;
- (8) is often easily distracted by external stimuli;
- (9) is often forgetful in the course of daily activities.

G2 At least three of the following symptoms of hyperactivity have persisted for at least six months, to a degree that is maladaptive and inconsistent with the developmental level of the child:

- (1) often fidgets with hands or feet or squirms on seat;
- (2) leaves seat in classroom or in other situations in which remaining seated is expected;
- (3) often runs about or climbs excessively in situations in which it is inappropriate (in adolescents or adults, only feelings of restlessness may be present);
- (4) is often unduly noisy in playing or has difficulty in engaging quietly in leisure activities;
- (5) exhibits a persistent pattern of excessive motor activity that is not substantially modified by social context or demands.

G3 At least one of the following symptoms of impulsivity has persisted for at least six months, to a degree that is maladaptive and inconsistent with the developmental level of the child:

- (1) often blurts out answers before questions have been completed;
- (2) often fails to wait in lines or await turns in games or group situations;
- (3) often interrupts or intrudes on others (e.g. butts into others' conversations or games);
- (4) often talks excessively without appropriate response to social constraints.

G4 Onset of the disorder is no later than the age of seven years.

G5 Pervasiveness

The criteria should be met for more than a single situation, e.g. the combination of inattention and hyperactivity should be present both at home and at school, or at both school and another setting where children are observed, such as a clinic. (Evidence for cross-situationality will ordinarily require information from more than one source; parental reports about classroom behaviour, for instance, are unlikely to be sufficient.)

G6 The symptoms in G1 and G3 cause clinically significant distress or impairment in social, academic, or occupational functioning.

G7 The disorder does not meet the criteria for pervasive developmental disorders (F84.-), manic episode (F30.-), depressive episode (F32.-), or anxiety disorders (F41.-).

Deficits in executive functions (defined as set of cognitive skills that are necessary to plan, monitor and execute a sequence of goal-directed complex actions and including inhibition, working memory, planning, and sustained attention) are common, although far from universal, in ADHD⁴.

Evidence from several clinical as well as epidemiological studies has well established that ADHD is frequently co-morbid with other psychiatric and neuro-developmental disorders. It has been reported that 54% to 84% of children and adolescents with ADHD may meet criteria for oppositional defiant disorder (ODD); a significant portion of these patients will develop conduct disorder (CD)^{5:6}. Fifteen percent to 19% of patients with ADHD will start to smoke⁷ or develop other substance abuse disorders⁸. About 25%-35% of patients with ADHD present with learning or language problem⁹. Anxiety disorders occur in up to one third of patients with ADHD⁹. The prevalence of mood disorders in patients with ADHD is more controversial, with an estimated prevalence between 0% to 33% of patients with ADHD meeting criteria for a depressive disorder⁹. The prevalence of mania among patients with ADHD remains a contentious issue⁹. It has also been reported a relationship between ADHD and Tourette's Syndrome (TS): about 10-30% of ADHD patients meet criteria for TS¹⁰. Mental retardation and borderline intellectual functioning are commonly mislabelled ADHD, although they often co-occur with ADHD¹¹. However, the relationship between ADHD and mental retardation/borderline intellectual functioning needs further investigation¹². Finally, although DSM-IV-TR criteria prohibits the co-diagnosis of ADHD and an autism spectrum disorder (ASD), recent studies indicate that co-occurrence of clinically significant ADHD and autistic symptoms is common, and that some genes may influence both disorders, suggesting that an eventual diagnosis of ADHD in patients with ASD may prompt the appropriate management of inattention, hyperactivity and impulsivity in patients with ASD¹³.

Because of inattention, hyperactivity/impulsivity, and eventual co-morbidities, children with ADHD suffer from various combinations of impairments in functioning at school, at home, and with peers. School-based problems include lower than expected or erratic grades, achievement test scores, and intelligence test scores, with possible grade retention. Behavioral difficulties related to

ADHD or to the combination with co-morbid conditions often lead to constant friction among the schoolmates, peers, the teacher, and the parents. Peers often quickly reject ADHD children, due to their impulsivity and noncompliance with rules¹¹.

1.1.2 Epidemiology

Considering that ADHD is a clinical diagnosis, the criteria adopted and validity of measures used play a major role in the results that are generated by epidemiological studies.

In the most recent and comprehensive meta-analysis¹⁴ including 102 studies comprising 171,756 subjects from several world regions, the aggregated prevalence of ADHD based on all studies was 5.29% (95% CI, 5.01–5.56). The pooled prevalence for children and adolescents were respectively 6.48% (4.62–8.35) and 2.74% (2.04–3.45). However, the estimate was associated with significant variability, with prevalences ranging from very low estimates of 0.2% to much higher estimates, up 27%. In the multivariate meta-regression model, diagnostic criteria, source of information, requirement of impairment for diagnosis, and geographic origin of the studies were significantly associated with ADHD prevalence rates. As expected, studies without a definition of impairment had significantly higher ADHD prevalence rates than those with a definition of impairment. Studies based on DSM-III-R or ICD-10 criteria, respectively, had significantly lower ADHD prevalence rates than those using DSM-IV criteria. Studies that relied on information provided by parents, teachers, and “or rule” (a symptom is considered present if it is endorsed by parents or teachers), respectively, were associated with significantly higher ADHD prevalence rates than those relying on a “best-estimate procedure” (merging of parental and teacher reports), whereas those relying on information provided using the “and rule” criterion (a symptom is considered present only if it is endorsed by both parents and teachers) were associated with significantly lower ADHD prevalence estimates. Geographic location was associated with significant variability only between estimates from North America and both Africa and the Middle East. No significant differences were found between Europe and North America. Therefore, geographic location plays a limited role in the reasons for the large variability of ADHD prevalence estimates worldwide. Instead, this variability seems to be explained primarily by the methodological characteristics of studies

Until cross-national studies are performed using parallel diagnostic interview methods, identical or comparable sampling frames, and similarly defined populations, determining whether meaningful differences exist among countries will not be possible.

1.1.3 Etiology

The etiology of ADHD includes genetic and environmental factors

Genetic factors

According to twin and adoption studies, genes play a relevant role in initiating ADHD. It has been estimated that the heritability of ADHD is about 0.76, meaning that genetic factors account for about 80% of the etiology of ADHD⁴.

Genome-wide scans that have been conducted thus far show divergent findings. Moreover, so far, candidate gene studies have shown that, for ADHD, genes with moderately large effects are unlikely to exist. The variability in findings and lack of replication is presumed to be due, at least in part, to diagnostic heterogeneity¹⁵.

However, the following genes have been reported to have a small but significant effect: the dopamine D4 receptor gene (*DRD4*), the dopamine D5 receptor gene (*DRD5*), the dopamine transporter gene (*SLC6A3*), the synaptosomal-associated protein of 25kD gene (*SNAP25*), and the serotonin 1B receptor gene (*HTR1B*). This is consistent with the idea that the genetic vulnerability to ADHD is mediated by many genes of small effects¹⁶.

By contrast with these positive findings, studies of other genes possibly involved in the neurobiology of ADHD on theoretical bases, have produced equivocal or negative results. They include: the dopamine D2 and D3 receptor gene (*DRD2* and *DRD3*), the dopamine beta-hydroxylase gene (*DBH*), the catechol-O-methyltransferase gene (*COMT*), the monoamine oxidase-A (*MAO-A*), the tryptophan hydroxylase-2 gene (*TPH-2*), the norepinephrine transporter gene (*SLC6A2*), the serotonin transporter gene (*SLC6A4*), the nicotinic acetylcholine receptors (*CHRNA4*), the glutamate receptor gene (*GRIN2A*), the 2A, 2C, and 1C norepinephrine receptors¹⁶, and the brain-derived neurotrophic factor gene (*BDNF*).

Environmental factors

Environmental factors may be classified as pre-natal, peri-natal, and post-natal in origin.

Proposed pregnancy- and birth-related risk factors include maternal smoking, exanthema, maternal anemia, breech delivery, prematurity, low birth weight, hypoxic-ischemic encephalopathy, small head circumference, cocaine and alcohol exposure, lead exposure, and iodine and thyroid deficiency. Childhood illnesses associated with occurrence of ADHD include viral infections, meningitis, encephalitis, otitis media, anemia, cardiac disease, thyroid disease, epilepsy, and autoimmune and metabolic disorders. Other causative factors might include head injury involving the frontal lobes, toxins and drugs, and nutritional disorders, the involvement of many being controversial (e.g., food additives, food allergies, sucrose, gluten sensitivity, and fatty acids)¹⁷.

Among all these proposed risk factors, maternal smoking has attracted the greatest attention in the recent literature. A systematic search of the literature found 24 studies on maternal tobacco smoking published between 1973 and 2002, all of which indicated an increased risk of ADHD in the offspring¹⁸.

In contrast to the risk of ADHD with prenatal nicotine exposure, the results of studies that have linked alcohol with ADHD are less uniform, and the findings reported from 9 alcohol studies have been contradictory¹⁷.

Although the results of the available studies have been variable, lead, and particularly prenatal exposure to lead, seems to be a risk factor for ADHD¹⁷.

The role of prematurity and peri-natal hypoxic-ischemic encephalopathy have been extensively investigated. About one third of premature infants with birth weights of <1500 g have been reported to have ADHD^{4;18}.

Viral infections during pregnancy, at birth, and in early childhood have been linked to an increased risk of developing ADHD. Measles, varicella, rubella, HIV, enterovirus 71, and varicella zoster encephalitis have been reported to have a significant association with ADHD. Although typical ADHD seems to have a weak association with otitis media, some researchers consider a history of significant middle-ear disease in early childhood to be a risk factor for hyperactivity and

especially speech and language disorders in children who present with learning problems in school¹⁷.

In a high proportion of post natal factors, a genetic factor is a likely basic cause, and the environmental factor is likely to act as a trigger.

Studies have provided variable results on zinc deficiency, omega-3 fatty acids, and iodine deficiency and therefore their significance is controversial¹⁷

It has also been shown that chronic conflict, reduced family cohesion, and exposure to parental psychopathology (especially maternal psychopathology) were more common in ADHD families compared with control families. In these studies, the differences between children with or without ADHD were not accounted for by social class or by parental history of major psychopathology⁴.

Gene-environment interaction is increasingly recognized as an important mechanism in the etiology and development of ADHD, with some genes (e.g., *DAT1*) affecting the individual sensitivity to environmental etiologic factors^{4;16;17}.

1.1.4 Pathophysiology

The pathophysiology of ADHD is not completely understood, although imbalances in dopaminergic and noradrenergic systems have been implicated in the core symptoms that characterize this disorder¹⁹.

The overt behavioral signs of ADHD (i.e., excessive inattention, hyperactivity, and impulsivity) are thought to result from underlying deficits in response inhibition, delay aversion, and executive functioning²⁰. In turn, these neuropsychological deficits are presumed to be linked to structural and functional brain abnormalities in frontal-striatal-cerebellar circuits²⁰.

So far, research has shown that the complex pathophysiology of ADHD, like that of other psychiatric disorders, is reflected in microscopic and quantitative, rather than macroscopic and qualitative, differences in brain development.

Imaging studies carried out in the last 20 years have helped elucidating the brain structures potentially involved in the pathophysiology of ADHD.

According to the largest study performed to date compared regional brain volumes of 152 children and adolescents who had ADHD and 139 controls children with ADHD, overall cerebral volumes were 3.2% smaller in children with ADHD than controls, affecting all four major lobes (frontal, parietal, temporal, and occipital)²¹. Further studies have confirmed these data and suggested that this effect may not be accounted for by age, height, weight, or IQ²⁰.

Beyond differences in brain volume, there is more conflicting evidence than certainties. Although converging findings from studies on the neuropharmacology, genetics, neuropsychology, and neuroimaging of ADHD attribute a central role to fronto-striatal pathway disruption in ADHD, current theories suggest that the disorder may result from a disruption in a more distributed circuitry, including the frontal brain regions, as well as the basal ganglia, the cerebellar hemispheres, and the cerebellar vermis.

According to a recent meta-analysis of structural MRI studies by Valera et al.²², the pooled data from available imaging studies suggest that children with ADHD present with a significant

global brain reduction compared to control subjects, with the largest differences observed in cerebellar vermis, corpus callosum, total and right cerebral volume, and right caudate.

Frontal cortex

Numerous structural neuroimaging studies have reported significantly smaller volumes of the frontal cortex in children who have ADHD compared with healthy controls^{22;23}. This volume decrease appears to be particularly significant in the right prefrontal cortex, reducing the typical right greater than left asymmetry of the prefrontal cortex in children who have ADHD.

Moreover, functional imaging studies have shown that the expected increase in prefrontal metabolism during response inhibition tasks is reduced markedly in ADHD subjects²³. A recent meta-analysis²⁴ of 16 published functional neuroimaging studies of ADHD revealed that significant patterns of frontal hypoactivity are detected across studies in patients who have ADHD, affecting anterior cingulate, dorsolateral prefrontal, and inferior prefrontal cortices. More recently, Shaw and colleagues²⁵ examined the relationships among cortical thickness measures, baseline diagnosis, and clinical outcome in the NIMH cohort of children and adolescents who had ADHD. Results confirmed previous findings of cortical abnormalities, prominently in medial and superior prefrontal and precentral regions, which are important for attentional control and motor output. The same group also reported a longitudinal comparison of cortical thickness between patients with ADHD and healthy controls. They showed that rather than a deviation from typical development, ADHD exhibited a marked delay in reaching peak thickness in most cortical regions.

Basal ganglia

As the caudate nuclei and associated subcortical circuits receive inputs from cortical regions implicated in executive functioning and attentional processes, research has also focused on the role of the basal ganglia on the pathophysiology of ADHD.

One of the earliest studies reported decreased metabolism in the striatal region, particularly the right caudate²⁶. Later studies have shown size reductions of the right globus pallidus and smaller volumes of left and right caudate²⁷. Indices of asymmetry are intrinsically less reliable: right greater

than left and left greater than right asymmetries and symmetric caudate volumes have all been reported to be associated with ADHD²⁰. A study by Castellanos and colleagues²¹, however, found that such volumetric differences may be transient and possibly related to developmental changes in symptoms of ADHD with increasing age.

Cerebellum

A significantly smaller posterior inferior lobe (lobules VIII-X) of the vermis consistently has been replicated in ADHD²⁰. In a longitudinal study²¹, the global nonprogressive reduction in total volume was shown to be more specific to the superior cerebellar vermis, a region that exhibited a volumetric developmental trajectory associated with better or worse clinical outcomes²⁸.

1.1.5 Assessment

According to the practice parameters of the American Academy of Child and Adolescent Psychiatry⁹, screening for ADHD should be part of every patient's mental health assessment by specifically asking questions regarding the major symptom domains of ADHD (inattention, impulsivity, and hyperactivity) and asking whether such symptoms cause impairment. If a parent reports that the patient suffers from any symptoms of ADHD, then a full evaluation for ADHD is indicated⁹.

The history/intake interview focusing on the chief complaints about inattention, impulsivity, distractibility, and hyperactivity at school and at home, and specifically addressing the DSM-IV criteria for ADHD, is the cornerstone of the diagnostic assessment for ADHD⁹. The clinician should perform a detailed interview with the parent about each of the 18 ADHD symptoms listed in DSM-IV. For each symptom, the clinician should determine whether it is present as well as its duration, severity, and frequency. Age at onset of the symptoms should be assessed. The patient must have the required number of symptoms, a chronic course, and onset of symptoms during childhood. Presence of impairment should be distinguished from presence of symptoms. For instance, a patient's ADHD symptoms may be observable only at school but not at home. Nonetheless, if the patient must spend an inordinate amount of time completing schoolwork in the evening that was not done in class, then impairment is present in two settings. DSM-IV requires impairment in at least two settings (home, school, or job) to meet criteria for the disorder, but clinical consensus agrees that severe impairment in one setting warrants treatment⁹. After reviewing the ADHD symptoms, the clinician should interview the parent regarding other common psychiatric disorders of childhood. Formal structured and semi-structured interviews like the Schedule for Affective Disorders and Schizophrenia for School-Age Children²⁹, the National Institute of Mental Health (NIMH) Diagnostic Interview Schedule for Children³⁰, the DSM-based Diagnostic Interview For Children and Adolescents (DICA)³¹, and the Child and Adolescent Psychiatric Assessment³² are available. Questionnaires, such as the Conners Parent³³ and Teacher³⁴ Rating Scales–Revised (CRS-

R) are an important and efficient part of the diagnostic assessment but cannot be used in isolation to make a diagnosis of ADHD.

The most commonly used questionnaires to assess ADHD symptoms are reported in Tab. 3.

Tab. 3. Questionnaires for the assessment of ADHD symptoms (*From: Pliszka et al., JAACAP, 2007⁹*).

Name of Scale	Reference
Academic Performance Rating Scale (APRS)	The APRS is a 19-item scale for determining a child's academic productivity and accuracy in grades 1–6 that has 6 scale points; construct, concurrent, and discriminant validity data as well as norms ($n = 247$) available (Barkley, 1990).
ADHD Rating Scale-IV	The ADHD Rating Scale-IV is an 18-item scale using <i>DSM-IV</i> criteria (DuPaul et al., 1998).
Brown ADD Rating Scales for Children, Adolescents, and Adults	Psychological Corporation, San Antonio, TX (www.drthomasebrown.com/asses_tools/index.html) (Brown, 2001)
Child Behavior Checklist (CBCL)	Parent-completed CBCL and Teacher-completed Teacher Report Form (TRF) www.aseba.org/index.html
Conners Parent Rating Scale–Revised (CPRS-R) ^a	Parent and adolescent self-report versions available (Conners, 1997)
Conners Teacher Rating Scale–Revised (CTRS-R) ^a	Conners, 1997
Conners Wells Adolescent Self-Report Scale	Conners and Wells, 1997
Home Situations Questionnaire–Revised (HSQ-R), School Situations Questionnaire–Revised (SSQ-R)	The HSQ-R is a 14-item scale designed to assess specific problems with attention and concentration across a variety of home and public situations; it uses a 0–9 scale and has test-retest, internal consistency, construct validity, discriminant validity, concurrent validity, and norms ($n = 581$) available (Barkley, 1990).
Inattention/Overactivity With Aggression (IOWA) Conners Teacher Rating Scale	The IOWA Conners is a 10-item scale developed to separate the inattention and overactivity ratings from oppositional defiance (Loney and Milich, 1982)
Swanson, Nolan, and Pelham (SNAP-IV) and SKAMP Internet site ADHD.NET	The SNAP-IV (Swanson, 1992) is a 26-item scale that contains <i>DSM-IV</i> criteria for ADHD and screens for other <i>DSM</i> diagnoses; the SKAMP (Wigal et al., 1998) is a 10-item scale that measures impairment of functioning at home and at school.
Vanderbilt ADHD Diagnostic Parent and Teacher Scales	Teachers rate 35 symptoms and 8 performance items measuring ADHD symptoms and common comorbid conditions (Wolraich et al., 2003a). The parent version contains all 18 ADHD symptoms, with items assessing comorbid conditions and performance (Wolraich et al., 2003b).

Note: ADHD = attention-deficit/hyperactivity disorder.

^a The longer form should be used for initial assessment, whereas the shorter form is often used for assessing response to treatment, particularly when repeated administration is required.

Teachers, parents, and older children can/should all report on symptoms to assess for agreement/validity of diagnosis, to document that the ADHD symptoms occur in multiple settings, and to take advantage of the special information that each can provide. Multiple informants (parents, teachers, youths, and health care professionals) do not necessarily agree on diagnosis. If the teacher cannot provide such a rating scale or the parent declines permission to contact the school, then materials from school, such as work samples or report cards, should be reviewed or inquired about. Care must be taken to determine the best interpretation of the data if disagreements occur.

A thorough medical history is part of the initial evaluation. For example, as reported in the previous section, prematurity and frequent episodes of otitis with hearing loss may be risk factors of ADHD. A complete family history for ADHD and its common co-morbidities should be taken. ADHD is a highly heritable disorder, and, although a positive family history does not confirm the diagnosis, it can be supportive. Moreover, because patients with ADHD perform better in structured settings, any factors in the family that create an inconsistent, disorganized environment may further impair the patient's functioning.

After interviewing the parents, the clinician should interview the child or adolescent. For the preschool or young school-age child the interview may be done concurrently with the parent interview. Older children and adolescents should be interviewed separately from parents. The primary purpose of the interview with the child or adolescent is not to confirm or refute the diagnosis of ADHD. Young children are often unaware of their symptoms of ADHD, and older children and adolescents may be aware of symptoms but will minimize their significance. The interview with the child or adolescent allows the clinician to identify signs or symptoms inconsistent with ADHD or suggestive of other serious co-morbid disorders. This is achieved by means of a mental status examination, assessing appearance, sensorium, mood, affect, and thought processes.

Since several medical problems can be associated with ADHD, as previously discussed, the general medical examination is indispensable to corroborate some of these conditions³⁵. The neurologic examination is part of any complete diagnostic evaluation. In addition to the traditional neurologic examination, a number of standardized office examinations that tap developmental neurologic functions are available. Furthermore, the neurologic examination provides an opportunity to evaluate for commonly co-morbid neurologic problems of coordination like dyspraxia, and dysgraphia³⁵.

Neuropsychological testing is not a necessary part of the diagnostic assessment of ADHD, unless specific co-morbid or associated learning issues need to be evaluated. Results of neuropsychological testing, however, may lend support to the diagnosis. Although neuropsychological testing may be supportive of the ADHD diagnosis, it cannot be used in isolation to diagnose ADHD⁹. Executive functions should be particularly investigated, although deficits in executive functioning are by no means always present in ADHD children nor are they unique to ADHD. Intelligence should be assessed. Higher IQ ADHD children may compensate for their attention difficulties sufficiently to mask executive dysfunction on traditional measures.

If the patient's medical history is unremarkable, laboratory or neurological testing is not indicated. The measurement of thyroid levels and thyroid-stimulating hormone should be considered only if symptoms of hyperthyroidism other than increased activity level are present. Exposure to lead, either prenatally or during development, is associated with a number of neurocognitive impairments, including ADHD. If a patient has been raised in environment where exposure to lead paint or plumbing is probable, then serum lead levels should be considered. Serum lead level should not be part of routine screening.

Unless there is strong evidence of such factors in the medical history, neurological studies (EEG), magnetic resonance imaging, SPECT, or PET are not indicated for the evaluation of ADHD. Although differences between electroencephalograms, particularly quantitative ones, and evoked

responses in ADHD versus controls have been reported, the number and quality of such studies do not support the use of these measures for diagnostic purposes⁹.

1.1.6 Outcome

Follow-up studies have begun to delineate the life course of ADHD. A majority (60%-85%) of children with ADHD will continue to meet criteria for the disorder during their teenage years³⁶, clearly indicating that ADHD does not remit with the onset of puberty alone. Although only 40% of 18- to 20-year-old “grown up” ADHD patients met the full criteria for ADHD, 90% had at least five symptoms of ADHD and a significant impairment, as indicated by a Global Assessment of Functioning score below 60. Adults with a childhood history of ADHD have higher than expected rates of antisocial and criminal behaviour³⁷, injuries and accidents³⁷, employment and marital difficulties, and health problems and are more likely to have teen pregnancies³⁸ and children out of wedlock.

1.1.7 Treatment

The patient's treatment plan may consist of psychopharmacological and/or behavior therapy⁹. This plan should include parental and child psychoeducation about ADHD and its various treatment options, linkage with community supports, and additional school resources as appropriate. After reviewing all available evidence, the American Academy of Child and Adolescent Psychiatry⁹ concluded that it seems established that a pharmacological intervention for ADHD is more effective than a behavioral treatment alone. Behavior therapy may be recommended as an initial treatment if the patient's ADHD symptoms are mild with minimal impairment, the diagnosis of ADHD is uncertain, parents reject medication treatment, or there is marked disagreement about the diagnosis between parents or between parents and teachers³⁹. Several studies have shown short-term effectiveness of behavioral parent training³⁹. No evidence support nonpharmacological interventions other than behavior therapy⁹, including cognitive-behavioral therapy and dietary modification. The AACAP practice parameters recommend that the initial psychopharmacological treatment of ADHD should be a trial with an agent approved by the Food and Drug Administration for the treatment of ADHD. Table 4 lists the currently approved FDA drugs for ADHD.

Tab. 4. Medications Approved by the FDA for ADHD (Alphabetical by Class) (From: *Pliszka et al., JAACAP, 2007*⁹).

Generic Class/ Brand Name	Dose Form	Typical Starting Dose	FDA Max/Day	Off-Label Max/Day	Comments
Amphetamine preparations					
Short-acting					
Adderall ^a	5, 7.5, 10, 12.5, 15, 20, 30 mg tab	3–5 y: 2.5 mg q.d.; ≥6 y: 5 mg q.d.–b.i.d.	40 mg	>50 kg: 60 mg	Short-acting stimulants often used as initial treatment in small children (<16 kg), but have disadvantage of b.i.d.–t.i.d. dosing to control symptoms throughout day
Dexedrine ^a	5 mg cap	3–5 y: 2.5 mg q.d.			
DextroStar ^a	5, 10 mg cap	≥6 y: 5 mg q.d.–b.i.d.			
Long-acting					
Dexedrine Spansule	5, 10, 15 mg cap	≥6 y: 5–10 mg q.d.–b.i.d.	40 mg	>50 kg: 60 mg	Longer acting stimulants offer greater convenience, confidentiality, and compliance with single daily dosing but may have greater problematic effects on evening appetite and sleep Adderall XR cap may be opened and sprinkled on soft foods
Adderall XR	5, 10, 15, 20, 25, 30 mg cap	≥6 y: 10 mg q.d.	30 mg	>50 kg: 60 mg	
Lisdexamfetamine	30, 50, 70 mg cap	30 mg q.d.	70 mg	Not yet known	
Methylphenidate preparations					
Short-acting					
Focalin	2.5, 5, 10 mg cap	2.5 mg b.i.d.	20 mg	50 mg	Short-acting stimulants often used as initial treatment in small children (<16 kg) but have disadvantage of b.i.d.–t.i.d. dosing to control symptoms throughout day
Methylin ^a	5, 10, 20 mg tab	5 mg b.i.d.	60 mg	>50 kg: 100 mg	
Ritalin ^a	5, 10, 20 mg	5 mg b.i.d.	60 mg	>50 kg: 100 mg	
Intermediate-acting					
Metadate ER	10, 20 mg cap	10 mg q.a.m.	60 mg	>50 kg: 100 mg	Longer acting stimulants offer greater convenience, confidentiality, and compliance with single daily dosing but may have greater problematic effects on evening appetite and sleep Metadate CD and Ritalin LA caps may be opened and sprinkled on soft food
Methylin ER	10, 20 mg cap	10 mg q.a.m.	60 mg	>50 kg: 100 mg	
Ritalin SR ^a	20 mg	10 mg q.a.m.	60 mg	>50 kg: 100 mg	
Metadate CD	10, 20, 30, 40, 50, 60 mg	20 mg q.a.m.	60 mg	>50 kg: 100 mg	
Ritalin LA	10, 20, 30, 40 mg	20 mg q.a.m.	60 mg	>50 kg: 100 mg	
Long-acting					
Concerta	18, 27, 36, 54 mg cap	18 mg q.a.m.	72 mg	108 mg	Swallow whole with liquids Nonabsorbable tablet shell may be seen in stool.
Daytrana patch	10, 15, 20, 30 mg patches	Begin with 10 mg patch q.d., then titrate up by patch strength	30 mg	Not yet known	
Focalin XR	5, 10, 15, 20 mg cap	5 mg q.a.m.	30 mg	50 mg	
Selective norepinephrine reuptake inhibitor					
Atomoxetine					
Strattera	10, 18, 25, 40, 60, 80, 100 mg cap	Children and adolescents <70 kg: 0.5 mg/kg/day for 4 days; then 1 mg/kg/day for 4 days; then 1.2 mg/kg/day	Lesser of 1.4 mg/kg or 100 mg	Lesser of 1.8 mg/kg or 100 mg	Not a schedule II medication Consider if active substance abuse or severe side effects of stimulants (mood lability, tics); give q.a.m. or divided doses b.i.d. (effects on late evening behavior); do not open capsule; monitor closely for suicidal thinking and behavior, clinical worsening, or unusual changes in behavior

Note: FDA = U.S. Food and Drug Administration; ADHD = attention-deficit/hyperactivity disorder.

^a Generic formulation available.

The American Academy of Pediatrics⁴⁰, an international consensus statement⁴¹, and the Texas Children's Medication Project⁴² have recommended stimulants as the first choice. At present time, the following drugs have been approved by FDA: dextroamphetamine (DEX), D and D,L-methylphenidate (MPH), mixed salts amphetamine, and atomoxetine⁹. Several randomized controlled trials in the past 30 years have consistently reported the effectiveness of stimulants for ADHD symptoms (the effect size of stimulant treatment relative to placebo is rather large, averaging about 1.0, one of the largest effects for any psychotropic medication⁹). The physician is free to choose any of the two stimulant types (MPH or amphetamine) because evidence suggests the two are equally efficacious in the treatment of ADHD. There is at present, no method to predict which stimulant will produce the best response in a given patient. The most common side effects of stimulants are appetite decrease, weight loss, insomnia, or headache. Less common side effects of stimulants include tics and emotional lability/irritability⁹.

Atomoxetine is currently considered as a second line pharmacological treatment. Direct comparisons of the efficacy of atomoxetine with that of MPH⁴³ and amphetamine⁹ have shown a greater treatment effect of the stimulants, and in a meta-analysis of atomoxetine and stimulant studies, the effect size for atomoxetine was 0.62 compared with 0.91 and 0.95 for immediate-release and long-acting stimulants, respectively⁹. However, atomoxetine may be considered as the first medication for ADHD in individuals with an active substance abuse problem, comorbid anxiety, or tics. Atomoxetine is preferred if the patient experiences severe side effects to stimulants such as mood lability or tics. Side effects of atomoxetine that occurred more often than those with placebo include gastrointestinal distress, sedation, and decreased appetite.

If a patient fails to respond to trials of all of these agents after an adequate length of time at appropriate doses for the agent as noted in Table 2, then the clinician should undertake a review of the patient's diagnosis of ADHD⁹. If the diagnosis of ADHD is confirmed, second line non FDA approved drugs may be considered, including bupropion, tricyclic antidepressants (TCAs), and alpha-agonists. These agents may have effect sizes considerably less than those of the approved agents

and comparable with the effectiveness of behaviour therapy⁴⁴. Thus, it may be prudent for the clinician to recommend a trial of behavior therapy at this point, before moving to these second-line agents. Bupropion may cause mild insomnia or loss of appetite. Extremely high single doses (>400 mg) of bupropion may induce seizures even in patients without epilepsy. TCAs frequently cause anticholinergic side effects such as dry mouth, sedation, constipation, changes in vision, or tachycardia. Reduction in dose or discontinuation of the TCA is often required if these side effects induce impairment. Side effects of alpha-agonists include sedation, dizziness, and possible hypotension. According to the AACAP practice parameters⁹, if a patient with ADHD has a robust response to psychopharmacological treatment and subsequently shows normative functioning in academic, family, and social functioning, then psychopharmacological treatment of the ADHD alone is satisfactory. On the other hand, if a patient with ADHD has a less than optimal response to medication, has a co-morbid disorder, or experiences stressors in family life, then psychosocial treatment in conjunction with medication treatment is often beneficial.

1.2 Iron metabolism

1.2.1 Systemic iron

1.2.1.1 Introduction

Iron is an element which has the capacity to accept and donate electrons readily, interconverting between ferrous (Fe 2+) and ferric (Fe3+) forms⁴⁵. The ease with which iron can gain and lose electrons makes it a useful component of oxygen-binding molecules (i.e., hemoglobin and myoglobin), cytochromes and various nonhaem enzymes⁴⁶. Therefore, iron plays a central role in a multitude of biological functions, including cellular respiration, DNA synthesis, and key metabolic reactions in neurotransmitter production, collagen formation, and immune system function⁴⁷.

However, the same facile property that permits iron to gain and lose electrons can result in the donation of electrons to oxygen, causing the generation of superoxide anions and the hydroxyl radical (Fenton reaction). These oxygen metabolites react readily with biological molecules, including proteins, lipids and DNA. Therefore, iron loading can lead to free radical damage^{46;48}.

Thus, since both iron deficiency and iron overload are deleterious⁴⁵, the challenge for most organisms is to acquire adequate amounts of iron for critical biological processes yet avoid the toxicity associated with free iron⁴⁹. Consequently, all mammalian species tightly regulate the concentration of iron on both the systemic and the cellular levels^{46;50}. To achieve this, at the systemic level, a complex system of proteins involved in iron uptake, utilisation, storage and export is present⁵¹. The control of systemic iron levels occurs through a meticulous regulation of iron acquisition and storage because in the million years of evolution, nature has not developed a regulated pathway for excretion of iron in humans⁴⁶.

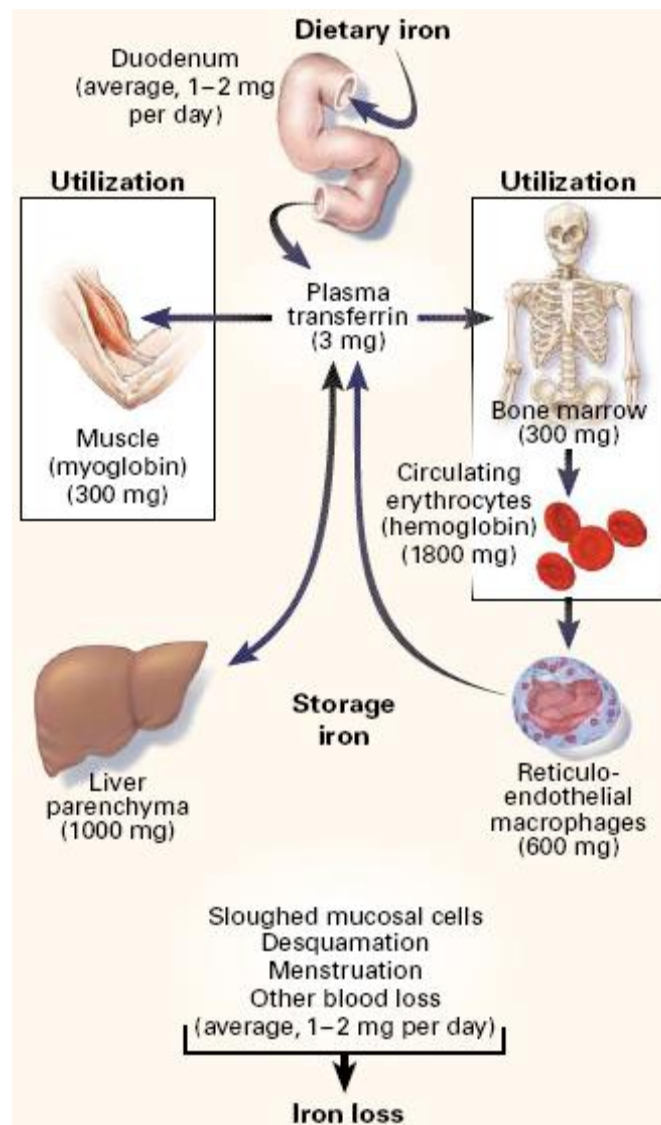
The last decade has seen a rapid advancement of knowledge in iron metabolism. Modern techniques in molecular biology and biochemistry are giving new insights through the identification and characterization of proteins involved in iron homeostasis⁴⁷.

1.2.1.2 Iron distribution in the body

In the body, iron is distributed in three pools: functional, storage and transport.

The distribution of iron in human tissues is shown in Fig. 1.

Fig. 1. Distribution of iron in human tissue (from Andrews, *NEJM*, 1999⁴⁵).



Adult men normally have 35 to 45 mg of iron per kilogram of body weight. Premenopausal women have lower iron stores as a result of their recurrent blood loss through menstruation⁴⁵.

About 0.1% of total body iron is circulating (bound to transferrin). Nearly 80% of iron is incorporated into hemoglobin in developing erythroid precursors and mature red cells. Approximately 10–15% is present in muscle fibers (myoglobin) and in other tissues as enzymes and cytochromes (functional iron)⁴⁷.

In the balanced state, 1 to 2 mg of iron enters and leaves the body each day⁴⁵.

Approximately 1 to 2mg of iron is lost each day by epithelial shedding in the gastrointestinal tract and the skin and through blood loss in menstruating women. There is no physiologic mechanism for excreting larger amounts of iron, even in severely iron-overloaded individuals. The normal losses are balanced by absorption of iron from the diet. Western diets contain a much greater amount of iron (10 to 20 mg) than what is absorbed daily under normal circumstances (1 to 2 mg).

Iron absorption increases several-fold in iron deficiency and is suppressed partly when iron stores are excessive⁴⁹.

1.2.1.3 Iron absorption

Iron absorption occurs predominantly in the apical surface of the duodenum and upper jejunum. The two forms of dietary iron, i.e. heme and non-heme iron, are absorbed by the enterocyte non-competitively. In diets rich in meat, heme accounts for approximately two-thirds of the dietary iron supply of the body⁵⁰.

Inorganic, nonheme iron is inefficiently absorbed but present in a wide variety of foodstuffs. Heme iron is more efficiently absorbed, primarily from animal sources in the form of hemoglobin or myoglobin⁴⁹.

Because there is little or no paracellular iron transport under normal circumstances, iron must traverse both the apical and basolateral membranes to gain access to the circulation⁴⁹.

A membrane protein called heme carrier protein 1 (HCP1) mediates heme uptake by cells. HCP1 is expressed at high levels in the duodenum⁵² and, upon binding of heme to HCP1 on the cell surface, the complex is internalized by receptor-mediated endocytosis. The resultant endosomal vesicles migrate to the endoplasmic reticulum, where iron is liberated from heme by the heme oxygenase-1 (HO1) that is found on the reticulum surface.

The non-heme iron mainly exists in the Fe³⁺ state. The ferric iron is reduced to ferrous iron by dietary components and duodenal cytochrome b reductase (Dcytb) which is highly expressed in the brush border of enterocytes⁵². Once the insoluble Fe³⁺ is converted to Fe²⁺, it enters the mucosal phase. However, ablation of the murine Dcytb homolog Cybrd1 results in no iron-deficient phenotype, suggesting that Dcytb is not essential for dietary iron uptake in the mouse and that another ferrireductase remains to be discovered⁵³. Fe²⁺ is transported across the apical membrane into the cell through a divalent metal transporter (DMT1). DMT1 is expressed at the duodenal brush border where it controls uptake of dietary iron. A pathway for Fe³⁺ transport has also been proposed, where it is transported as an integrin–mobilferrin (IM) complex across the enterocyte. The Fe³⁺ is then converted to Fe²⁺ by the inherent ferrireductase activity of paraferitin⁵⁴.

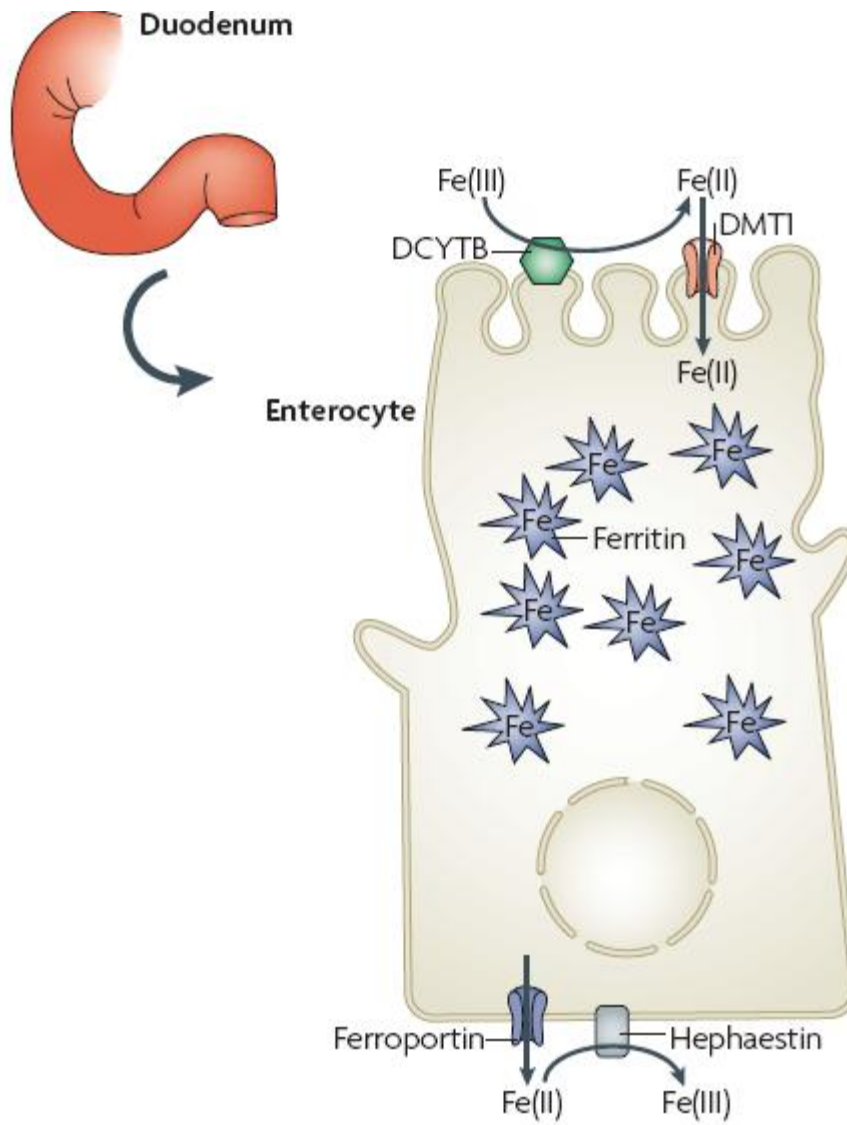
Iron liberated from heme or imported into the enterocyte by DMT1 then enters the hypothesized intracellular or 'labile' iron pool. The molecular character of this pool in enterocytes remains unknown, but it could consist of low molecular weight chelates or chaperone proteins that bind and transport iron⁵⁵. Some of the iron extracted from the diet is stored in ferritin within the enterocyte, and some is exported across the enterocyte's basolateral membrane. The retained iron is lost after two to three days owing to the sloughing of enterocytes into the gut lumen.

The Fe²⁺ inside the cell can undergo two fates: either it can be stored as ferritin or transported across the basolateral surface into the blood stream by a transport protein called ferroportin (FPN)^{56;57}. Ferroportin likely conducts Fe²⁺ ions. Cellular iron export requires an associated ferroxidase activity. In the intestine, ceruloplasmin, a circulating multicopper oxidase, and hephaestin, a homolog of ceruloplasmin, appear to supply this activity. The precise site of ferroxidase activity remains uncertain, but Fe²⁺ must be oxidized to Fe³⁺ by hephaestin to circulate bound to plasma proteins⁴⁹.

Studies have shown that plant and animal ferritin can also be absorbed in the intestine^{58;59}. Receptors for lactoferrin, an iron binding protein, are found in fetal enterocytes. Lactoferrin is viewed as the primary source of iron in infants and it may be a source in adult females also⁶⁰.

Fig. 2 illustrates iron transport across the enterocyte.

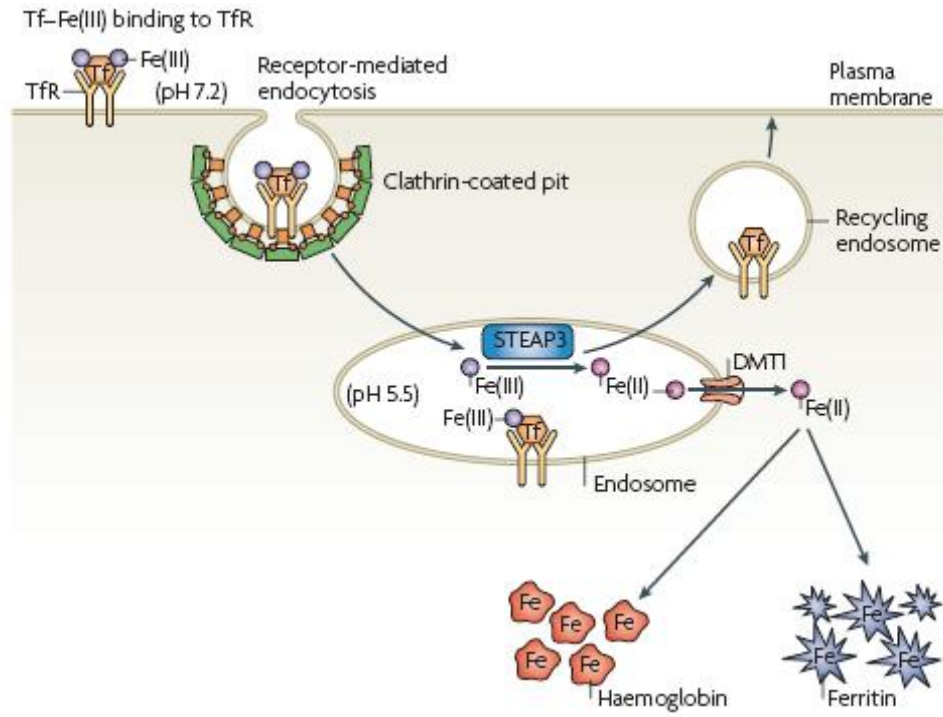
Fig. 2. Iron transport across the enterocyte (from De Domenico et al., *Nature Reviews, Molecular Cell Biology*, 2008⁴⁶).



1.2.1.4 Delivery of iron to tissues

Absorbed iron is rapidly bound to transferrin (TF), an abundant, high affinity iron-binding protein⁴⁹. Under normal circumstances, TF carries nearly all serum iron. Very small amounts of iron may be loosely associated with albumin or small molecules. In normal human subjects, iron occupies approximately 30% of the iron-binding sites on plasma TF. The saturation of TF by iron varies on a diurnal cycle and rapidly responds to local circumstances⁶¹. It is likely to be higher in the portal circulation, where recently absorbed iron from the intestine enters the circulation and passes through the liver⁴⁹. Conversely, TF saturation is likely lower than average in plasma leaving the erythroid bone marrow, where most of the iron present is extracted for use by erythroid precursor cells⁴⁹. Binding of iron to transferrin limits the ability of iron to generate toxic radicals. Then, transferrin directs iron towards cells that express transferrin receptors⁴⁶. The transferrin-Fe(III) complex in plasma is transported into cells through one of two cell-surface transferrin receptors. Transferrin receptor R-1 is expressed on all dividing cells and is particularly enriched on precursors of the erythron because these cells show the highest demand for iron. Transferrin R-2 is expressed primarily in the liver and binds the transferrin-Fe(III) complex at a much lower affinity than transferrin R-1 does⁴⁶. After binding to its receptor, the complex of Fe(III)-transferrin-transferrin R- is rapidly internalized by receptor-mediated endocytosis through clathrin-coated pits (Fig. 3).

Fig. 3. Endocytosis of the complex of iron, transferrin and the transferrin receptor (*from De Domenico et al., Nature Reviews, Molecular Cell Biology, 2008⁴⁶*).



Inside the cells, the internalized complex localizes to an endosome that is acidified by an AT dependent proton pump that lowers the luminal pH to ~5.5. Acidification produces a conformational change in both transferrin-Fe(III) and transferrin R-1 with the consequent release of iron. The endosomal Fe(III) is converted into Fe(II) by a ferrireductase that has been identified as STEAP3. Endosomal DMT1 transports the product of the STEAP3-catalysed reaction from the endosome to the cytosol. At acidic pH, apotransferrin remains bound to transferrin R-1 and the complex is recycled to the cell surface. At the more neutral pH of plasma, apotransferrin dissociates from transferrin R-1 and is free to bind iron and initiate further rounds of receptor-mediated endocytosis^{62;63}.

1.2.1.5 Iron utilization

The erythroid bone marrow is the largest consumer of iron. Normally, two-thirds of the body iron endowment is found in developing erythroid precursors and mature red blood cells⁴⁹. Although TFRs are widely expressed, most other cells apparently can use non-TFR mechanisms to assimilate iron; it is not clear why hematopoietic cells remain so dependent upon the TF cycle. Once iron leaves the endosome, it must move to the mitochondrion for incorporation into protoporphyrin IX by ferrochelatase to form heme. Heme biosynthesis begins and ends in the mitochondrion, but intermediate steps occur in the cytoplasm⁴⁹. Mitoferrin (also known as SLC25A37) carries out mitochondrial iron import⁶⁵. Murine mitoferrin is a homolog of the zebrafish protein *frascati* and the yeast proteins MRS3 and MRS4⁶⁵. Although muscle cells also require large amounts of iron to produce myoglobin, far less is known about how they assimilate iron⁴⁹. It is not clear whether mitoferrin is also involved in mitochondrial iron uptake in muscle and other nonhematopoietic tissues. Heme is then transported out of the mitochondrion for insertion into protein, such as cytochromes. However, the heme transporters(s) responsible for heme release remain unclear.⁴⁸ Three molecules have been identified as possible mitochondrial heme exporters or transporters: the breast cancer resistance protein (ABCG2)⁶⁶, the ABC-mitochondrial erythroid (ABC-me) transporter⁶⁴ and the feline leukemic virus subgroup C receptor (FLVCR)⁶⁵.

TFR plays an important, but poorly understood, role in lymphopoiesis. It has been shown that TFR is a marker of lymphocyte but is not clear whether TFR is needed for accelerated iron uptake by rapidly proliferating cells or for a different, unrelated function⁶⁶.

1.2.1.6 Iron recycling and storage

Intestinal absorption accounts for only a fraction of iron circulating bound to TF. Because there is no known regulated mechanism for iron excretion, and the amount of iron entering the body each day represents less than 0.1% of the total body iron endowment, most circulating iron must be derived from the recycling of iron already within the system. Quantitatively, recovery of iron from senescent erythrocytes contributes the most. Old and damaged erythrocytes are phagocytosed by tissue macrophages, particularly in the spleen. The cells are lysed, and hemoglobin is catabolized, presumably by heme oxygenase, to liberate iron. Some iron remains in storage in macrophages, although some is exported to plasma TF. FPN is critical for macrophage iron export and can be regulated to change the ratio between stored and released iron⁴⁹. Hepatocytes also serve as depots for iron storage, but they acquire their iron load in a different fashion. Although the TF cycle may be involved in hepatocyte iron acquisition to some extent, non-transferrin-bound iron (NTBI) uptake pathways become particularly important when serum iron levels exceed TF binding capacity⁴⁹.

The identity of the hepatocyte NTBI uptake system is not known. Candidates for the NTBI transporter include L-type calcium channels⁷⁰, transient receptor potential canonical protein TRPC6⁶⁷, and transporters already identified for their ability to transport other metal ions. Hepatocytes have a large capacity to store excess iron. Most storage iron is probably in the form of ferritin, which can be mobilized when needed elsewhere in the body. The substrate for ferritin is Fe(II), which is oxidized to Fe(III) within the ferritin shell, and Fe(III) is stored with ferritin. Ferritin is a multimer of 24 subunits comprised of H (for heavy or heart) and L (for light and liver) protein subunits⁶⁸. Ferritin shells show different combinations of the two subunits depending on the tissue in which they are expressed. The liver and spleen are rich in L subunits, whereas the heart is rich in H subunits.

1.2.1.7 Iron deficiency

If one considers that there is no physiologic pathway for iron excretion, it is easy to understand that iron deficiency (ID) will result from any condition in which dietary iron intake does not meet the body's demands. For this reason, rapidly growing children and premenopausal women are at highest risk. Worldwide, dietary insufficiency as a cause of iron deficiency is usually secondary to intestinal blood loss resulting from parasitosis. In such cases, dietary intake is unable to keep up with chronic losses. Tab. 5 summarizes the most common causes of iron deficiency, which are reviewed in detail and discussed in the recent paper by Borgna-Pignatti and Marsella⁶⁹.

Tab. 5. Causes of iron deficiency (*from Andrews, NEJM, 1999*⁴⁵).

Inadequate absorption

Poor bioavailability
Antacid therapy or high gastric pH
Excess dietary bran, tannin, phytates, or starch
Competition from other metals (e.g., copper or lead)
Loss or dysfunction of absorptive enterocytes
Bowel resection
Celiac disease
Inflammatory bowel disease
Intrinsic enterocyte defects

Increased loss

Gastrointestinal blood loss
Epistaxis
Varices
Gastritis
Ulcer
Tumor
Meckel's diverticulum
Parasitosis
Milk-induced enteropathy of early childhood
Vascular malformations
Inflammatory bowel disease
Diverticulosis
Hemorrhoids
Genitourinary blood loss
Menorrhagia
Cancer
Chronic infection
Pulmonary blood loss
Pulmonary hemosiderosis
Infection
Other blood loss
Trauma
Excessive phlebotomy
Large vascular malformations

During the development of iron deficiency, the human body prioritizes the use of iron in several ways. The erythron has relative priority as compared with other tissues. Red-cell production is unperturbed until iron stores are depleted, as reflected by low serum ferritin levels. The development of iron deficiency is gradual.

Depletion of iron stores is the first step. At the beginning, iron is still available for erythropoiesis. When the stores have been used up, the iron saturation of transferrin (a protein which transports iron in the plasma, as we will explain below) decreases and when its value is less than 15%, patients begin to show evidence of iron-deficient erythropoiesis. The first biochemical clues of iron deficiency are increased levels of free protoporphyrin and zinc protoporphyrin in erythrocytes. The levels of soluble transferrin receptor, a protein-cleavage product that is present in plasma, increase when the lack of iron limits the production of new red cells. Frank anemia with microcytosis is detected later.

On the basis of the temporal course and characteristics of iron deficiency, iron deficient states are defined in the following way:

1. Iron deficiency (ID) is a reduction in body Fe to the extent that cellular storage Fe required for metabolic/physiological functions is fully exhausted, with or without anaemia.
2. Iron deficiency anaemia (IDA) is defined as ID and a low haemoglobin (Hb).
3. Iron-deficient erythropoiesis (IDE) is defined as laboratory evidence of a reduced supply of circulating Fe for erythropoiesis, indicated by either reduced Fe saturation of plasma transferrin or signs of ID in circulating erythrocytes. IDE is not synonymous with ID or IDA; IDE can occur despite normal or even increased storage Fe, due to impaired release of Fe to the plasma(21). IDE is often associated with malignancy or inflammation.

1.2.1.8 Iron regulation

Regulation of cellular iron

Individual cells must maintain internal iron homeostasis to ensure that there is adequate iron for basal functions but no free iron that could promote formation of reactive oxygen species. Cells accomplish this by at least two mechanisms:

First, all mammalian cells produce ferritin, an iron storage protein. Each ferritin polymer can accommodate up to 4500 iron atoms. Ferritin acts as a depot, accepting excess iron and allowing for the mobilization of iron when needed⁴⁹.

The second protective mechanism involves iron regulatory proteins (IRPs), which, when iron is limiting, bind to RNA stem-loop iron regulatory elements (IREs) found in the untranslated regions of mRNAs involved in iron transport and storage. Two IRPs have been identified: IRP1 (approved gene symbol ACO1) and IRP2 (approved gene symbol IREB2)⁵¹. IRP1 can incorporate an iron-sulfur cluster, which acts as an iron sensor⁷⁰. IRP2 does not contain an iron-sulfur cluster, but it is rapidly ubiquitinated and degraded in the presence of excess iron⁷¹. The binding of IRPs to IREs serves either of two purposes, depending upon the location of the IREs. IRP binding to IREs found in the 5' untranslated regions of mRNAs encoding ferritin, sterically blocks the initiation of translation, thus stopping the production of ferritin when iron storage would be counterproductive. On the other hand, IREs found in the 3' untranslated region of TFR1 stabilize the molecule, thus allowing more TFR1 to be produced when iron is limiting. However, the roles of IREs and IRPs *in vivo* remain uncertain. IRP2 binds consensus IRE sequences with an affinity and specificity similar to that of IRP1 (Refs 2, 5), but it has been shown to recognise an exclusive subset of IRE-like motifs⁵¹. IRP2 is specifically modulated in response to stimuli and agents other than iron, such as hypoxia and is more sensitive than IRP1 to variations of iron in the diet, is expressed at high levels in most cell lines and, when abundantly or uniquely expressed, can act as the major or only modulator of intracellular iron metabolism, as also indicated by studies in cells in which either IRP1, IRP2 or both were knocked down⁵¹. Mice lacking either IRP1 or IRP2 are viable, thus

indicating that the two IRPs can compensate for each other. Over the past decade it has been shown that other genes involved in iron uptake, release and utilisation are also controlled by the IRE–IRP regulatory network⁵¹. In addition to modulating ferritin and TFR1 levels, IRPs can regulate the mRNAs for other proteins. IRE structures have been detected in several mRNAs encoding proteins related to iron utilisation (eALAS; mitochondrial aconitase, approved gene symbol ACO2; succinate dehydrogenase, gene symbol SDHB), uptake [divalent metal transporter 1 (DMT1), official symbol SLC11A2] and release (ferroportin, gene symbol SLC40A1)⁵¹.

Regulation of systemic iron balance

Iron balance must be meticulously regulated to provide iron as needed but avoid the toxicity associated with iron excess.

The regulation of systemic iron metabolism involves iron absorption but also macrophage iron recycling and hepatocyte iron mobilization.

For several days after a dietary iron bolus, absorptive enterocytes are resistant to acquiring additional iron. This phenomenon has previously been called “mucosal block.” This blocking action probably results from the accumulation of intracellular iron, leading the enterocyte to believe that its set-point requirements have been met. It may occur even in the presence of systemic iron deficiency. A second regulatory mechanism also senses iron levels but responds to total body iron, rather than dietary iron. This mechanism has been termed the stores regulator. It is capable of changing the amount of iron absorbed to a limited extent: iron absorption is modulated by a factor of only two to three in iron deficient states as compared with iron-replete states. Although the molecular details of the stores regulator are not known, it probably acts at the level of crypt-cell programming, in response to the saturation of plasma transferrin with iron. The third regulatory mechanism, known as the erythropoietic regulator, does not respond to iron levels at all. Rather, it modulates iron absorption in response to the requirements for erythropoiesis. Thus, the stimuli known to modulate the iron homeostatic mechanism are erythroid iron needs, hypoxia, iron deficiency, iron overload, and inflammation.

Hepcidin, a circulating peptide hormone, controls much of systemic iron regulation⁷². Hepcidin expression is altered in response to each of the stimuli known to affect iron homeostasis: it is increased in response to increased serum iron, iron overload, and inflammation and diminished in response to increased erythroid drive, hypoxia, and iron deficiency⁴⁹. Hepcidin interrupts cellular iron export in at least two sites: the intestinal epithelium and tissue macrophages by binding directly to ferroportin and triggering its internalization and degradation within lysosomes⁷³. Under basal conditions, hepcidin expression depends upon signaling through a bone morphogenetic protein. Regulation of hepcidin also occurs in inflammatory conditions. Interleukin-6 (IL-6) and possibly other inflammatory cytokines induce transcription of the hepcidin gene in hepatocytes *in vitro* and *in vivo*⁷⁴. There are 3 sites in the mammalian body that are excluded from the liver-dependent macroregulatory axis: the central nervous system (CNS), testis, and retina⁷⁵.

1.2.1.9 Assessment of peripheral iron status

Tissue iron concentrations

The liver and bone marrow are important and relatively accessible storage sites and the amount of iron present in a tissue biopsy can be estimated either visually, using the Prussian blue reaction on tissue sections, or chemically. There is generally a good correlation between iron concentrations in liver and bone marrow. Assessing marrow iron histologically distinguishes between 'true' iron deficiency and other chronic disorders in which there is impaired release of iron from reticuloendothelial cells⁷⁶.

Therefore, bone-marrow examination to establish the absence of stainable iron remains the gold standard for the diagnosis of iron deficiency, particularly when performed and reviewed under standardized conditions by experienced investigators. However, marrow examinations are expensive, uncomfortable, and require technical expertise, and are not performed routinely in clinical practice⁷⁷.

Haemoglobin

Haemoglobin (Hb) is a widely used screening test for ID, but used alone has low specificity and sensitivity. Its sensitivity is low because individuals with baseline Hb values in the upper range of normal need to lose 20–30% of their body Fe before their Hb falls below the cut-off for anaemia⁷⁸. Its specificity is low because there are many causes of anaemia other than ID. Cut-off criteria differ with the age and sex of the individual, between laboratories, and there are ethnic differences in normal Hb^{79:80}.

Mean corpuscular volume and reticulocyte Hb content (CHr)

Measured on widely-available automated haematology analysers, the mean corpuscular volume (MCV) is a reliable, but relatively late indicator of ID, and its differential diagnosis includes thalassemia. The reticulocyte Hb content has been proposed as a sensitive indicator that falls within days of the onset of IDE⁸¹. However, false normal values can occur when the MCV is

increased or in thalassemia; its wide use is limited as it can only be measured on one model of analyzer. For both MCV and CHr, low specificity limits their clinical utility⁸².

Erythrocyte zinc protoporphyrin

Erythrocyte zinc protoporphyrin (ZnPP) increases in IDE because zinc replaces the missing iron during formation of the protoporphyrin ring⁸³. Therefore, ZnPP may also be a sensitive test for detecting iron deficiency. However, the specificity of ZnPP in identifying iron deficiency may be limited, because ZnPP can be increased by lead poisoning, anaemia of chronic disease, chronic infections and inflammation, haemolytic anaemias, or haemoglobinopathies⁷⁷.

Transferrin saturation

Transferrin saturation is a widely used screening test for iron deficiency, calculated as the ratio of plasma Fe to total Fe-binding capacity. Although relatively inexpensive, its use is limited by diurnal variation in serum iron and transferrin (transferrin shows a diurnal variation ranging from 17 to 70%⁸⁴) as well as the many clinical disorders that influence transferrin levels⁷⁸.

Serum ferritin

Serum ferritin (SF) may be the most useful laboratory measure of iron status; a low value is diagnostic of iron deficiency anemia in a patient with anaemia. In healthy individuals, SF is directly proportional to iron stores. It is widely available, well-standardized, and has repeatedly been demonstrated to be superior to other measurements for identifying iron deficiency anemia. Serum ferritin concentrations are normally within the range 15-300 mcg/l (this is a “statistical” range; we will discuss the issue of the clinical implication of a cut-off value of serum ferritin later in the thesis). Values are lower in children than adults, and from puberty to middle age mean concentrations are higher in men than in women. Good correlations have been found between serum ferritin concentrations and storage iron mobilised by phlebotomy, stainable iron in the bone marrow and the concentration of both non-haem iron and ferritin in the bone marrow. In patients with iron deficiency anaemia, serum ferritin concentrations are less than 12-15 mcg/l (depending on the assay) and a reduction in the level of reticuloendothelial stores is the only common cause of a low

serum ferritin concentration⁷⁶. This is the key to the use of the serum ferritin assay in clinical practice. However, because it is an acute phase protein, SF is increased independent of Fe status by acute or chronic inflammation. It is also unreliable in the setting of malignancy, hyperthyroidism, and heavy alcohol intake⁷⁷. The other major influence confounding the use of the serum ferritin assay to determine iron stores is liver disease. The liver contains much of the storage iron in the body and any process that damages liver cells will release ferritin. Starvation, or even fasting for a short period, can elevate the serum ferritin concentration and vitamin C deficiency may reduce it⁷⁶.

Red cell ferritin

The ferritin in the circulating erythrocyte is a residue of that present in its nucleated precursors in the bone marrow. Red cell ferritin levels reflect the iron supply to the erythroid marrow and tend to vary inversely with red cell free-protoporphyrin levels. Red cell ferritin levels do not therefore necessarily indicate levels of storage iron⁷⁶. High levels of red cell ferritin are found in thalassaemia, megaloblastic anaemia and myelodysplastic syndromes, indicating the disturbance of erythroid iron metabolism associated with abnormalities in globin synthesis in these conditions. Because it is necessary to have fresh blood in order to prepare red cells free of white cells (which have much higher ferritin levels) the assay of red cell ferritin has seen little routine application despite possible diagnostic advantages.

Serum transferrin receptor

The serum transferrin receptor (TfR) is a transmembrane glycoprotein that transfers circulating Fe into developing red cells. A circulating, soluble form of TfR consists of the extracellular domain of the receptor. Serum TfR concentration appears to be a specific indicator of IDE that is not confounded by inflammation⁸⁵. In normal subjects the serum transferrin receptor level also provides a sensitive indicator of functional iron deficiency in subjects with absent iron stores but who have not yet developed iron deficiency anaemia⁷⁶. However, normal expansion of the erythroid mass during growth, as well as diseases common in developing countries, including thalassaemia, megaloblastic anaemia due to folate deficiency, or hemolysis due to malaria, may

increase erythropoiesis and TfR independent of iron status. Thus, the diagnostic value of TfR for iron deficiency anemia is uncertain in children from regions where inflammatory conditions, infection and malaria are endemic⁸⁶.

TfR/SF ratio

The ratio of TfR/SF can be used to quantitatively estimate total body Fe⁷⁸. The logarithm of this ratio is directly proportional to the amount of stored Fe in Fe-replete subjects and the tissue Fe deficit in ID. However, it cannot be used in individuals with inflammation because the SF may be elevated independent of iron stores, and is assay specific.

Quantitative phlebotomy

A direct way of measuring iron stores is by quantitative phlebotomy (removing up to 500 ml blood week until anaemia develops). This gives a measure of the amount of iron available for haemoglobin synthesis. Quantitative phlebotomy has been applied to validate the concept that serum ferritin concentrations in normal subjects reflect the level of available storage iron⁷⁶.

Non-invasive methods for determining tissue iron concentrations

Magnetic resonance imaging is a widely available technique which has been applied to the determination of liver and heart iron concentrations⁷⁶. We will discuss extensively the use of MRI to assess brain iron status later in the thesis.

Assessing iron status using multiple indices

The major diagnostic challenge is to distinguish between IDA in otherwise healthy individuals, and the anaemia of chronic disease (ACD)⁷⁷. The distinction between ACD and IDA is difficult, as an elevated SF in anaemia does not exclude IDA in the presence of inflammation. A widely-used marker of inflammation is the C-reactive protein (CRP). If anaemia is present and the CRP is elevated, IDA can usually be diagnosed in individuals with inflammatory disorders/anaemia of chronic disease by an elevated TfR and/or ZnPP. However, it has recently been pointed⁷⁷ out that sensitivity and specificity of TfR and ZnPP in identifying iron deficiency and IDA in children is modest, regardless of the diagnostic cut-offs chosen, because of the overlap in the distributions of

these indicators in a comparison of children with IDA with those with normal iron status. Because each test of iron status has limitations in terms of its sensitivity and specificity, they have been combined in models to define iron deficiency. Examples include the model based on low transferrin saturation and high ZnPP, and the ferritin model based on low SF and transferrin saturation and high ZnPP. With these models, specificity increases but sensitivity is low, and they tend to underestimate ID. When it is feasible to measure several indices, the best combination is usually Hb, SF and, if CRP is elevated, TfR and/or ZnPP⁷⁷. Overlap in laboratory values between anaemic and non-anaemic individuals means that using haemoglobin measurements alone leads to an overestimate of the prevalence of iron deficiency anaemia. Similarly, the use of only one indicator of storage iron levels or tissue iron supply - ferritin, transferrin saturation or erythrocyte protoporphyrin - may overestimate the number of individuals with absent iron stores. Cook et al.⁸⁷ showed that lower figures for the prevalence of iron deficient erythropoiesis and iron deficiency anaemia were obtained by using multiple criteria (abnormal values for any two of serum ferritin, transferrin saturation or erythrocyte protoporphyrin).

Methodological and biological variability of assays

The blood assays vary greatly in both methodological and biological stability. Haemoglobin concentrations are stable and the simple and well standardised method of determination ensures relatively low day-to-day variation in individuals⁷⁶. The more complicated immunoassays for ferritin have higher methodological variation (coefficient of variation, CV, of at least 5%) and this, coupled with some physiological variation, gives an overall CV for serum ferritin for an individual over a period of weeks of the order of 15%. There is little evidence of any significant diurnal variation in serum ferritin concentration. Serum iron shows high physiological variability giving an overall 'within subject' CV of approximately 30% when venous samples are taken at the same time of day. Moreover, serum iron concentrations are generally higher in the morning than in late afternoon.

There is no information on seasonal factors influencing most of these analyses although seasonal changes in red cell parameters have been reported⁷⁶.

1.2.2 Brain iron

1.2.2.1 Introduction

A number of recent studies, especially the new discovery of mutations in the genes associated with brain iron metabolism, have made important contributions to our understanding of homeostatic mechanisms of brain iron metabolism⁸⁸. In this section, we summarize recent advances in studies on physiological aspects of brain iron metabolism.

1.2.2.2 Iron transport into the brain

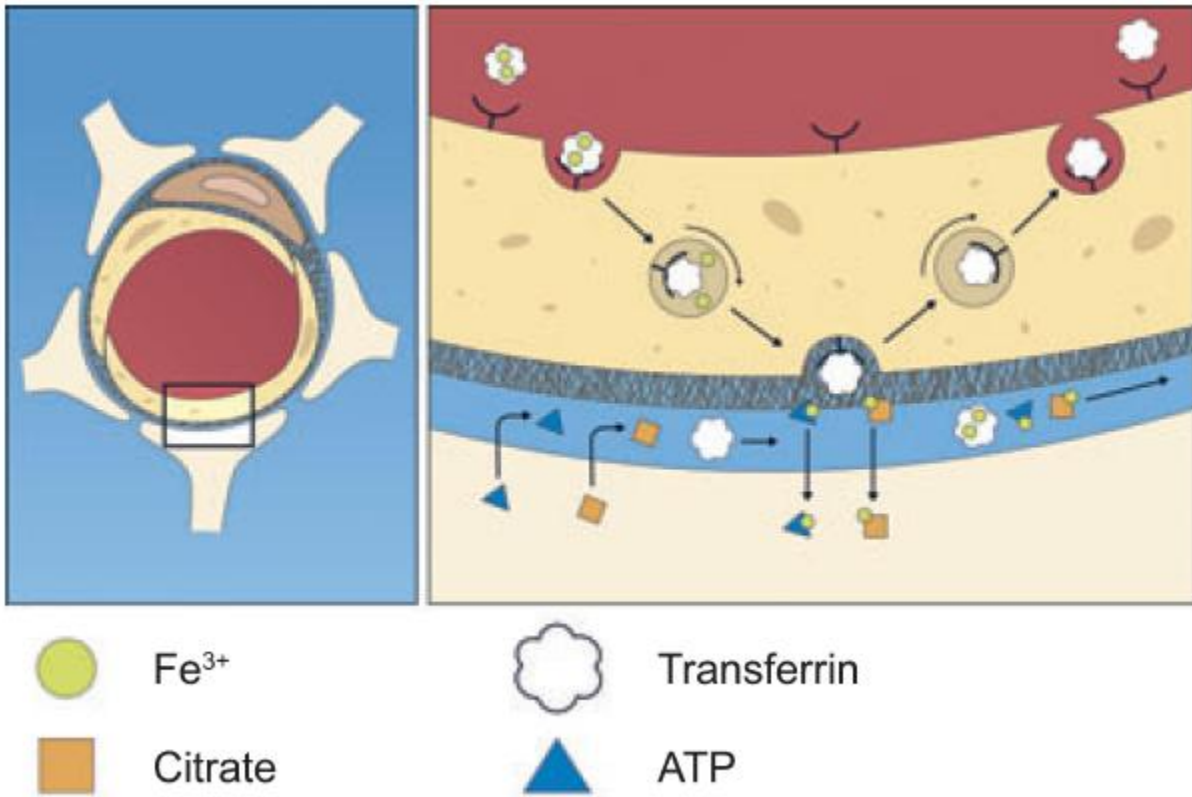
Iron transport across the blood–brain barrier (BBB)

For many years, it was believed that iron entered the brain mainly during infancy before the blood-brain barrier (BBB) matured. However, in the last decade, it has become apparent that brain-iron uptake is possible throughout the BBB of adult animals⁷⁵.

How iron crosses the BBB has not been completely clarified. Available data suggest that the transferrin/transferrin receptor (Tf/TfR) pathway may be the major route of iron transport across the luminal membrane of the capillary endothelium^{88;89}. It has been hypothesized that the uptake of transferrin-bound iron (Tf-Fe) by TfR-mediated endocytosis from the blood into cerebral endothelial cells includes several steps: 1. Binding of Tf to the extracellular portion of TfR; 2. Endocytosis of the complex of iron–Tf–TfR and formation of endosome; 3. Acidification of the microenvironment within endosome; 4. Dissociation of iron from Tf and reduction of Fe³⁺ to Fe²⁺; 5. Translocation of iron (Fe²⁺) across the endosomal membrane probably by a divalent metal transporter 1 (DMT1, previously referred to as Nramp2 or DCT1)-mediated process⁹⁰. Most of the Tf will return to the luminal membrane with TfR. However, Moos and colleagues⁹¹ pointed out that DMT1 is absent from brain capillary endothelial cells. They suggest that transferrin receptor-containing vesicles are transported through the brain capillary endothelial cells (BCECs) and fuse with the abluminal side, thus offering iron detached from transferrin and ready to be transported further into the brain. According to Moos et al.⁹¹, the released iron atoms would probably be in their ferric form, and the process would not require the function of ferroportin. Indeed, in vitro studies of BCECs grown in polarized conditions do indicate that transferrin-containing vesicles fuse with the abluminal surface, confirming the hypothesis of Moos and co-workers⁹¹. Moreover, the astrocytes probably have the ability to take up Fe²⁺ from endothelial cells through their end feet processes on the capillary endothelia. Moos and colleagues⁹¹ proposes that the low Ph microenvironment of the abluminal surface of BCECs contributes to the release of iron from transferrin. ATP and other nucleotides are released from astrocytes and other brain cells⁹⁴⁻⁹⁶ and could act as mediators of iron

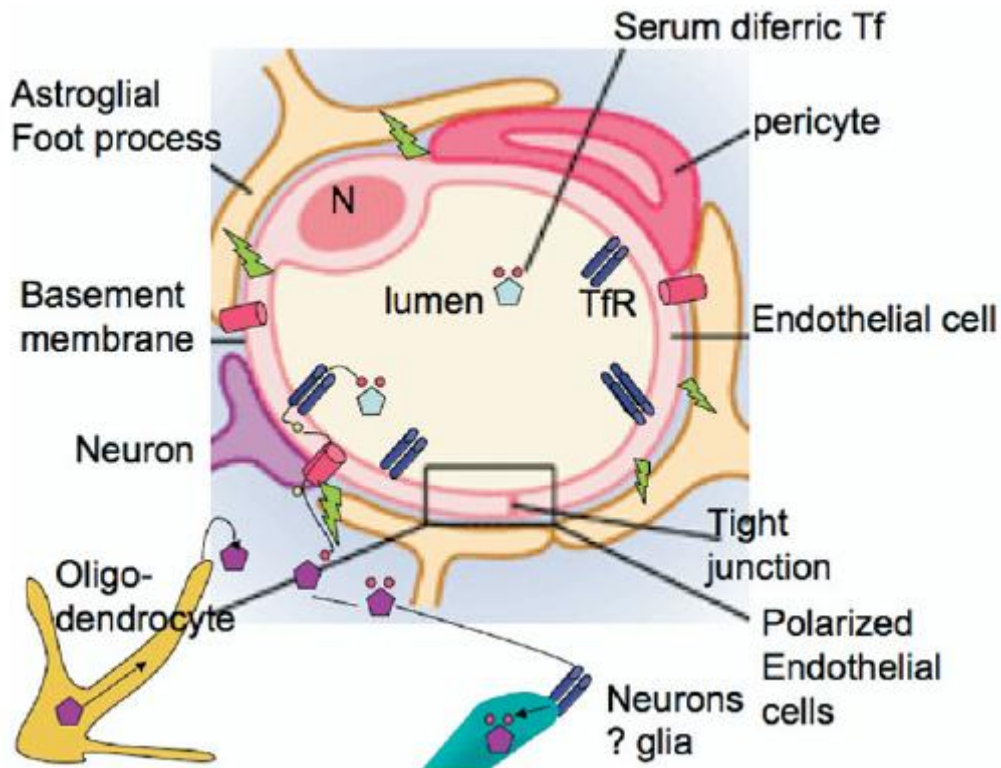
release from transferrin. Also, citrate, another such mediator, is released from astrocytes⁹² and is present in brain interstitial fluid in relatively high concentrations (Fig 4). The transferrin would mainly remain bound to its receptor which has a high affinity for apotransferrin at acidic pH⁹³ and would recycle to the plasma. The iron would pass into the brain interstitium to be bound by citrate, ascorbate⁹⁴ or the transferrin present in the interstitial fluid.

Fig. 4. Transport of iron across the brain capillary according to Moos et al. (from Moos et al., *J Neurochem*, 2007⁹¹).



On the contrary, Siddappa et al. reported the presence of DMT1 in BCECs^{95;96}. This may be consistent with an export of iron across the abluminal membrane of BCECs by means of ferroportin 1 (FP1)/ hephaestin (Hp) and/or hephaestin-independent (FP1/CP) iron export systems, similarly to the process of export found in the enterocytes. Indeed, a number of recent studies have confirmed that the brain, including capillary endothelium of the BBB, neurons and astrocytes, has the ability to express FP1 and Hp⁹⁷⁻¹⁰¹ although one study did not find evidence of FP1 in brain capillary endothelial cells¹⁰² (Fig. 5).

Fig. 5. Transport of iron across the brain capillary according to Rouault et al. (from Rouault and Cooperman, 2006⁷⁵)



The blood-brain barrier is composed of endothelial cells joined by tight junctions surrounded by a basement membrane in which pericytes are found and which is in close apposition with astrocytic foot processes. TfR1 is expressed on the luminal membrane of endothelial cells, which have nuclei (N) and which likely express ferroporin (cylinders). Astrocytic foot processes express GPI-linked ceruloplasmin (lightening). On endocytosis of the Tf- TfR complex, ferric iron (dots) is reduced to ferrous iron (pale dot); exported to cytosol, most likely by DMT1; exported from the cell, presumably by ferroporin; and oxidized to ferric iron by GPI-linked ceruloplasmin within brain interstitium. Transferrin synthesized by oligodendrocytes in the brain binds ferric iron, and neurons and probably many other brain cells acquire iron by expressing the TfR, although TfR expression is low in nonneuronal cells. To exit the brain interstitial fluid and cerebrospinal fluid, iron must cross the arachnoid membrane (not shown), a tight epithelial layer that brain Tf does not cross.

Therefore, given the contrasting findings on the modalities of iron export from BCECs, this issue needs further investigation.

Moreover, the lactoferrin receptor (LfR)/lactoferrin (Lf¹⁰³) and GPI-anchored melanotransferrin (MTf)/soluble melanotransferrin (sMTf)¹⁰⁴ pathways might play a role in iron transport across the BBB. It is also possible that a small amount of iron might cross the BBB in the form of intact Tf-Fe complex by receptor mediated transcytosis¹⁰⁵. The existence of the a non-transferrin binding iron transport across the BBB cells has also been reported¹⁰⁶.

Iron transport across the blood–cerebrospinal fluid barrier

Choroid plexus epithelial cells simultaneously constitute a barrier: the blood–cerebrospinal fluid (CSF) barrier (the blood–CSF barrier) for circulating plasma proteins from the brain^{112;113}.

A major difference between the choroid plexus (blood–CSF barrier) and the brain endothelium (BBB) barrier is that the choroid plexus of the lateral and third ventricle synthesize Tf, which may be of importance for the transport of iron across the choroid plexus⁸⁸. However, available studies indicate that the contribution of iron transported through the blood–CSF barrier to the brain may be negligible as compared to that transported through the BBB. It is possible that the blood–CSF barrier is more useful for iron removal from brain other than iron transport into the brain.

Iron transport by the circumventricular organs

The circumventricular organs that receive neuronal projections, mainly from hypothalamic nuclei, have been suggested to play a role for iron transport in the developing brain. Some motor neurons that project to peripheral organs devoid of blood barrier express TfR and retrograde axonal transport iron into brain¹⁰⁷. The physiological significance of bypassing the brain barrier iron transport deserves further investigation.

1.2.2.3 Iron transport within the brain

After the iron has been transported across the BBB or blood– CSF barrier most of iron (Fe^{2+}) will be oxidized to Fe^{3+} and then bind to Transferrin (Tf) before being acquired by brain cells^{99;115;116}. It has been reported that the main source of brain transferrin is represented by oligodendrocytes¹⁰⁸. Unlike Tf found in blood, Tf in CSF and IF is fully saturated with iron. The excess iron will bind to other transporters. Hence, it is possible that there are two transport forms of iron in CSF and IF in the brain: Tf-Fe and NTBI^{99;116}. The latter probably includes citrate- Fe^{3+} or Fe^{2+} , ascorbate- Fe^{2+} , and albumin-Fe ($2+$ or $3+$) and also Lf- Fe^{3+} and sMTf- Fe^{3+} . The Tf-Fe or probably Lf-Fe and sMTf-Fe will be taken up by brain cells via TfR or LfR and GPI-anchored MTf-mediated processes, respectively^{109;110}. NTBI will be acquired by neuronal cells or other brain cells, probably via DMT1 (iron carrier) or trivalent cation-specific transporter (TCT)¹¹¹-mediated mechanisms¹¹². However, the relevant mechanisms have not yet been detailed. In neurons, the internalization of Tf is followed by detachment of iron within recycling endosome and transport into the cytosol via DMT1. Moreover, the presence of LfR on neurons indicates that a Lf/LfR-mediated pathway may be involved in iron uptake by neurons. TfR mediated endocytosis has been anticipated to be involved in Fe^{3+} uptake in oligodendrocytes^{108;113}. In addition, based on the recent identification of a ferritin receptor (FtR) predominantly on oligodendrocytes, it has been suggested that these cells may obtain iron via ferritin/FtR pathway^{114;115}. It has yet to be determined whether oligodendrocytes can take up iron by interchanging with the axon of vicinity neurons. In the astrocytes, the existence of TfR expression and TfR-mediated iron uptake has been reported^{125;126}. However, little is known about the relevant molecular mechanisms. Several findings suggest that GPI-anchored MTf (melanotransferrin receptors) may deliver iron across the BBB by a mechanism analogous to that mediated by the TfR, and play a role in iron uptake by microglial cells¹⁰⁹. The presence of DMT1 on astrocytic endfeet in the cerebral cortex and hippocampus of monkeys suggests that astrocytes may take up iron from endothelial cells through DMT1¹¹⁶.

Neurons are thought to regulate their iron levels so that iron not used for metabolic purposes is released from the cells. Iron-export mediated by ferroportin is a permanently active mechanism, which ensures iron-homeostasis inside the neuron. Iron is thought to undergo axonal and dendritic transport, and as ferroportin is found in the somata, axons, and dendrites of neurons, it probably plays an important role to regulate iron levels everywhere in the neuron¹⁰². Neurons of some forebrain nuclei, however, also contain ferritin, showing that neurons are capable of storing iron¹¹⁷. As for iron efflux from brain cells, the detection of Ferroportin 1 (FP1) in most rat brain regions and cells, including the endothelial cells of the blood–brain barrier, neurons, oligodendrocytes, astrocytes, the choroid plexus as well as ependymal cells of the normal and IRP knockout mice^{101;118;119} implies that this protein may be involved in brain iron efflux, in accordance to the role of this protein in iron efflux from enterocytes. A role for Hephaestin (Hp) in iron efflux from brain cells has also been hypothesized, but further data are needed to support this hypothesis.

Increasing amount of data suggest that ceruloplasmin (CP) might also be involved in iron release from the brain cells¹²⁰⁻¹²² in particular from astrocytes, which do not contain ferroportin¹²³. However, the possibility that CP might play a role not only in iron efflux from, but also iron influx into the brain cells via its ferroxidase activity has been also suggested¹²⁴. It has been suggested that the physiological function of CP is provided by its ferroxidase activity. This activity plays a role in both iron efflux and iron influx¹²⁵, but it is highly likely that the major role of CP in brain cellular iron balance might be “uptake”, and “release” plays a minor role. Recently, interest has been focused on the potential role of Heme oxygenase-1 (HO-1), which has been linked to the efflux of iron from cells for cellular protection under stress. HO-1 expression in the normal brain is confined to a small group of scattered neurons and neuroglia¹²⁶. There is preliminary evidence that HO-1 has a dual role in iron metabolism: it can offer neuroprotection by facilitating the cellular iron export in some stress conditions but exert a neuroendangering effect by promoting iron deposition in mitochondria in other circumstances⁸⁸.

1.2.2.4 Iron localization in the brain

Regional level

Iron can be detected in the ex vivo brain using chemical methods and histochemistry and in vivo using magnetic resonance imaging (MRI)¹²⁷.

Iron is present in the brain as heme iron and non heme iron¹²⁸. Heme containing proteins include the mitochondrial electron transport cytochromes (b, c1, c, a, a3). Non heme proteins, in addition to the storage pool of nonheme iron as ferritin, include a large number of enzymes in various subcellular compartments that contain iron not associated with the protoporphyrin ring. Some of these nonheme enzymes include mitochondrial ac-glycerophosphate dehydrogenase, aldehyde oxidase, succinate dehydrogenase, and various other Fe-S centers in the electron transport system. Other examples include xanthine oxidase, tyrosine 3-hydroxylase, tryptophan hydroxylase, and ribonucleoside diphosphate¹²⁸. According to Worm-Petersen, only about 10% of the iron content of the brain is in hemoglobin¹²⁹.

The study of non heme iron in the brain was made possible by the histochemical ferrocyanide-Prussian blue reaction, eponymically referred to as “Perls’ stain.” (The Perls’ stain was introduced in 1867 by the Hungarian pathologist, Dr M. Perls)^{130;131}. The Perls’ stain is based on detection of ferric ferrocyanide (Prussian blue), which is formed when ferric ions released by hydrochloric acid react with potassium ferrocyanide¹³⁰. The distinction of “heme-” and “non-heme-” iron in the brain must be attributed to Zaleski (cited by¹³¹) who observed that the iron in hemoglobin did not react with potassium ferrocyanide, hence the blue colour had to arise from non-heme iron. Guizzetti and Spatz (cited by¹³¹) used immersion of fresh or fixed animal and human brain slices in Perls’ solution to visualize iron in various gray and white matter structures of the brain and theirs are the two most classically cited articles on brain iron localization. In particular, the long paper by Spatz offered the first colour illustrations of the unusual differential distribution of iron. Spatz found that globus pallidus and substantia nigra gave the most intense iron reaction, followed by red nucleus, putamen, caudate nucleus, dentate nucleus and the subthalamic body. The

cerebral and the cerebellar cortex, the anterior nucleus of the thalamus, the mamillary body, and the central grey matter of the third ventricle gave only a variable and weaker reaction. The reaction was less intense in the thalamus but was still stronger than in the cerebral cortex and the subcortical white matter. Further histochemical studies have in general confirmed that most of the iron is found in the extra-pyramidal structures (e.g.:^{129;132-137}) with the globus pallidus, substantia nigra, zona reticulata, red nucleus and myelinated fibres of the putamen showing the highest iron concentration, followed by the striatum, thalamus, cortex and deep white matter, substantia nigra zona compacta, and cerebellar cortex. The brain-stem and spinal cord generally showed low iron concentration.

However, Perls (for Fe^{3+}) and other histochemical techniques, such as Turnbull's (to detect Fe^{2+}), although inexpensive and available, remain relatively insensitive, even with diaminobenzidine intensification. Therefore, some researchers¹³⁰ have subsequently proposed other approaches to quantify brain, such as *X-ray* Energy Spectrometry, Proton-induced x-ray emission (PIXE), secondary ion mass spectrometry (SIMS), and Laser microprobe technology, which, however, have been scarcely used to quantify iron in normal brain.

In the last decade, MRI has provided a useful estimation of brain iron. MRI studies have confirmed that most of brain iron is found in extra-pyramidal structures.

Importantly, both histochemical techniques and MRI have also show, in post-mortem and in vivo studies respectively, that in normal individuals, iron levels increase with age in subcortical and some cortical gray matter regions¹³⁸⁻¹⁴³. However, the rates of iron accumulation are different in various brain structures¹⁴⁰. The following graphs, from Hallgren and Sourander seminal work¹⁴⁰, illustrate the rates of brain iron accumulation during the life span.

Fig. 6. Non heme iron in sensory cortex (from Hallgren and Sourander, *J Neurochem*, 1958¹⁴⁰)

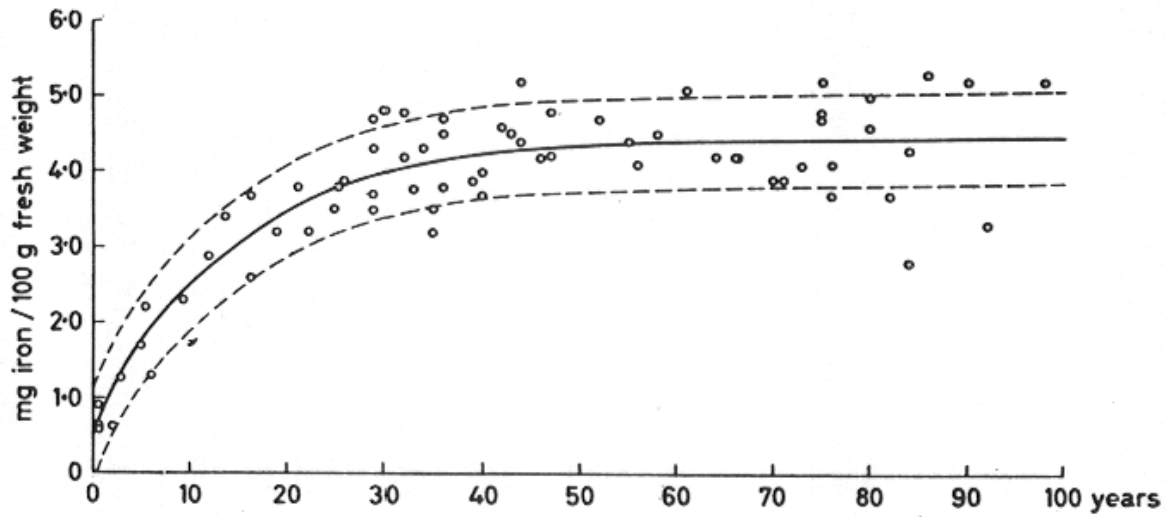


Fig. 7. Non heme iron in the medulla oblongata (from Hallgren and Sourander, *J Neurochem*, 1958¹⁴⁰)

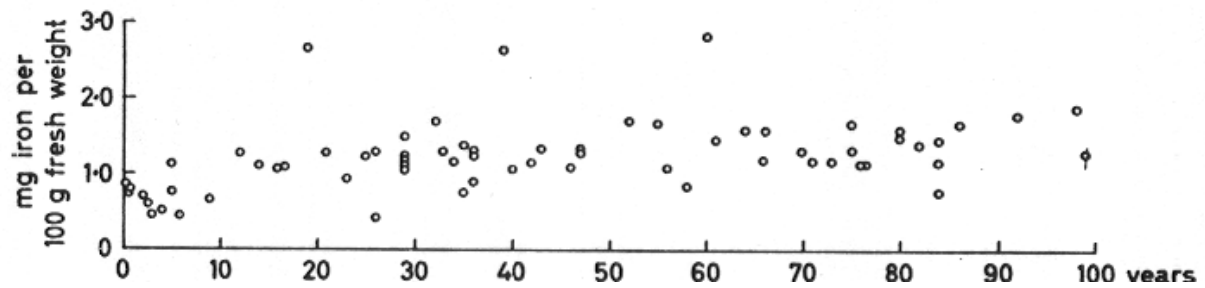


Fig. 8. Non heme iron in the thalamus (from Hallgren and Sourander, *J Neurochem*, 1958¹⁴⁰)

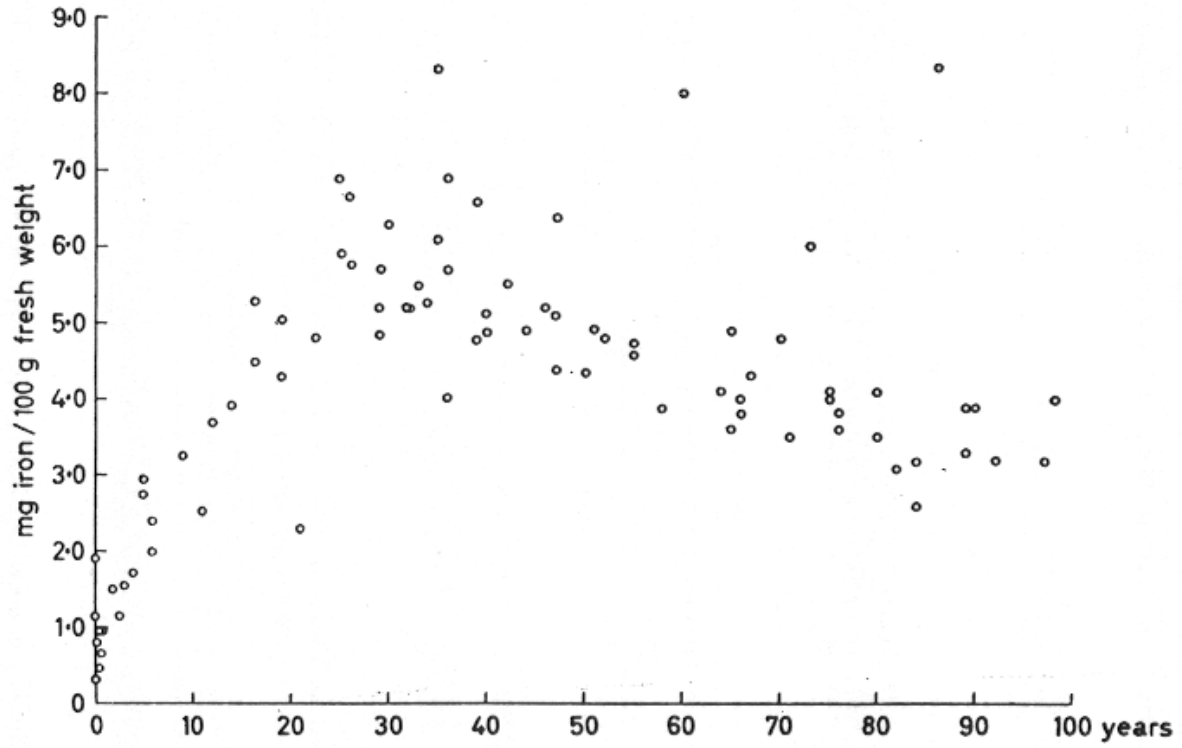


Fig. 9. Non heme iron in the globus pallidus (dotted lines denote standard estimates) (from Hallgren and Sourander, *J Neurochem*, 1958¹⁴⁰)

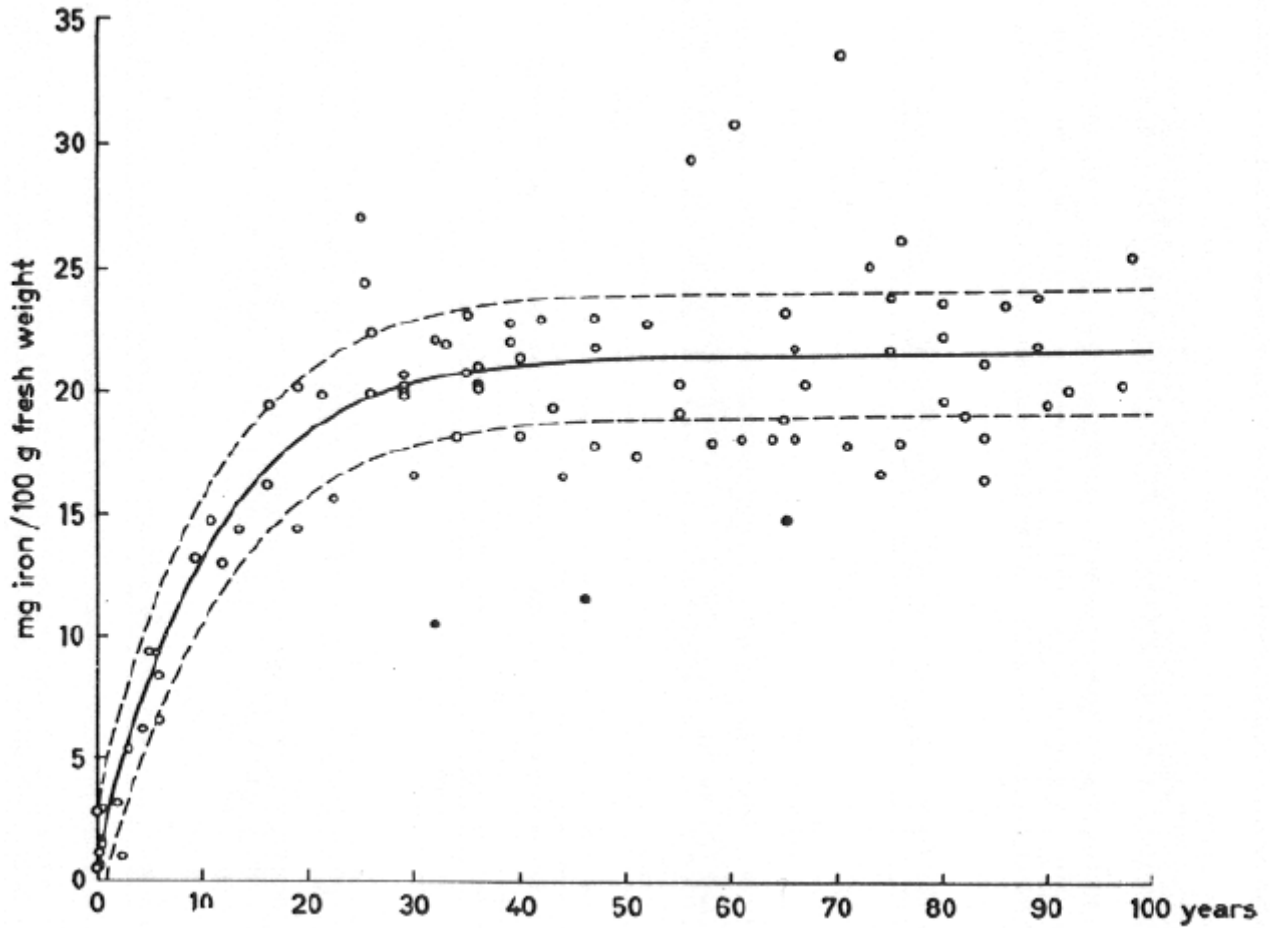


Fig. 10. Non heme iron in the putamen (from Hallgren and Sourander, *J Neurochem*, 1958¹⁴⁰)

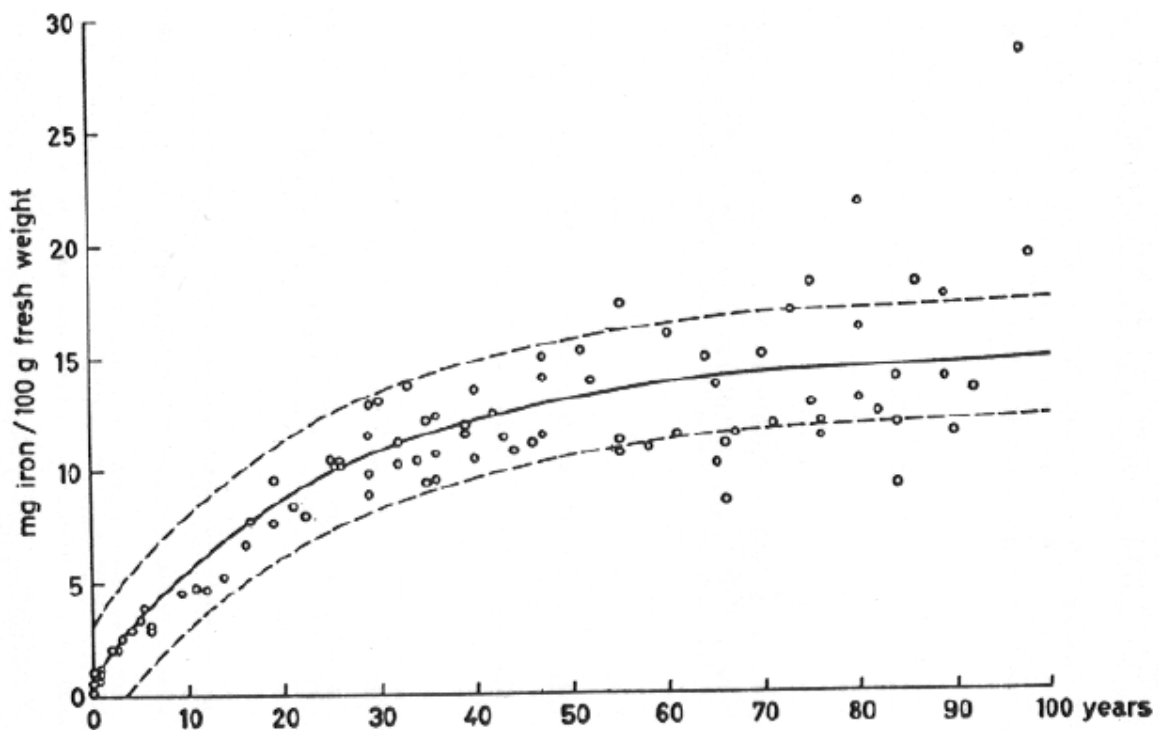


Fig. 11. Non heme iron in the motor cortex (from Hallgren and Sourander, *J Neurochem*, 1958¹⁴⁰)

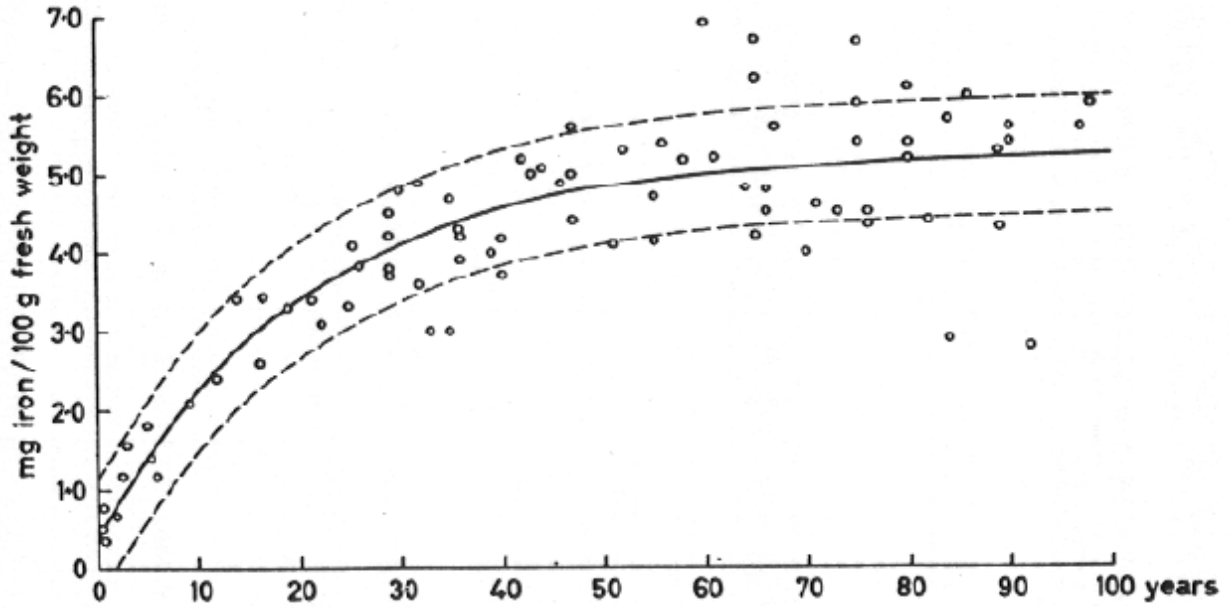
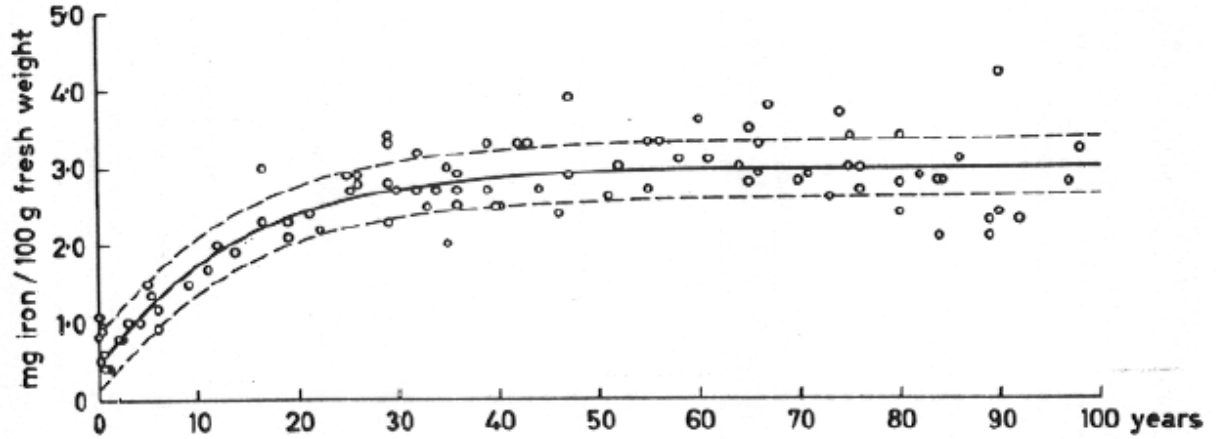


Fig. 12. Non heme iron in the prefrontal cortex (from Hallgren and Sourander, *J Neurochem*, 1958¹⁴⁰)



The globus pallidus has a rich supply of iron during the first two decades and no further increase seems to occur after 30 years of age. Although the iron values in the red nucleus and substantia nigra show considerable scattering, a rapid increase in iron content during the first two decades is also clearly demonstrated. In other structures, such as the putamen, iron deposits increase more slowly, and iron concentrations reach maximal values at about the sixth decade.

Very little is known about gender differences in brain iron. Recently, Bartzokis et al.¹⁴⁴ first reported that women had lower brain iron levels than men in the caudate, thalamus, and frontal white matter. The only available MRI study to assess hemispheric differences in iron content showed that the left hemisphere had higher iron deposition than the right in the putamen, globus pallidus, substantia nigra, thalamus and frontal white matter¹⁴⁵.

Importantly, it has been pointed out that the non heme iron in the different parts of the brain seems to be largely independent of the amounts of storage iron in the iron depots. For example, Hallgren et al. failed to find any significant correlation when they plotted non heme brain iron levels against iron levels in the liver¹⁴⁰.

Cellular level

In 1966, Perl's reaction was modified to enhance visualization of the reaction product with 3,3 diaminobenzidine(DAB)¹⁴⁶. This modification was a major advancement in the ability to detect iron and made study of cellular iron distribution in the animal brain possible.

Using DAB intensification, an iron staining reaction product is seen predominantly within oligodendrocytes but is not generally appreciated within neurons^{147;148}. However, in rat brain, positive DAB-intensified iron staining has been reported within neuronal perikarya in the islands of Calleja, the dentate nucleus of the hippocampus, the lateral septal nucleus, and the central amygdale^{147;149}. Considering the lack of specificity of the stain and the extreme limitations of its ability to detect iron except in massive deposition, the negative findings with respect to intraneuronal staining are difficult to interpret. It has also been pointed out that with age there is an

increase in stainable iron in neurons without a concomitant increase in neuronal ferritin immunostaining, suggesting a ferritin independent accumulation of neuronal iron with age.¹⁵⁰

Although predominant in the white matter, iron-positive oligodendrocytes are found throughout the brain including in the iron-rich brain nuclei, such as the substantia nigra and striatum^{127;150}.

Intracellularly, iron in oligodendrocytes is found in the perikaryal cytoplasm and processes^{150;151}.

The distribution of iron-positive oligodendrocytes in the white matter tracts is not uniform. The white matter tracts that stain with iron have a patchy appearance resulting from areas of robust cell staining that extends into processes in the neuropil admixed with areas of no detectable iron staining despite the presence of cells¹⁵².

The distribution of iron in oligodendrocytes changes according to developmental phases. In rats models, during normal development, iron first appears in microglia in myelinogenic foci in white matter¹⁵³. As development proceeds, iron appears in oligodendrocytes and is no longer detectable in microglia. The appearance of iron (and the iron storage protein ferritin) in oligodendrocytes corresponds spatially and temporally to the appearance of myelin. The appearance of transferrin receptors on oligodendrocytes during development in the optic nerve is clearly associated with cells near blood vessels¹⁵⁴, supporting the hypothesis that iron accumulation by oligodendrocytes and myelin production are biologically linked.

In addition to oligodendrocytes, microglia are the cell type most often seen to accumulate stainable quantities of iron in the brain.

Ferritin and iron have also been detected in tanycytes and transferrin has been found in the choroid plexus and ependyma surrounding the third ventricle, suggesting that a transport system may exist for the transfer of iron between the brain and cerebrospinal fluid¹⁵⁰.

1.2.2.5 Iron transport out the brain

The quantity of iron in the brain increases with age but not as rapidly as would be expected from measurement of the rate of iron uptake from the blood at the different ages. Hence some iron must be exported⁹¹.

Knowledge about how iron leaves the brain is far from complete. Tf-Fe readily exits the brain via the venous system and returns to the systemic circulation through the arachnoid granulations^{99;138}.

Endothelial cells of the BBB also have a potential capacity to mediate the export of elemental iron from brain interstitium to the systemic circulation, although there is no evidence so far for direct export of iron from the brain¹⁵⁵.

Within the CSF transferrin is fully saturated with iron^{94;156}. Nevertheless, the concentration of transferrin in CSF is very low so that the capacity to export iron by this route is limited. The CSF also contains lactoferrin, ferritin and non-protein-bound iron^{116;139}, which represent additional means of iron export from the brain.

1.2.2.6 Iron and neurotransmitters

The effects of iron deficiency on neurotransmitters have been mainly studied by means of rat and, more recently, monkey models¹⁵⁷. The first rodent studies induced ID predominantly at weaning^{157;158}, at a time period roughly corresponding to the last part of the brain growth spurt in the human. More recent rodent models have emphasized the maternal-fetal unit and the role of maternal ID in determining postnatal iron status in the offspring. One such model induces ID during fetal life and continues an ID diet during lactation until weaning at around postnatal day (P) 21¹⁴². This gestational/lactational model is designed to mimic the common worldwide human condition where mothers are ID during pregnancy and breastfeed their infants for 1 to 2 years, with widespread ID in this age period. Another model focuses on fetal and neonatal ID. ID diet is used as in the gestational/lactational model through about P7, with an iron-sufficient diet thereafter (P10 is considered to be the rodent “brain equivalent” of term human birth). The purpose of this model is to mimic human conditions such as intrauterine growth restriction (IUGR) or gestational diabetes mellitus where the fetus is ID during gestation but then receives adequate dietary iron after birth¹⁵⁷. Given that the rate-limiting enzymes for the synthesis (tyrosine and tryptophan hydroxylases) and catabolizing enzymes (monoamine oxidase) of the aminergic neurotransmitters (dopamine, serotonin, and noradrenaline) were shown to be dependent on iron for their full enzymatic activity, it was hypothesized that iron is involved in the metabolism of monoamine neurotransmitters and that iron deficiency leads to alterations in the production and functioning of these neurotransmitters¹⁵⁹. Moreover, it has been reported that iron is co-localized with dopaminergic neurons throughout the brain^{159;160}.

In part contrary to this hypothesis, Youdim and colleagues demonstrated that, whereas iron deficiency induced in rats by feeding them a diet low in iron resulted in the reduction of iron-dependent enzymes (monoamine oxidase, phenylalanine hydroxylase, succinic dehydrogenase, cytochrome oxidase to mention a few) in peripheral tissues, none of the brain enzymes containing iron (including tyrosine hydroxylase, tryptophan hydroxylase hydroxylase, and monoamine

oxidase) as a cofactor was changed^{161;162} (Table 6) despite the fact that adult (48 days old) rat brain iron was reduced by 30% to 40% in the striatum, hippocampus, cortex, and raphe nucleus (Table 6).

Tab. 6. Effects of iron deficiency on brain iron enzymes (*from Youdim, Nutrition, 2000¹⁵⁹*).

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

Moreover, Youdim et al. also reported unaltered brain (striatum, caudate nucleus, and raphe nucleus) levels and turnover of dopamine, noradrenaline, and serotonin¹⁶² (Table 7).

Tab. 7. Neurotransmitters and their precursor levels in brains of iron-deficient rats (*from Youdim, Nutrition, 2000¹⁵⁹*).

	Concentration	Turnover
Serotonin	Decreased	Unchanged
Dopamine	Unchanged	Increased
Noradrenaline	Unchanged	Unchanged
Tryptophan	Unchanged	—
Tyrosine	Unchanged	—
5-Hydroxyindole acetic acid	Decreased	—

However, the same group¹⁶² interestingly reported an alteration in the dopamine receptors: by employing radioligand analysis of dopamine D1 and D2 receptors, they observed an increase in K_a of dopamine D1 and a decrease of dopamine D2 B_{max} in the striatum of iron deficient rats. Interestingly, this effect was related to iron deficiency and not to the anemia resulting from it because haemolytic anemia induced by chronic treatment of rats with phenylhydrazine does not alter brain dopamine D2 receptor number or their behavioral responses^{146;147}. Alterations in D1 and D2 receptors have also been replicated by other authors^{163;164}. In line with these observations, Nelson et al. reported that the response to dopamine reuptake blockade was significantly attenuated in iron deficient rats compared with control¹⁶⁵.

Tab. 8 summarizes the biochemical consequences of reduced dopamine D2 receptor in the brains of iron-deficient rats.

Tab. 8. Biochemical and behavioral consequences of reduced dopamine d2 receptor in the brains of iron-deficient rats (*from Youdim, Nutrition, 2000¹⁵⁹*).

	Response
Monoamine oxidase inhibitor plus tryptophan	Decreased
Monoamine oxidase inhibitor plus L-dopa	Decreased
Monoamine oxidase inhibitor plus 5-hydroxy-tryptophan	Decreased
5-methoxy- <i>N,N</i> -dimethyltryptamine	Decreased
D-amphetamine	Decreased
Apomorphine	Decreased
Learning processes	Decreased
Thyrotropin-releasing hormone and its analog	Unchanged
Phenobarbitone sleeping time	Increased
Serum prolactin	Increased
Serum testosterone	Increased
Liver prolactin receptor	Increased
Antinoceptive response to β -endorphin and enkephalins	Increased
Dynorphin and met- and leu-enkephalin concentrations in the globus pallidus, substantia nigra, caudate nucleus, and central gray	Increased

In the brain regions where iron does not fall, there are no changes in DA receptors¹⁶⁶.

However, further studies have reported an alteration of neurotransmitters themselves. It has been reported that the effects of early iron deficiency on brain iron and neurotransmitters depend on when they are assessed and on the severity of the iron deficiency¹⁶⁷. For instance, modest iron deficiency during gestation does not lead to marked changes in rat pup brain iron content until the latter part of lactation¹⁵¹. There are, however, elevations in striatal dopamine transporters, D2R, and catecholamines that precede measurable changes in regional iron. By weaning, both brain iron and catecholamines are reduced to levels 20% to 30% below controls, suggesting that earlier elevations are an “adaptive” response to iron deficiency. Others have interpreted the elevations in the levels of catecholamines as the expression of a defect of their uptake and catabolism¹⁶⁸. In line with this hypothesis, Mackler et al. have reported decreased activity of aldehyde oxidase and consequent increased levels of serotonin in brain of iron deficient rats¹⁶⁹. Some of these neurotransmitter alterations may relate to the recently observed effects of early iron deficiency on the neuronal surface protein Thy1, which is iron responsive. In the dietary iron-deficiency model, Thy1 was decreased in the brains of iron-deficient rats¹⁷⁰. Thy1 deficiency may affect the release of neurotransmitters and the synaptic efficacy and could contribute to a variety of abnormal neuron-neuron communications in the iron-deficient rat.

Further research also showed that iron deficiency resulted in a reduction of dopamine transporter in striatum¹⁷¹ or in the nucleus accumbens and prefrontal cortex¹⁶³. Erikson et al. have suggested that that elevated levels of extracellular dopamine reported in some studies assessing extracellular levels of dopamine in iron deficient rats in the striatum of iron-deficient rats are likely to be the result of decreased DAT functioning and not increased rates of release. Moreover, it has been reported that the impact of iron deficiency on dopamine transporter is more pronounced in males than females¹⁷².

Moreover, experiments with pheochromocytoma (PC12) cells and neuroblastoma cells demonstrated a dose response relationship between iron chelation and expression of the DA transporter¹⁶⁰.

The mechanism by which ID brings about the reduction of dopamine D2 receptor number and thus dopamine neurotransmission in rat brains is not fully understood. It has been hypothesized that iron could be part of the dopamine-receptor site to which agonists (apomorphine) and antagonists (phenothiazines) bind¹⁵⁹.

Although most of the research activity on brain iron and neurotransmitters has focused on DA, there is evidence that both serotonin and norepinephrine (NE) metabolism are also altered in brain iron insufficiency. The serotonin transporter density was significantly lower in brains of iron-deficient¹⁷³ mice whereas in vivo micro dialysis in rats provided evidence for decreased rates of uptake of NE¹⁶⁸. Studies by Beard and co-workers showed that iron deficient anemic women and rats had elevated plasma NE levels¹⁷⁴.

Recent studies have also showed that the serotonin transporter and norepinephrine transporter densities are altered by dietary iron deficiency^{175 176 177 178;178}.

As for other neurotransmitters, Youdim et al. have also reported a reduction of GABA receptors in iron deficient rodents¹⁷⁹, confirming the observations by Li¹⁸⁰, Taneja et al.¹⁸¹, and Shukla et al.¹⁸² that iron deficiency in the brain could change the utilization and metabolism of GABA.

Interesting, brain-iron distribution is related to the distribution of GABA systems. The high-iron regions of the brain receive GABAergic input, and inhibition of GABA degradation reduces brain iron.

The group by Youdim also noted interesting dopamine opiate interactions in iron deficient rodents¹⁷⁹. The brain regions known to contain the highest concentrations of iron (globus pallidus, substantia nigra, caudate nucleus, and putamen) are also enriched with the densest population of opiate-peptide (enkephalins, endorphins, and dynorphin B) neurons. During the past few years it has

become evident that endogenous opiopeptides are involved in memory and learning¹⁷⁹ because central injection of such peptides cause amnesia and forgetfulness. Consistent with this finding is that administration of naloxone (opiate antagonist) improves memory¹⁷⁹. Indeed, ID rats show a highly significant increase in opiate-analgesic response which can be blocked by naloxone¹⁸³. The increase of analgesic response in ID rats has been attributed to the highly significant elevation of the opiate peptides (dynorphin Band met- and leu-enkephalin) as measured in the globus pallidus, substantia nigra, caudate nucleus, and central grey by radioimmunoassay. The mechanism of increased opiopeptides and their action in ID animals are not fully understood. However, a link with the diminished central dopaminergic neurotransmission, as seen in ID rats, has been postulated because dopamine is inhibitory to opiates^{183;184}

The evidence for alterations in dopamine or norepinephrine in humans is limited. Voorhess et al¹⁸⁵ showed that urine of iron-deficient infants was particularly high in norepinephrine and returned to normal with the restoration of iron adequacy. Webb et al.¹⁸⁶ also found high levels of norepinephrine in iron deficient children and suggested that this is linked to decrease activity of monoaminoxidase. Beard et al. (18) showed alterations in plasma norepinephrine levels in iron-deficient women during cold stress¹⁷⁴.

1.2.2.7 Iron and myelination

Oligodendrocytes are the glial cells in the CNS that produce myelin. Oligodendrocytes stain for iron more robustly than any other cell in the normal adult brain^{150;151}. The highest period of iron uptake in the CNS coincides with the peak myelination^{151;187}. There is evidence that iron deficiency leads to reduced myelination¹⁸⁸. The iron acquisition by oligodendrocytes at the peak of myelination is likely linked to their energy metabolism. Many of the enzymes involved in ATP production, cholesterol and fatty acid synthesis, which are precursors of myelin, are iron dependent. These include HMG-CoA reductase, succinic dehydrogenase, NADH dehydrogenase, dioxygenase and glucose-6-phosphate dehydrogenase, and most of these are enriched in oligodendrocytes compared to other cells in CNS¹⁸⁹. Beard and colleagues have reported that both CNPase activity and concentration (marker of oligodendrocytes metabolic activity) and myelin basic protein (MBP) concentration were reduced in iron deficient rats¹⁶⁰. Moreover, Wu et al. have reported lower density of 2-,3-cyclic nucleotide 3-phosphohydrolase (CNPase, marker of oligodendrocyte) in iron deficient rats¹⁹⁰. Interestingly, a single apo-transferrin intraparenchymal injection administered to myelin deficient rat pups enabled mutant oligodendrocytes to synthesize myelin basic protein and to myelinated axons, indicating that transferrin availability is essential for oligodendrocyte maturation and function¹⁹¹. Iron deficiency effects on myelin production suggest that iron requirements for myelin are related to metabolic processes underlying general myelin production rather than limited to lipid or protein biosynthesis.

Several studies have shown that apoTf acts on pro-oligodendrocytes inducing their rapid differentiation from glial precursor cells^{192;193}. It has been pointed out that iron may affect oligodendrocyte development at stages during early embryogenesis rather than during later development.

Emerging data suggest that adequate iron in the brain, at least at some point during the intervention, are required for the full apoTf effect. It appears that there is a “critical temporal window” in the mouse brain development in which oligodendrocytes are responsive to

apotransferrin. Rats models show that when oligodendrocytes reach maturation their complete downregulation of transferrin receptor makes them no longer responsive to exogenous transferrin¹¹⁴. Despite their lack of transferrin receptor, mature oligodendrocytes continue to remain the principal cell in the brain that stains for iron. Presumably there is a continued need for iron uptake given that iron deficiency in the adult can lead to loss of myelin¹⁹⁴. Therefore, uptake of iron into oligodendrocytes may continue in the absence of transferrin¹⁹⁵. Preliminary data suggest that H-ferritin may act as iron delivery protein for oligodendrocytes¹¹⁵. Connor and co-workers suggested that two alternative iron delivery mechanisms exist in the adult brain: transferrin in grey matter and neurons, and ferritin in white matter and oligodendrocytes, while in the perinatal period, the main source of iron for oligodendrocytes would be still transferrin, given the reported spatial and temporal association between myelinogenesis and transferrin^{154;196;197}. The advantage of a separate iron delivery for oligodendrocytes in form of H-ferritin may be avoiding competition for transferrin-bound iron with the neurons and other brain cells that primarily use transferrin system while still preserving iron supply to oligodendrocytes in form of H-ferritin.

1.2.2.8 Iron and neuronal metabolism

Iron is an essential component of cytochromes a, b, c, cytochrome oxidase, and the iron sulphur complexes of the oxidative chain¹²⁷.

Dallman¹⁹⁸ demonstrated that iron deficiency had primary effects in the brain tissue, independently of anaemia, by documenting reductions in brain cytochrome *c* concentrations. He proposed that reduced brain tissue iron concentrations altered cerebral energy metabolism through loss of cytochromes and inefficient ATP generation and electron transport. These findings are supported by regionally distributed losses of cytochrome *c* oxidase, a marker of neuronal energy status, particularly in the hippocampus and frontal cortex¹⁹⁹. Hippocampal and striatal abnormalities in energy metabolism, as assessed by sequential magnetic resonance spectroscopy studies of live rodents, indicate acute (while iron-deficient) and persistent (during iron repletion) changes^{200;201}.

1.2.2.9 Iron and neuronal structure

In iron deficient rats, significant delays and abnormalities in the structural development of the dendrites of CA1 area neurons are found, as assessed by MAP2 (microtubule-associated protein 2) expression²⁰². This protein, which is important for dendritic scaffolding, may be affected secondarily by lack of adequate energy to support complex dendritic growth or by a direct effect on *MAP2* gene expression²⁰³. Accompanying this altered structure were magnetic resonance spectroscopy findings consistent with intracellular sequestration of the neurotransmitter glutamate²⁰¹, reduced concentrations of the activity-dependent signalling molecule CaMKII α (Ca²⁺/calmodulin-dependent protein kinase II α), and reduced transcript and protein concentrations of an important post-synaptic density protein, PSD-95.

1.2.2.10 Iron and genes expression

Although the above mentioned effects of iron deficiency may well be due to the lack of post-translational incorporation of iron into functional haemoproteins and iron–sulfur proteins, two recent studies have defined the effects of early iron deficiency on gene expression of proteins involved in myelination, dendritic morphology, the neurometabolome and cellular energetics in the whole brain²⁰³ and in the hippocampus²⁰⁴. For example, in the hippocampus, 250 known gene transcripts were identified by microarray to be altered by iron deficiency at P15; 30% were involved in primary metabolism, 20% in signal transduction, and 11% in establishment of localization²⁰⁴.

Specific genes and proteins critical for hippocampal neuron differentiation and plasticity were involved [40]. The transcripts for BDNF (brain-derived neurotrophic factor) III and IV, as well as the transcript and protein levels of its receptor, are downregulated throughout the period of iron deficiency²⁰⁵.

1.2.2.11 Consequences of iron deficiency on behavior and cognitive functions

A large body of research is available on the relationship between iron deficiency (with, and, to less extent, without anemia) and behavior cognition, both in human and in animal models. Since it is not possible to cite here all the published papers on this topic, we refer to the two most comprehensive and recent systematic reviews and to the most relevant papers cited by these reviews^{167;206}.

Studies examining alterations in behavior or cognition during or soon after the period of iron deficiency

In almost all case-control studies (for a review, see:^{207;208}) comparing otherwise healthy full-term infants with iron deficiency anemia to infants with better iron status, their mental development test scores averaged 6 to 15 points lower. One preventive trial²⁰⁹ has showed the positive effects of iron supplementation on global mental functioning. Among case-control studies that included an assessment of motor development, most found that infants with iron-deficiency anemia received lower motor test scores, averaging 6 to 17 points lower¹⁶⁷. Among five preventive trials with well-nourished term infants, three showed motor benefits of iron supplementation²⁰⁹⁻²¹¹.

Every case-controlled study that examined social-emotional behavior found differences in iron-deficient anemic infants (e.g., more wary, hesitant, solemn, unhappy, kept closer to their mothers)¹⁶⁷. Two of three randomized trials to prevent iron deficiency that assessed this domain reported social-emotional differences as well^{209;212}. One preventive trial did not find social-emotional effects²¹¹.

Many of the case-controlled studies have included assessments before and immediately after iron therapy. Most studies report that differences in behavior and development persist in the majority of iron-deficient anemic infants even after a full course of iron treatment (2–6 months, depending on the study)²⁰⁹. However, three studies reported improvements, sometimes dramatic, in mental and/or motor test scores after iron therapy²¹³.

Two studies related perinatal iron deficiency to newborn temperament-like behaviors. One reported higher levels of irritability in infants whose mothers were iron deficient²¹⁴. In the other²¹⁵, lower levels of neonatal Hb and serum iron (and ferritin to a lesser degree) were correlated with higher levels of negative emotionality and lower levels of alertness and soothability. A small randomized, controlled trial involving breast-fed infants in Canada showed a benefit of early (between 1 and 6 months of age) iron supplementation on motor development and visual acuity at 12 months²¹⁰.

Long-Lasting Effects of Iron deficiency Beyond Infancy: Global Outcomes

These studies, most of which have been conducted at late preschool or early school age, followed children who had anemia (presumably due to iron deficiency), iron-deficiency anemia, or other indications of chronic severe iron deficiency in infancy.

There are at least six follow-up studies at late preschool age. In general, most of these studies reported a significant correlation between iron deficiency anemia and global IQ or equivalent measures of global outcome¹⁶⁷.

Only one study has related a direct measure of iron status at birth (cord ferritin concentration) to development years later. In this sample of 278 US children, those with cord ferritin concentrations in the lowest quartile received lower scores for language ability, fine-motor skills, and tractability at 5 years²¹⁶.

There are at least four reports of outcome in school-aged children¹⁶⁷. All found significant lower learning in formerly anemic children.

There is only one follow-up study past 10 years of age²¹⁷. Children who had been treated for severe, chronic iron deficiency in infancy still tested lower in arithmetic and writing achievement and motor function compared with 114 children with good iron status in infancy before and/or after treatment. Affective and social/emotional differences were still observed. The parents and teachers of formerly iron deficient children rated their behavior as more problematic in several areas, agreeing in increased concerns about anxiety/depression, social problems, and attention problems.

Neurocognitive and Neurophysiologic Outcomes

Two prospective studies, conducted respectively in Chile and Costa Rica¹⁶⁷, have assessed neurocognitive and neurophysiologic outcomes in formerly anemic children. In both Chile and Costa Rica, children with iron deficiency anemia or some other indication of chronic, severe iron deficiency in infancy had poorer performance on tests of some specific cognitive functions. For the Costa Rica sample at 11 to 14 years, poorer performance was observed for tachistoscopic threshold, spatial memory, and selective attention. At 5 and 10 years in the Chile follow-up and 19 years in the Costa Rica follow-up, formerly iron-deficient children did worse on executive function tasks, particularly ones that require inhibition and planning.

Of note, iron deficiency typically occurs in disadvantaged environments, but the findings from most of the above mentioned studies have been statistically significant after controlling for background factors in a wide variety of different settings. Nonetheless, the confluence of iron deficiency with a disadvantaged environment means that causal inferences cannot be made with confidence from case-controlled, long-term follow-up studies.

However, a note of caution has been expressed in a systematic review by McCann and Ames²⁰⁶. After reviewing the methodology of the above mentioned studies, they concluded that, although most of the 5 conditions of causality (association, plausible biological mechanisms, dose response, ability to manipulate the effect, and specificity of cause and effect) are partially satisfied in humans, animals, or both, a causal connection between iron deficiency and alteration in behavior/cognition has not been clearly established. Moreover, the same authors pointed out that most of the available studies included children with iron deficiency plus anemia. According to McCann and Ames the question if iron deficiency without anemia leads to cognitive and behavioural dysfunctions is still unsolved. In rats, anemia *per se* does not result in biochemical brain effects associated with iron deficiency anemia^{206:218}. However, it cannot be excluded that the poor performance of iron deficient children (or animals) without anemia on cognitive or behavioral

tests is confounded by generalized effects of anemia on physical energy levels as opposed to specific effects of iron deficiency in the brain.

However, despite an unfortunately small number of studies involving iron deficiency without anemia, some evidence, both direct and indirect, does suggest that cognitive or behavioural performance and brain function could be affected, particularly if deficiency occurs during critical stages of development.

Effects on cognitive, behavioral, or motor activity in rodents and monkeys

Animal studies provide important corroborative evidence and other information that is difficult or impossible to obtain in human studies. For example, some types of experiments cannot be done in humans, such as mechanistic studies that correlate changes in biochemical indicators of brain function to performance deficits on cognitive or behavioral tests. Also, much greater flexibility of design is possible in animal experiments, which avoids some serious confounders in human studies²⁰⁶. In rodents model of iron deficiency, motor or exploratory activity was most frequently measured, using activity monitors, the hole board, or home orientation and open-field tests. However, some methods target cognitive functions, such as learning and memory, more directly, such as the Morris water maze and passive or active avoidance tests.

Twenty rodent and 2 monkey studies that examined cognitive, behavioral, or motor activity in iron-restricted animals were identified in the review by McCann and Ames²⁰⁶. Regardless of the degree to which animals were iron-restricted, regardless of whether they were iron deficient with or without anemia at the time of testing and regardless of the degree of undernutrition or whether it was controlled for, changes in activity or behavior compared with that in controls were reported on at least some tests in all studies. Dietary intake of iron was restricted either during gestation or before weaning or after weaning. In all cases, statistically significant performance differences between iron restricted and control groups on at least some performance tests were reported.

Some long-term behavioral alterations in the developmental rat model of early deficiency closely parallel prior experimental studies of specific early dopaminergic insults (perseveration,

behavioural inflexibility, increased anxiety, lifelong hyperreactivity to novel objects and experiences in an unfamiliar environment, but normal responses to the same stimuli in the home cage). Thus, rats models show that early iron deficiency is associated with impairment in attentional and affective responses. Specific perinatal damage to nigrostriatal dopamine neurons or intrinsic striatal or corticostriatal neurons, whether induced by early iron deficiency or pharmacologic injury, results in fine motor, gross motor, motor sequencing, and sensory deficits in rats. These data further support the hypothesis that iron deficiency in infancy leads to a chronic disorder of dopaminergic and corticostriatal function²¹⁷.

Important differences exist between animals and humans that must be taken into account in evaluating the implications of results for humans. For example, in rats and mice, offspring are born at an earlier stage of development than are humans, with the period of the brain growth spurt generally considered to begin at birth and extend up to weaning (usually postnatal day 21). In humans, the brain growth spurt begins in the last trimester of pregnancy and extends through the first 2 y of life. Another important difference between rodents and humans is that rats have a greater requirement for dietary iron during development than do humans because the rate of development is relatively much more rapid. Also, in the great majority of the studies discussed below, the dietary supply of iron was much more severely restricted than in human studies. Thus, species and dosimetry differences must be considered when results in animals are extrapolated to humans²⁰⁶.

Neurophysiologic alterations

Evidence on neurophysiologic effects of iron deficiency in children is limited but growing. One study²¹⁹ has compared auditory brainstem response (ABR) and visual evoked potentials (VEP) in formerly iron deficient anemic children and controls. The formerly iron-deficient anemic group (n = 41) showed longer ABR and VEP latencies compared with children who had been non-anemic in infancy (n = 43). The magnitude of effects was large: 1 to 1.2 SD. Another study (cited in ¹⁶⁷) has evaluated polysomnographic recordings during an overnight sleep study for 27 formerly iron-

deficient anemic children and 28 children who had been non-anemic in infancy. There were significant differences in measures of the sleep/wake cycle, including REM latency, duration of REM episodes and their pattern through the night, the duration of slow-wave sleep episodes early in the night, both the isolated REMS and non-isolated REMS indices, and inter-REM intervals. It has also been reported an overall increase in intra-individual variability of spontaneous activity, measured with actigraphy, in formerly iron-deficient anemic children, especially for the duration of different states during both day and night¹⁷⁵.

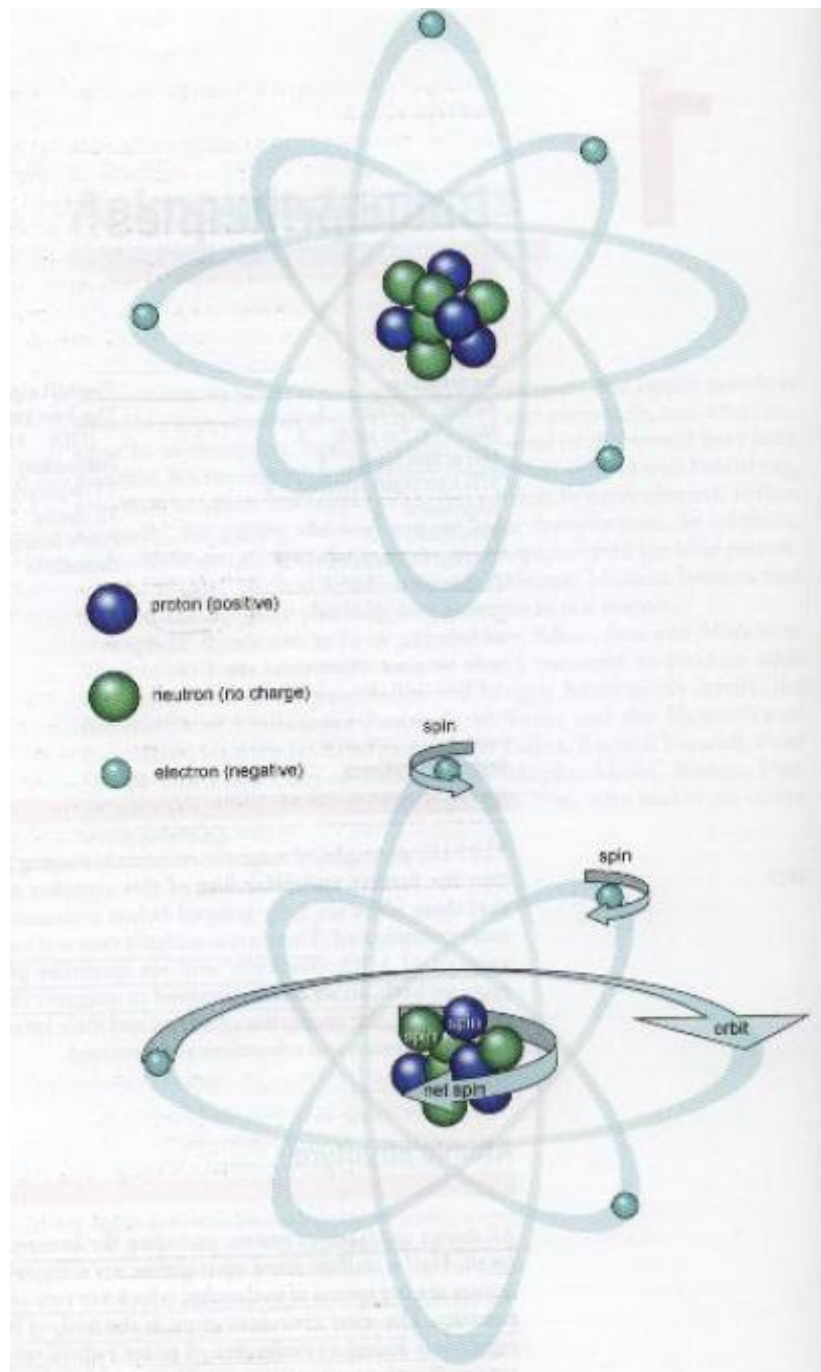
1.2.2.12 Estimation of brain iron by means of MRI

Physical principles of relaxometry (The contents and figures of this section are taken from: Westbrook, Roth, Talbot. MRI in practice. Blackwell Publishing. 2005²²⁰, chapters 1 and 2)

All things are made of atoms, including the human body. The most abundant atom in the body is hydrogen. The atom consists of a central nucleus (subdivided into protons and neutrons) and orbiting electrons. Three types of motion are present within the atoms: 1. Electrons spinning on their own axis; 2. Electrons orbiting the nucleus; 3. The nucleus itself spinning about its own axis.

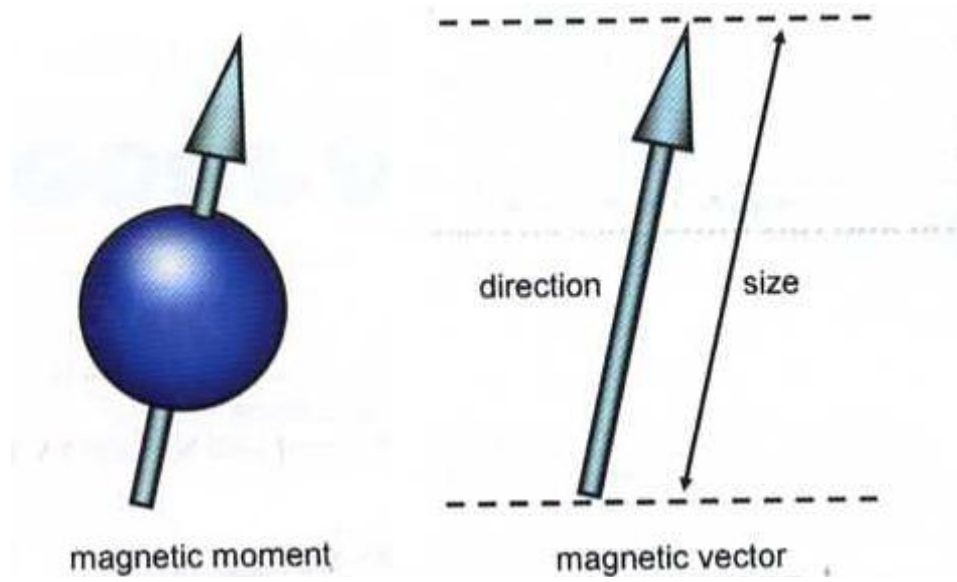
(Fig. 13).

Fig. 13. The atom and motions present within the atom (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).



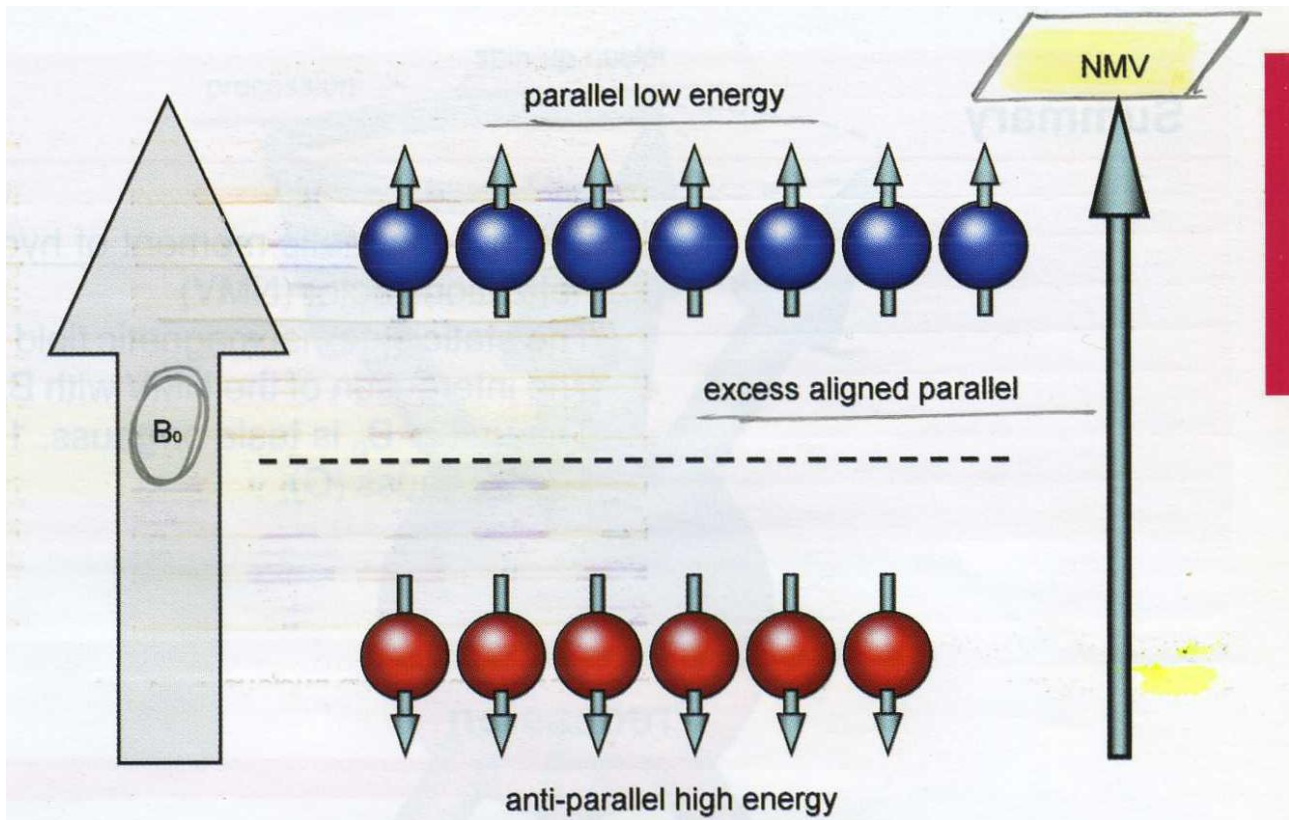
The spin of the nucleus derives from the individual spins of protons and neutrons within in the nucleus. Pairs of subatomic particles automatically spin in opposite direction. In nuclei that have an even mass number, i.e. the number of protons equals the number of neutrons, half spin in one direction and half in the other. The nucleus itself has not net spin. In nuclei with odd mass number, spin directions are not equal and opposite, so the nucleus itself has a net spin or angular momentum. These are know as MR active nuclei. MR active nuclei are characterize by their tendency to align their axis of rotation to an applied magnetic field, thus acquiring a magnetic moment (Fig. 14).

Fig. 14. The magnetic moment of hydrogen nucleus (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).



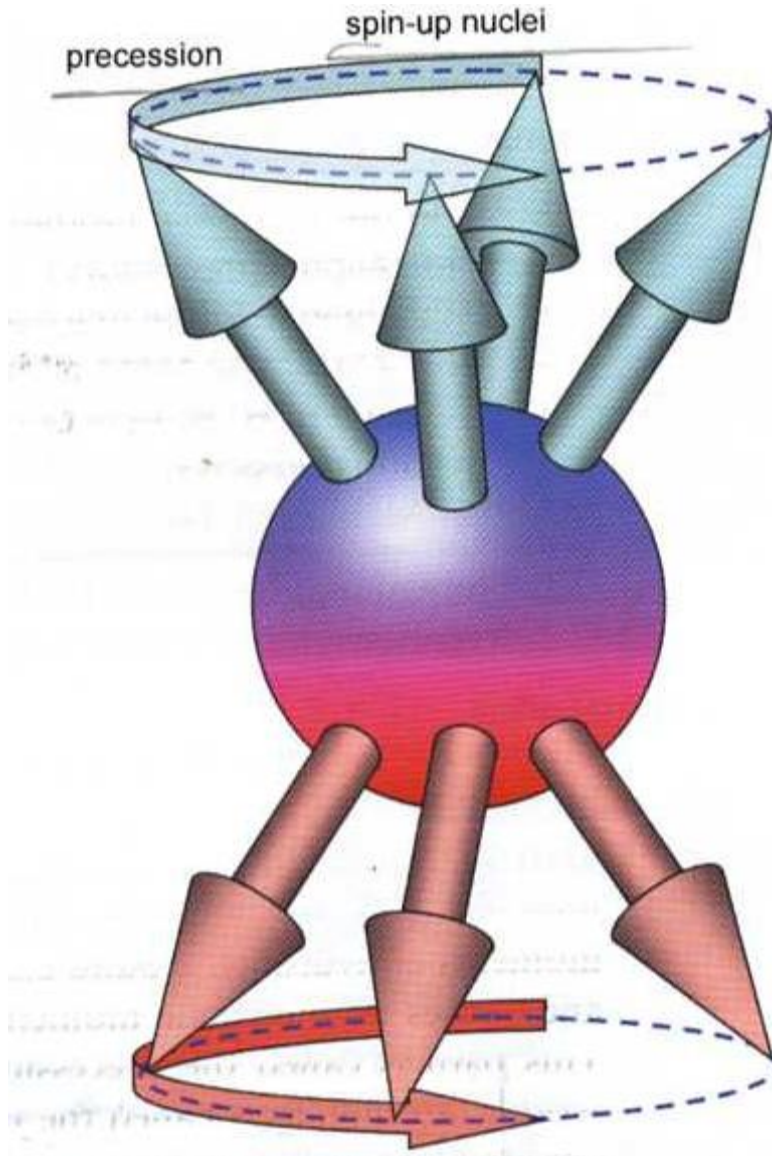
The hydrogen nucleus is the MR active nucleus used in clinical MRI. It is used because it is very abundant in the human body and because its solitary proton gives it a relatively large magnetic moment. In the absence of an applied magnetic field, the magnetic moments of the hydrogen nuclei are randomly oriented. When placed in a strong static external magnetic field, termed B_0 , the magnetic moments of the hydrogen nuclei align parallel with the magnetic field. According to quantum theory (first described by Max Planck in 1900), hydrogen nuclei possess energy in two discrete quantities or populations termed low and high. Low energy nuclei align their magnetic moments parallel to the external field (spin up nuclei), while high energy nuclei align in the anti-parallel direction (spin-down nuclei). As there is a larger number aligned parallel, there is always a small excess in this direction that produces a net magnetic moment (NMV) (Fig. 15).

Fig. 15. The net magnetization vector (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).



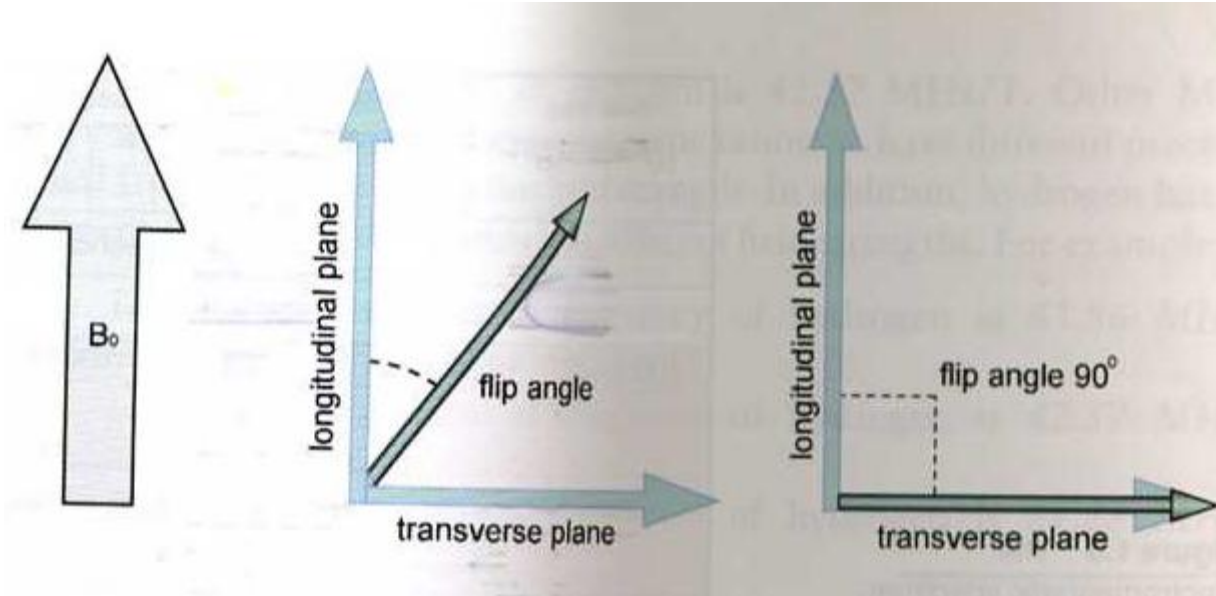
The influences of B_0 produces an additional spin, called precession, and causes the magnetic moments to follow a circular path around B_0 , called precession. The speed at which the magnetic moments wobble around B_0 is called precessional frequency, which is proportional to B_0 , according to the equation: $\omega_0 = \lambda B_0$, where ω_0 is the precessional frequency, B_0 is the magnetic field strength of the magnet, and λ is a constant (the gyro-magnetic ratio) (Fig. 16).

Fig. 16. Precession of the spin-up and spin-down populations (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).



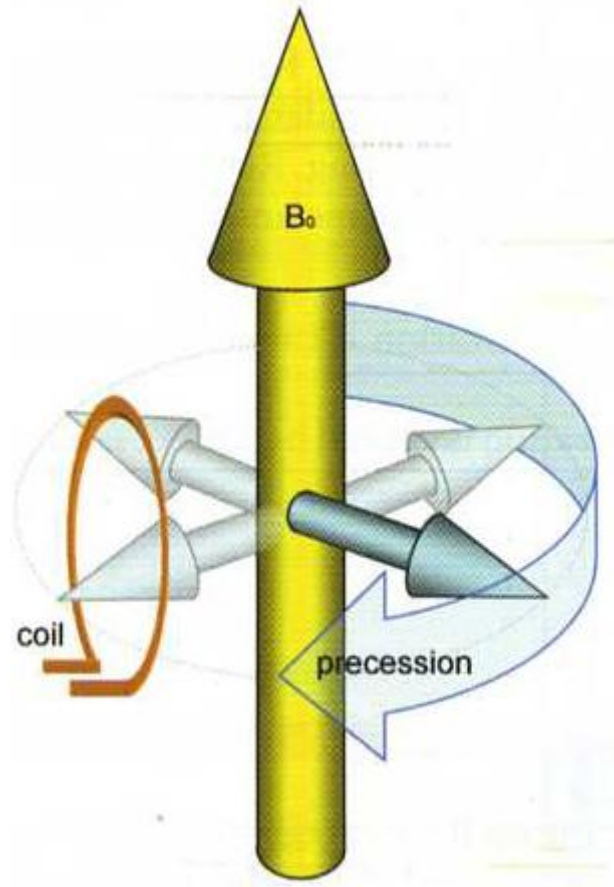
The application of a radio frequency (RF) pulse causing resonance makes NMV to move out of alignment away from B_0 (usually the flip angle is 90° , i.e. the NMV moves from the longitudinal to the transverse plane) (Fig. 17).

Fig. 17. The flip angle (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).



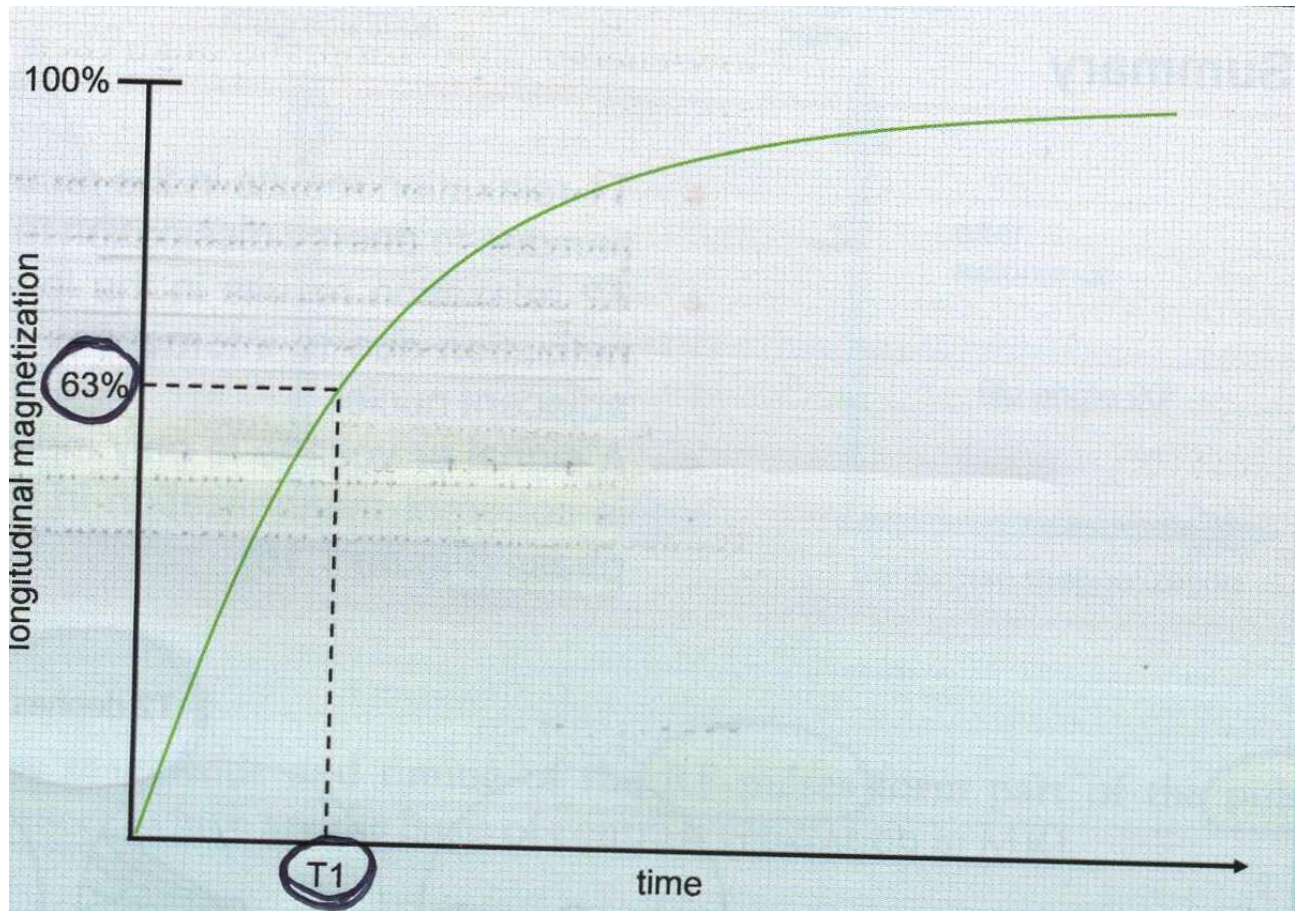
The other result of resonance is that the magnetic moments of hydrogen nuclei move into phase with each other. The MR signal is produced when coherent (in phase) magnetization cuts across a receiver coil (Fig. 18).

Fig. 18. Generation of the signal (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).



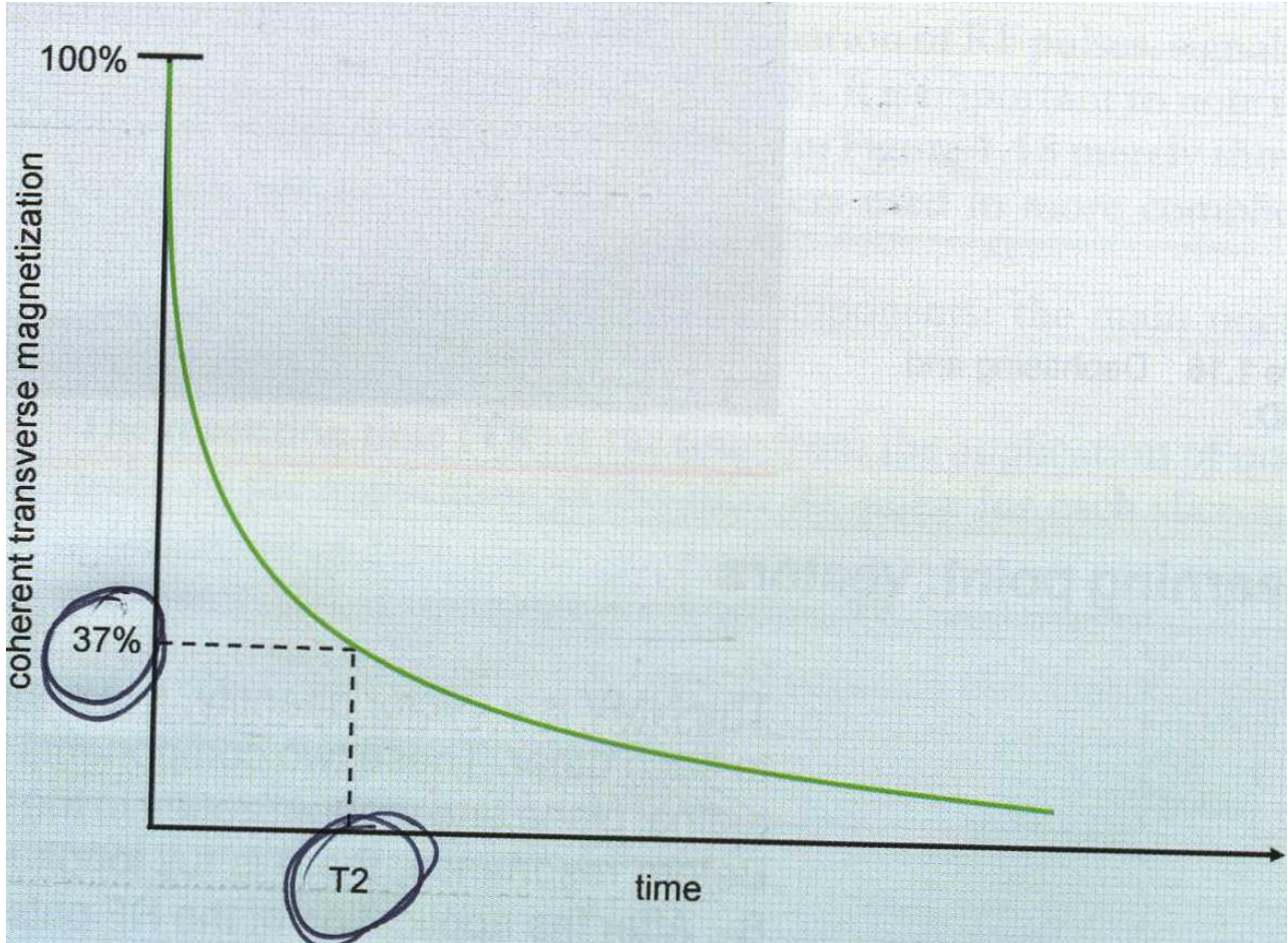
When the RF pulse is switched off, the NMV is again influenced by B_0 and it tries to realign with it. This process is called relaxation. Two processes occur: 1. The amount of magnetization in the longitudinal plane gradually increases (T1 recovery); 2. At the same time but independently, the amount of magnetization in the transverse plane gradually decreases (T2 decay). T1 recovery is caused by nuclei giving up their energy to the surrounding environment or lattice. T1 is the time it takes 63% of the longitudinal magnetization to recover in the tissue (Fig. 19).

Fig. 19. The T1 recovery curve (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).



T2 decay is caused by nuclei exchanging energy with neighbouring nuclei. T2 is the time it takes 63% of the transverse magnetization to be lost (Fig. 20). T2* decay is faster than T2 and is a combination of: T2 decay itself and dephasing due to magnetic field inhomogeneities.

Fig. 20. The T2 decay curve (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).



Water and fat have different T1 and T2, as reported in Fig. 21-22

Fig. 21a. T1 recovery in fat (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).

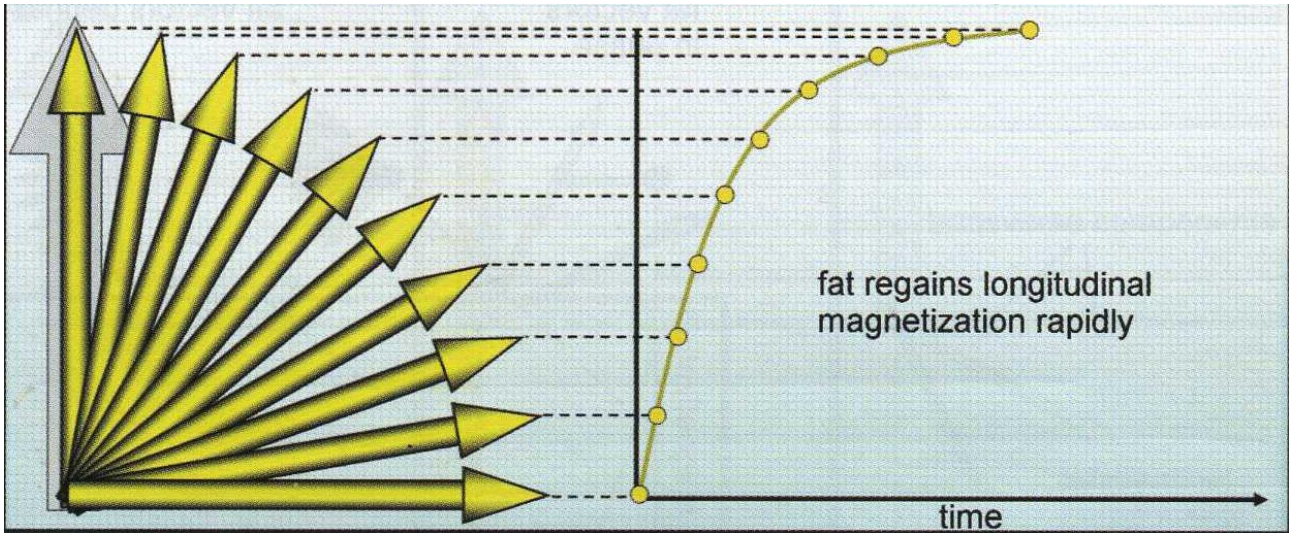


Fig. 21b. T1 recovery in water (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).

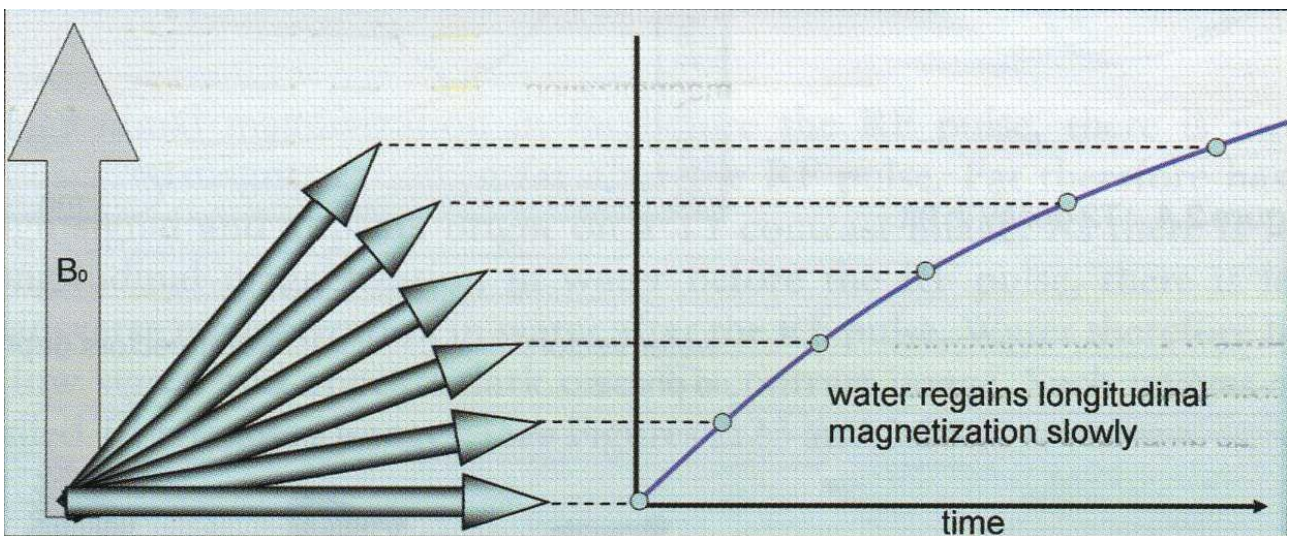


Fig. 22a. T2 decay in fat (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).

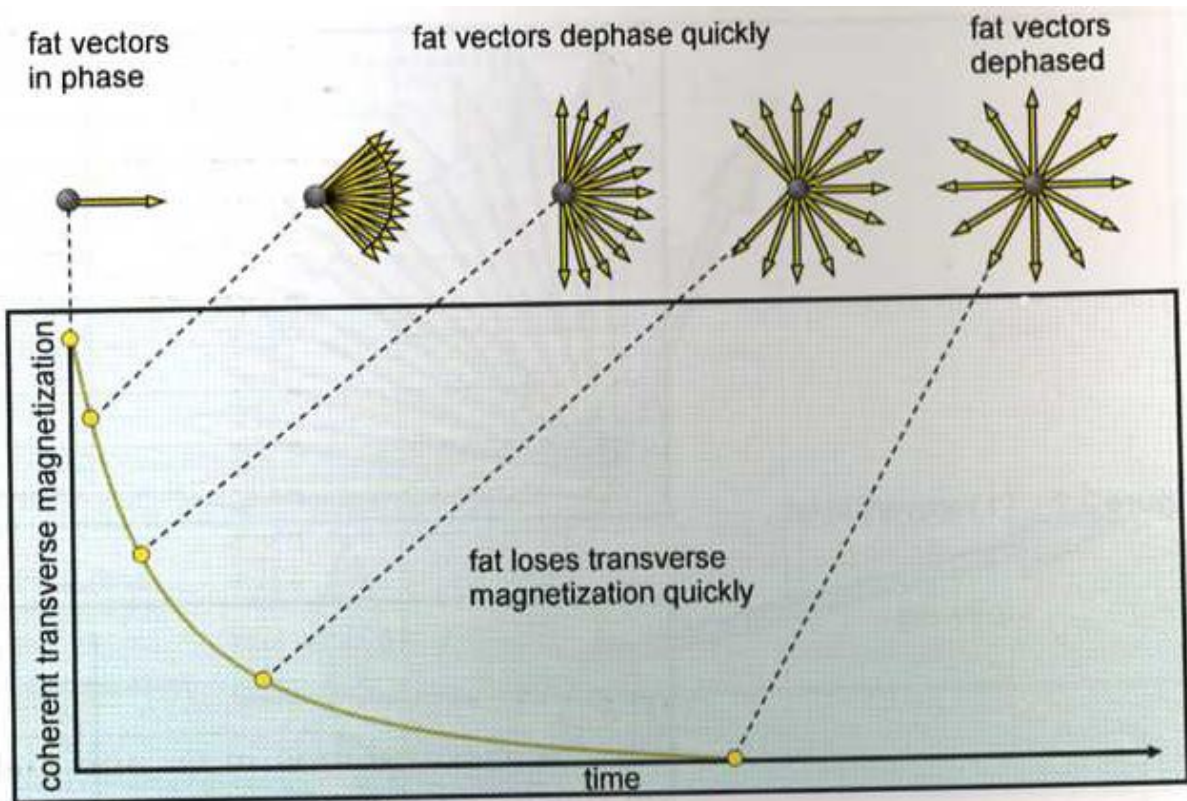
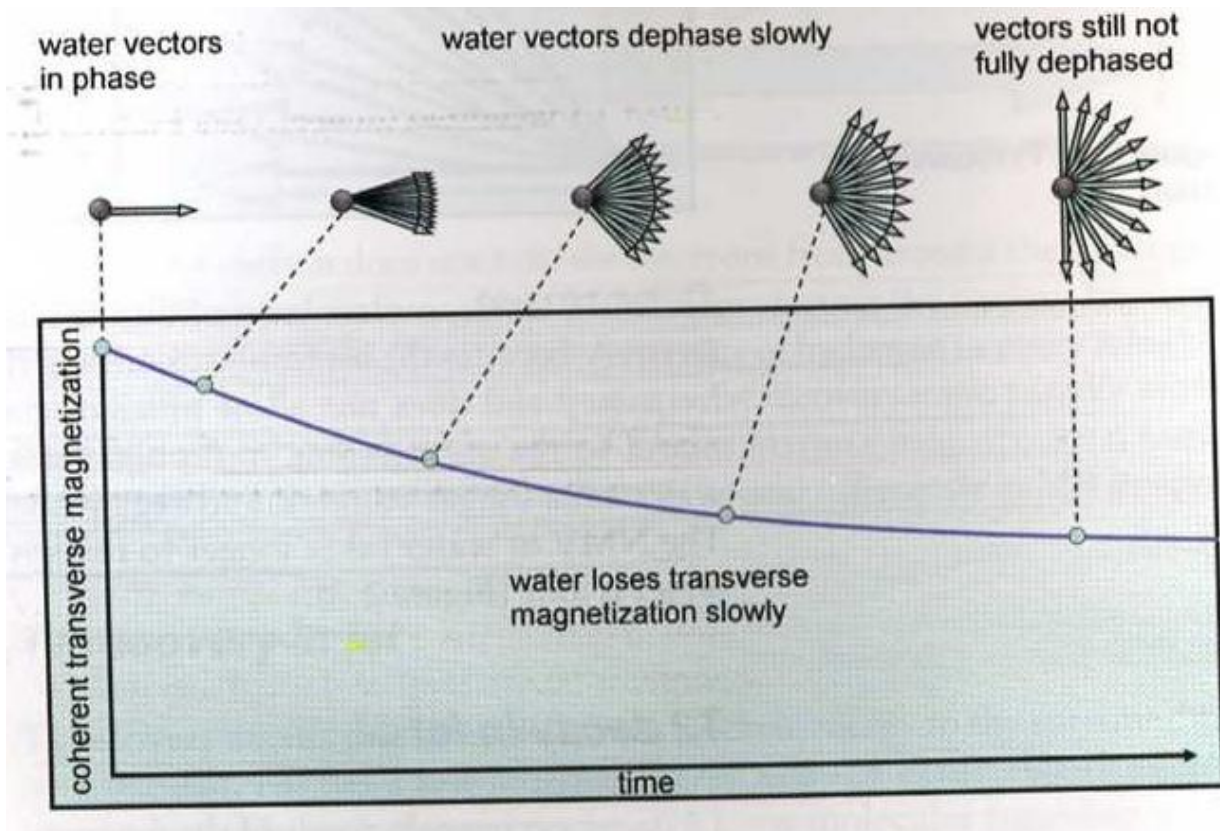


Fig. 22b. T2 decay in water (from: Westbrook, Roth, Talbot. *MRI in practice*. Blackwell Publishing. 2005²²⁰).



This is useful to image and differentiate them in biological tissues. As the T1 time of fat is shorter than that of water, the fat vector (representing magnetic moment) realigns with B_0 faster than water vector. After a certain time that is shorter than the total relaxation times of the tissues, the next RF excitation pulse is applied. The RF excitation pulse flips the longitudinal components of magnetization of both fat and water into the transverse plane. As there is more longitudinal magnetization in fat before the RF pulse, there is more transverse magnetization in fat after the RF pulse. Fat therefore has a high signal and appears bright on a T1 contrast image. As there is less longitudinal magnetization in water before the RF pulse, there is less transverse magnetization in water after the RF pulse. Water therefore has a low signal and appears dark on a T1 contrast image. Such images are called T1 weighted images.

The T2 time of fat is shorter than that of water, so the transverse component of magnetization of fat decays faster. The magnitude of transverse magnetization in water is large. Therefore, water has a high signal and appears bright on a T2 contrast image. On the other hand, the magnitude of transverse magnetization in fat is small. Fat therefore has a low signal, and appears dark on a T2 contrast image. Such images are called T2 weighted images.

Magnetic properties of iron

Iron in biologic systems is found with a variety of oxidation states, reduction potentials, magnetic properties, degrees of aggregation, solubilities, and mobilities²²¹. Each of these variables influences not only biologic activities and toxicities but also the detection and quantification of iron. As previously stated, there are two categories of iron in the brain: heme iron, in hemoglobin and some enzymes like peroxidases, and nonheme iron. The types of nonheme iron in the brain include 1. Low-molecular weight complexes; 2. Metalloproteins like transferrin, melanotransferrin and lactoferrin; 3. Storage proteins such as ferritin and hemosiderin and 4. Ionic iron. Iron in the form of biogenic magnetite has also been reported²²².

At least one third of the nonheme iron in the brain is in the form of ferritin, but concentrations can be much greater in iron-rich gray matter²²². For example, Dedman et al.²²³

suggested that 85–88% of the nonheme iron in the parietal cortex is located in ferritin. Ferritin and hemosiderin are considered to be the only forms of nonheme iron present in sufficient quantities to affect MR contrast in the human brain²²⁴. As discussed above, transferrin concentrations in brain tissue are at least 10 times lower than ferritin and it can only bind two iron atoms compared to thousands of iron atoms within each ferritin molecule. The concentrations of other iron species, like free iron aqua ions, the labile iron pool, or nontransferrin-bound iron are also considered to be too low to affect MR contrast²²⁴. The human brain also contains other ions with magnetic properties, like copper and manganese. However, Schenck²²⁴ suggests logical concentrations of copper and manganese are too small to produce detectable MR contrast.

Ferritin has a large spherical protein coat, about 12 nm in diameter, that surrounds a crystalline core of hydrous ferric oxide $[5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}]^{225}$. There can be up to 4500 iron atoms stored in the 8-nm-diameter internal cavity of one ferritin protein. Iron (II) passes into the core through six channels in the protein coat and is oxidized to iron (III) for storage. It was at first suggested that ferritin has paramagnetic (see Tab. 9 for a definition of “paramagnetic”) properties. Aggregates of paramagnetic particles that are largely shielded from the water by a protein shell exert their effect on MR relaxation by dephasing the magnetic spins of the protons diffusing in their vicinity. This is appropriately called the “outer sphere mechanism”. It results in T2 relaxation time reduction and produces hypointense regions on T2-weighted MR images²²⁶. On the other hand, individual magnetic ions, or small ion clusters, in direct exposure to the solvent water molecules have their major effect on T1 via the so-called inner sphere mechanism and reduce T1 to produce hyperintense regions in T1-weighted images²²⁶.

As ferritin acts primarily through the outer sphere mechanism, the major effect of brain iron on MR images is a reduction in T2 and much reduced effect on T1 relaxation rates²²⁴. However, it has been pointed out by Bizzi and colleagues²²⁷ that relaxation rates attributed to ferritin are generally found to be too high to be explained by simple paramagnetism. In fact, the ferrihydrite

crystal structure of ferritin is antiferromagnetic and, because of the nanometric size of the grains, ferritin is also superparamagnetic.

One of the properties of small antiferromagnetic crystals is that they saturate (see Tab. 9 for the definition of saturation) at much lower magnetic field strengths than paramagnetic substances do. In an early report, a theoretical calculation for fully loaded ferritin (4500 iron atoms), according to Neel's theory²²⁸, predicts that saturation will begin at around 1.0 T at body temperature. However, a number of recent studies^{138;229;230} have reported a strong linear correlation between the relaxation rate and the field strength (1.0 T and up) in either ferritin solutions or iron rich brain areas. No saturation effect has been observed. The size of the ferritin core may be a factor since the field strength needed for saturation depends inversely on crystal size. Thus, only when the iron content of the particle is sufficiently high would it lead to magnetic saturation at lower field strengths.

Table 9. Magnetism: Glossary (from Bizzi et al., Radiology, 1990²²⁷).

-
- *Paramagnetism.* Magnetism found in substances with unpaired electrons, arising mostly from the electron spin. Paramagnetic substances include iron, manganese, nickel, cobalt, chromium, the rare earths (e.g., gadolinium), and molecular oxygen. In the absence of an applied magnetic field, the spins point in random directions, and there is no observable magnetism. In the presence of an external field, the individual magnetic moments become partially aligned and create an additional reinforcing magnetic field. The degree of alignment is proportional to the applied field strength, but at normal MR imaging field strengths and temperatures, it is less than 1% of maximum
 - *Ferromagnetism.* A much stronger magnetism created when paramagnetic atoms in a crystal lattice become locked into *magnetic alignment* by interatomic forces. The best known example is metallic iron, in which field strengths well over 1T can be found at room temperature.
 - *Antiferromagnetism.* A variation of ferromagnetism, found in certain crystals, in which interatomic forces create an *antiparallel magnetic alignment*; that is, neighboring atoms point magnetically in opposite directions. In general, the number of paramagnetic atoms pointing in each direction is the same, and there is no net magnetization.
 - *Superparamagnetism.* When the crystal size is very small, less than about 10 nm, surface effects create an inequality in the two spin populations, and hence a net permanent magnetic moment of the crystal. The population inequality is on the order of the square root of the number of paramagnetic atoms, so this moment is much smaller than that of a comparably sized ferromagnet but still large compared with a single atom. These large crystalline moments align with an applied field, as do paramagnetic moments, but, because of their strength, the degree of alignment is much greater. Therefore, superparamagnetic materials have positive magnetic susceptibilities that are greater than paramagnetic materials and less than those of ferromagnetic materials
 - *Saturation.* A condition in which individual magnetic moments, whether paramagnetic atoms or superparamagnetic crystals, are completely aligned along the applied magnetic field, producing 100% of the possible magnetization. Further increases in applied field strength therefore do not increase the magnetization. Saturation is seen in paramagnetic substances only at very low temperatures, but superparamagnetic crystals, because of their larger magnetic moments, may saturate at room temperature
-

Proposed relaxometry metrics to estimate brain iron

At present time, there is a lack of consensus on the most desirable MRI method for accurately estimating iron content in the brain. Given the magnetic properties of ferritin, the 3 primary relaxometry metrics (the transverse relaxation rates or reciprocals of the transverse relaxation times ($R2 = 1/T2$, $R2^* = 1/T2^*$, and $R2' = 1/T2'$, where $R2' = R2^* - R2$))have been proposed as pertinent to the detection of brain iron²³¹.

There is considerable debate and controversy as to which MRI technique, if any, most closely estimates iron deposition with acceptable sensitivity and specificity^{231;232}.

R2

R2 is a transverse relaxation rate measured from a series of spin echoes acquired at different echo times and generated with RF refocusing pulses as opposed to gradient reversals.

Several studies have assessed the usefulness of R2 to estimate brain ferritin levels (e.g.^{227;229;230;233-244}).

Both in vitro (e.g.^{230;244}) and in vivo (e.g.^{227;233-235;241}) data on healthy subjects show strong linear correlations between R2 in gray matter regions and iron concentrations derived from the literature¹⁴⁰ (as in ^{233;234;241}) or measured directly from the tissue sample (as in ^{227;235}). Schenker et al.²⁴¹ also demonstrate that the correlation between R2 and age in healthy subjects follows the same relationship to that of iron and age. Drayer et al.²³³ pointed out that iron is the only trace metal in the brain that can prominently and preferentially decrease the T2 relaxation time (thus explaining the correlation between iron levels and R2) and localize in precisely the topographic basal ganglia regions seen on the high field strength MR images. Calcium may increase with age in the globus pallidum and dentate nucleus, but it does not accumulate in significant quantities in the red nucleus or substantia nigra. The structures that have decreased signal intensity on the T2-weighted images have an intensity comparable to cerebral gray matter on non-T2- weighted images. Melanin has only a mild effect on T₁ relaxivity and a different topographic distribution. Lipofuscin has a similar anatomic localization, but will not significantly and specifically affect T2 relaxivity.

Although the effect on T2 has been classically attributed to paramagnetic properties of ferritin, Bizzi et al.²²⁷ noted that the dependence of T2 shortening on field strength was not quadratic, as expected for paramagnetic iron, but instead showed a marked levelling off at higher field strengths. This magnetic “saturation” is explainable by antiferromagnetism and superparamagnetism of the ferritin core and has been observed in ferritin solutions at low temperatures. This T2 shortening is not to be confused with spin-spin paramagnetic relaxation (e.g., from gadopentetate dimeglumine), which affects both T1 and T2²²⁷.

Clearly, not all T2 is influenced by ferritin. Schenker et al.²⁴¹ pointed out that the relaxation time in a biological tissue is complex. It can be expressed as a sum of the reciprocal T2 values of the different contributing factors:

$$\frac{1}{T2 \text{ (tot)}} = \frac{1}{T2 \text{ (Fe)}} + \frac{1}{T2 \text{ (rest)}}$$

where T2 (tot) = measured T2, T2 (Fe) = T2 of iron and T2 (rest) = T2 of all other factors such as water, protein, etc.. According to Schenker et al.²⁴¹, T2 (rest) seems to be constant and is not believed to be responsible for changes of T2 (tot) values under normal conditions. With increasing age, however, the T2 (rest) becomes more important due to higher individual variations of the tissues, reflecting structural changes in the basal ganglia, on the one hand, and saturation of the iron concentration [and therefore constant T2 (Fe)], on the other.

However, opinions on the correlation between R2 and iron diverge when gray and white matter regions are considered together in healthy adults (e.g.^{245;246}) or when certain neurodegenerative diseases (HD, PD, AD) are considered (e.g.^{247;248}). These discrepancies arise because, although R2 is strongly affected by iron concentration, the water content of brain tissue also influences R2. On average, there is a 12% difference in water content between white matter (roughly 72%), with its high lipid content, and gray matter (roughly 84%)²⁴⁹. The lower water

content results in a higher R2 value in white matter tissue compared to gray matter regions with the same iron concentration and could explain why some studies (e.g. ^{245;246;250}) did not obtain a strong correlation with iron. Moreover, Chen et al.²⁴⁶ pointed out that the relationship between tissue iron concentrations and T2 values depends not only on the total amount but also on the physical state, form, and microscopic distribution of iron. For example, iron in the arteriolar walls is effectively sequestered from free water and may be a less effective relaxation enhancer, accounting, in part, for the inconsistency in the observation made between iron-stained brain sections and in vivo MRI images.

To overcome the limitations of R2 in disease, the Bartzokis group adopted the field-dependent rate increase (FDRI) technique^{138;139;144;251-255}, which measures the difference in R2 of a set tissue when applying 2 different field strength instruments by taking into account that R2 is higher when measured at a higher magnetic field strength. The difference of the R2 in tissue between field strengths correlates well with the quantity of ferritin iron in normal adults and it is claimed to be specific for ferritin. Bartzokis and colleagues have shown increased iron levels in the basal ganglia of patients with Alzheimer, Parkinson, and other diseases^{144;252-255}, compared with healthy controls using FDRI.

R2'

Some authors^{248;256-258} have reported that R2' (R2*-R2) is more specific for iron than R2, since it depends on the susceptibility effect associated with iron and is independent of other parameters which affect R2. However, Hikita et al.^{248;256-259} measured R2' and R2 in the basal ganglia of 13 healthy volunteers and correlated these relaxometric measures with iron content derived from historical postmortem data. A linear correlation was identified between R2 and iron concentration and between R2' and iron concentration; but the correlation between MRI and iron levels in the gray matter was higher for R2 than R2'. The R2' measurements were affected by field

inhomogeneity related to the skull and thus were less sensitive to interregional differences (e.g., iron in the cortex vs. putamen).

*R2**

There has also been some works that found a significant correlation between brain iron and $R2^*$ ^{248;256-258;260-264}. Interestingly, Zywicke et al.²⁶⁴ have shown that $R2^*$ mapping may also be used to visualize reduced brain iron, not only brain iron overload.

Recently, P eran and colleagues²⁶⁰ have published a voxel-based analysis of whole-brain $R2^*$ maps without defining anatomical structures a priori.

However, $R2^*$ has its own difficulties related to other local background sources of magnetic field variation that cause signal loss unrelated to the internal iron content of the tissue²⁶⁵.

Other MRI metrics used to estimate brain iron levels include:

R1

Vymazal et al.²⁴³ and Ogg and Steen²⁶⁶ have reported that ferritin does shorten T1, resulting in increased signal intensity on T1-weighted images. It is not clear whether this effect derives mainly from isolated iron atoms attached to the protein shell or from the loaded core.

Phase shifts

Ogg et al.¹⁴³ and Haacke et al.²⁶⁷ have shown a direct correlation between phase shift and brain iron. Phase shift has also been used in a recent study¹⁸⁸ to assess age, gender, and hemispheric differences in iron deposition in the human brain.

Magnetic field correlation (MFC)

The Jensen group²⁶⁸ proposed this new technique to quantify brain iron. MFC is based on the influence of MR signal by magnetic field inhomogeneities (MFI) (due to spatial variations in

magnetic susceptibility). These changes can be due to macroscopic structures such as cavities, bones and vessels, microscopic structures such as capillaries, and the presence of paramagnetic substances (e.g., metals, contrast agents). MFC imaging is based on a theoretical model of MR relaxation in the presence of magnetic field inhomogeneities and uses a non-monoexponential decay curve. It differs from R_2 and R_2^* in having a simpler and more direct connection to MFIs and in being, by definition, independent of relaxation mechanisms, such as dipolar interactions, unrelated to MFIs.

T2 rho

A newer relaxometry measure, T2 rho, has been developed which is more sensitive to diffusion and exchange of water protons in environments with different local magnetic susceptibilities.

Wheaton et al.²⁶⁹ demonstrated an increase in iron related contrast when comparing images created with T2 rho to conventional T2 in the normal human brain. Using a 4T magnet with a rotating frame and applying adiabatic pulses.

Michaeli et al.²⁷⁰ demonstrated a statistically significant shortening of relaxation times in substantia nigra tissue in patients with PD compared to normal controls with T2 rho but not with routine T2. This correlates with postmortem observations of excessive iron deposition associated with the disease and suggests that this novel technique generates better iron related contrast than conventional T2 methodology.

Several newer MRI methods for estimating iron content are under investigation. These include susceptibility-weighted imaging, magnitude imaging, phase imaging, and advanced analysis methods.

2. THE STUDY

2.1 Rationale

Several lines of evidence, reviewed in detail in the first part of the thesis, suggest that iron deficiency might contribute to the etiopathogenesis and pathophysiology of ADHD, at least in a subset of patients.

First, iron is a co-factor of enzymes necessary for the synthesis and catabolism of the aminergic neurotransmitters (dopamine, serotonin, and noradrenaline)¹⁵⁹, which have been shown to be involved in the pathophysiology of ADHD⁴.

Second, iron deficiency is associated with a decrease in the dopamine transporter (in particular in accumbens and striatum)²⁷¹, whose gene has been demonstrated to have a significant effect in the genetic vulnerability for ADHD¹⁶.

Third, it has been reported that iron deficiency is associated with a dysfunction in the basal ganglia¹⁷⁹, which are believed to play a significant role in the pathophysiology of ADHD²⁰.

Fourth, there is a large body of research showing that ID in childhood is associated with cognitive and behavioral impairments, including poor attention and hyperactivity¹⁶⁷.

Moreover, two specific and recently developed areas of research further suggest a possible link between iron deficiency and ADHD/ADHD symptoms.

1) The first is the potential association between ADHD and Restless Legs Syndrome (RLS). RLS is a sensorimotor disorder characterized by an irresistible urge to move the legs, often accompanied by uncomfortable sensations, relieved by movement and worse in the evening or night and at rest²⁷². Although clear delineation of dopaminergic pathology in RLS has not yet emerged²⁷³, several studies, in particular pharmacologic ones, indicate that a dysfunction of dopamine should be present in RLS²⁷⁴. Increasing evidence both from peripheral (i.e. serum ferritin levels)^{275:276} and central measures of iron status (i.e. MRI²⁷⁷⁻²⁸⁰ and autopsy studies²⁸¹) suggests that iron deficiency is a contributing factor to RLS. Although RLS has been traditionally considered a disorder of middle to older age, it may occur in children²⁸². As we concluded in a review of published clinical

studies²⁸³, up to 44% of subjects with ADHD have been found to have RLS or RLS symptoms, and up to 26% of subjects with RLS have been found to have ADHD or ADHD symptoms. Given that most of the reviewed studies are hampered by methodological issues (lack of representative samples and standardized assessment of ADHD as well as of RLS), a definitive conclusion on the nature of the relationship between ADHD and RLS symptoms is not possible. We have suggested^{283;284} that several hypotheses may explain the relationship between RLS and ADHD/ADHD symptoms. Sleep disruption associated with RLS might lead to inattentiveness, moodiness and paradoxical overactivity. Diurnal manifestations of RLS, such as restlessness and inattention, might mimic ADHD symptoms. Alternatively, RLS might be co-morbid with “true” ADHD. Subjects with RLS and a subset of subjects with ADHD might share a common dopamine dysfunction, caused, at least in part, by iron deficiency.

2) The second is provided by the even more recent interest for the relationship between ADHD and obesity/overweight. In a systematic review of empirically based evidence on this issue, we concluded that there is a bi-directional link between ADHD and overweight: obese patients referred to obesity clinics present with higher than expected prevalence of ADHD (diagnosed according to standardized criteria) and subjects with ADHD (both from clinical and population based sample) present with significantly higher than the average mean Body Mass Index standardized scores. Interestingly, an inverse correlation between iron status and adiposity has been reported²⁸⁵. This may be due to the effects of hepcidin, which, as reviewed in the first part of the thesis, inhibits enterocyte iron absorption as well as the release of non-heme iron from macrophage²⁸⁵.

Therefore, evidence from different areas of research suggests that iron status should be explored in children with ADHD.

To date, two published studies^{286;287} from two independent groups have suggested a possible role of ID in childhood ADHD. However, both these studies are based on the measure of serum ferritin, a *peripheral* marker of ID. However, how well peripheral iron indices correlate with central

(i.e. brain) iron content is still unclear. Although low peripheral iron levels may negatively impact on brain iron, several authors failed to find a significant correlation between peripheral and brain iron (e.g.:¹⁴⁰). To date, no published study has assessed brain iron levels in children with ADHD by means of MRI.

2.2 Objectives

In order to fill the gaps in current literature and advance the field, the aims of the present research project are:

Primary: To compare brain iron levels, estimated by means of MRI, in a sample of children with ADHD and in a group of healthy controls. Iron levels were estimated in four regions which have been shown to contain iron: thalamus, putamen, pallidum, and caudate. In order to assess if a potential central ID is a specific finding in ADHD or it is also present in other psychiatric disorders, brain iron levels were also estimated in a group of children with various psychopathologies (depressive, anxiety, and psychotic disorders).

Secondary: To assess the relationship between serum ferritin levels and estimated brain iron levels (in the four above mentioned regions) in children with ADHD as well as in healthy controls.

2.3 Methods

2.3.1 Subjects

Patients with ADHD, as well as those with other psychiatric disorders, were recruited from the outpatient consultation and from the inpatient unit of the Child and Adolescent Psychopathology Service of the Hospital Robert Debré in Paris, in the period January 2007-June 2008. Healthy controls were recruited from relatives of hospital employees, during the same period. All the subjects were recruited by the doctoral candidate during his clinical activity.

Inclusion criteria were as follows:

Children with ADHD: 1. Diagnosis of ADHD according to DSM-IV-TR criteria and confirmed by the semi-structured interview Kiddie-SADS-PL; 2. Age range: 6-14 years.

Children with other psychopathologies: 1. Diagnosis of a psychiatric disorder different from ADHD according to DSM-IV-TR criteria and confirmed by the semi-structured interview Kiddie-SADS-PL; 2. Age range: 6-14 years.

Healthy control group: 1. Age range: 6-14 years.

Exclusion criteria were the following:

Children with ADHD: 1. Mental retardation (global IQ < 70 on the Wechsler Intelligence Scale for Children Fourth Edition plus impairment in adaptive function); 2. Other co-morbid psychiatric disorders; 3. Neurological diseases; 4. Current or past iron supplementation; 5. Current psychotropic treatment; 6. Anemia (according to age-related cut-offs suggested by Yip et al.²⁸⁸); 7. Chronic diseases impairing iron absorption.

Children with psychopathologies other than ADHD: 1. ADHD according to DSM-IV DSM-IV-TR criteria and the semi-structured interview Kiddie-SADS-PL; 2. Mental

retardation; 3. Neurological diseases; 4. Current or past iron supplementation; 5. Current psychotropic treatment; 6. Anemia 7. Chronic diseases impairing iron absorption.

Healthy control group: 1. Any psychiatric disorder according to DSM-IV DSM-IV-TR criteria and the semi-structured interview Kiddie-SADS-PL; 2. Mental retardation; 3. Neurological diseases; 4. Iron supplementation; 5. Current psychotropic treatment; 6. Anemia ; 7. Chronic diseases impairing iron absorption.

All the subjects and their parents provided written informed consent for the participation in the study.

2.3.2 Assessment and measures

Plan of the study visits:

V0: Screening visit

V1: Clinical psychiatric interview

V2: Collection of blood for the assessment of peripheral measures of iron status and MRI examination.

Subjects and their parents were interviewed by the doctoral candidate during a screening visit to assess inclusion and exclusion criteria. An accurate developmental history (conception, pregnancy, delivery, birth weight, neonatal status, fine and gross motor development, cognitive and school functioning, toilet training and any lapses, eating behavior and attitudes, and sleep patterns), medical history (prenatal and perinatal complications, illnesses, medication, hospitalizations, serious injuries, operations) and familial history were obtained. After the clinical interview, a clinical psychologist administered the Wechsler Intelligence Scale for Children Fourth Edition to estimate the intellectual quotient (IQ). It is currently one of the most widely used intelligence scales for children. It is made up of 4 parts: Verbal Comprehension, Perceptual Reasoning, Working Memory, and Processing Speed. The full scale score is a composite of the 4 areas.

If the subjects meet inclusion criteria and did not present exclusion criteria, they were invited to take part to V1 and V2.

During V1, the diagnosis of ADHD or other psychiatric disorders was assessed according to DSM-IV criteria and confirmed by the semi-structured interview Kiddie- SADS lifetime version (Kiddie-SADS-PL)²⁸⁹. The Kiddie-SADS-PL is a semi-structured diagnostic interview designed to assess current and past episodes of psychopathology in children and adolescents according to DSM-III-R and DSM-IV criteria. It is administered by interviewing the parent(s), the child, and finally achieving summary ratings which include all sources of information (parent, child, school, chart, and other). Questions relatives to the following diagnoses were used: Major Depression, Dysthymia, Mania, Hypomania, Cyclothymia, Bipolar Disorders, Panic Disorder, Agoraphobia,

Separation Anxiety Disorder, Avoidant Disorder of Childhood and Adolescence, Simple Phobia, Social Phobia, Overanxious Disorder, Generalized Anxiety, Obsessive Compulsive Disorder, Attention Deficit Hyperactivity Disorder, Conduct Disorder, Oppositional Defiant Disorder, Transient Tic Disorder, Tourette's Disorder, Chronic Motor or Vocal Tic Disorder, Alcohol Abuse, Substance Abuse, Schizophrenia. The French version of the Kiddie SADS was used in this study²⁹⁰.

Data on child's behavior were obtained by the school to corroborate the diagnosis of ADHD, previous consent of the parents, by means of the Revised Conners Teacher Rating Scale³⁴ (French Version).

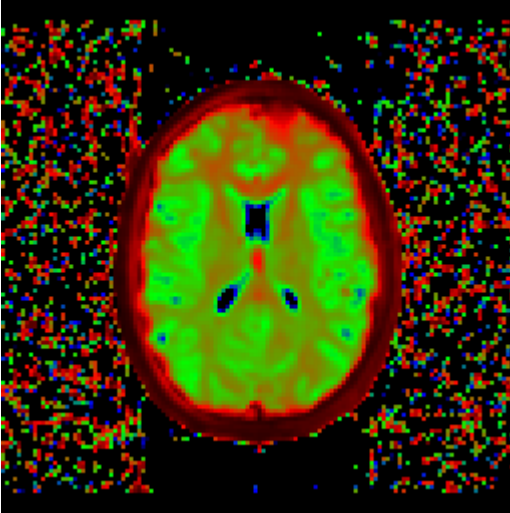
During the first part of the V2, in the morning, blood samples were collected from each subject to obtain a complete blood count, and measurement of serum ferritin levels, as well as of serum iron and hemoglobin. Serum ferritin and iron levels were determined with commercial kits: ferritin levels by the Tinaquant method and iron levels by the Ferrozine method (Roche, Basel, Switzerland).

After the collection of the blood samples, MRI examination was performed. As discussed in detail in the first part of the thesis, several relaxometry metrics have been proposed to estimate brain iron and, at present time, there is a lack of consensus on the most reliable MRI method to accurately estimate brain iron content. We chose to estimate brain iron by means of T2* because it has been reported as a reliable measure not only of iron overload but also of iron deficiency²⁶⁴. We used the same sequence reported in a recent paper by a group of French colleagues²⁹¹ who gave us advice on the selection of the sequence.

Brain MRI examination was performed on the base of the transverse relaxation rates (R2*) or its inverse, T2*. Changes in R2* (or T2*) reflect variations in local iron concentration. R2* are directly correlated with iron stores. MRI examinations were performed on a 1.5 T Philips Unit. Deep gray structures were localized by using a T2*-weighted echoplanar sequence. For each selected section, iron monitoring was performed using a single-slice multigradient echo sequence (field of view 28 cm, 160/160 matrix; slice thickness: 4 mm; TR: 618 ms, 17 echoes at TE from 0

ms to 80 ms; flip angle: 50° ; acquisition time: 4 min). The determination of $T2^*$ was performed from data of the multigradient echo sequences using the equation: $S_i = S_0 \cdot \exp(-R2^* \cdot TE_i)$.

Fig. 23 shows a cartography of $T2^*$ (section at caudatum level) in a patient of the research protocol.



2.3.3 *Statistical analysis*

Sample size and power analysis: based on data provided by Allen et al.²⁷⁷, we calculated that the inclusion of about 10 patients in each of the three groups (children with ADHD, children with other psychopathologies, and healthy controls) would have allowed a detection of a difference of 2 points in T2* with a power of about 85%.

Since we planned to compare children with ADHD vs. children with other psychopathologies and children with ADHD vs. healthy controls, as well as children with ADHD vs. children with other psychopathologies plus healthy controls, we planned to include about 20 children with ADHD, 10 children with other psychopathologies, and 10 healthy controls.

One way analysis of variance (ANOVA) with post-hoc Bonferroni analysis for multiple comparisons was used to compare age, serum ferritin levels, and T2* values in the regions of interest among the three study groups. The correlation between serum ferritin levels was assessed using Spearman correlation.

All statistical analyses were performed using the SPSS v. 15.0 software for Windows package for personal computers (SPSS, Inc., Chicago, IL, USA).

2.4 Results

-- DATA FROM THIS STUDY ARE CURRENTLY SUBMITTED TO A PEER REVIEW
JOURNAL--

2.5 Discussion

--DISCUSSION OF THE RESULTS OF THIS STUDY IS CURRENTLY SUBMITTED TO A
PEER REVIEW JOURNAL--

2.6 Conclusions and future perspectives

In spite of the above mentioned limitations, we believe that our study provides a significant contribution to our understanding of the pathophysiology of ADHD, suggesting that brain iron deficiency might contribute to the pathophysiology of ADHD via its impact on thalamic functioning, which is part of neuronal circuits subserving attention and alertness.

Our study also confirms the relevance of MRI assessment of brain iron to gain insight into the relationship between iron deficiency and ADHD. In fact, previous studies on iron deficiency in ADHD have relied only on the measure of serum ferritin levels, which, according to our results, may be a proxy of brain iron levels but can not estimate them accurately.

Further larger studies are necessary to replicate our results.

Moreover, since we used T2* values to estimate brain iron and this metric presents some limitations (these values may be hampered by local background sources of magnetic field variation)²³², further studies should assess brain iron levels by means of novel approaches (including susceptibility-weighted imaging, magnitude imaging, and phase imaging²³¹) which may estimate brain iron in a more accurate way.

Furthermore, it would be interesting to compare estimated brain iron levels in different types of ADHD (“predominantly inattentive”, “predominantly hyperactive-impulsive”, “combined”) and to assess the correlation between estimated brain iron levels and severity of ADHD.

Finally, since it has been reported that children with ADHD present with higher activity levels than controls not only during daytime but also in sleep²⁹², and since we²⁹³ have recently reported that low serum iron levels correlate with high nighttime activity in ADHD, it would be noteworthy to assess the correlation between nighttime activity and estimated brain iron levels.

All this body of research might contribute to advance our knowledge on the pathophysiologic pathways underlying the cluster of ADHD symptoms. The approach which underlies the rationale of these studies is an innovative one in the field of ADHD, and, in general, in child psychiatry, moving from the description of syndromes to pathophysiology-based disorders.

This approach would also lay the groundwork for interesting treatment applications. In fact, ADHD medications act on the dopaminergic and noradrenergic system. Interestingly, it has been reported that the effect of drugs acting on the dopaminergic and noradrenergic systems is reduced in the presence of iron deficiency¹⁵⁹. Therefore, if further research confirms low brain iron levels in ADHD, it would provide a strong rationale for trials assessing the effects of ADHD medications plus iron supplementation for daytime ADHD symptoms and associated nighttime hyperactivity.

This might contribute to improve current clinical practice, allowing for a better quality of life for children with ADHD and their families. Therefore, given the tremendous social and personal impairment associated with ADHD, further research on the relationship between iron deficiency and ADHD is worthwhile.

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